Les peptides : un levier pour orienter les fonctionnalités de matrices riches en caséines

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« Le savant n'est pas l'homme qui fournit les vraies réponses

c'est celui qui pose les vraies questions »

de Claude Lévi-Strauss, extrait de Le Cru et le cuit.

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Summary

Abbreviations

(-)	Cheese without NaCl addition
(+)	Cheese with NaCl addition
Abs	Absorbance
AI	Aliphatic index
CA	Correspondance analysis
CD	Circular dichroism spectroscopy
CN	Casein
CMS	Casein micelle suspension
CV	Concentration volume factor
D	Day of ripening
DH	Degree of hydrolysis
F	Cheese fabrication
FTIR	Fourier transform infrared spectroscopy
G’	Elastic modulus
G”	Viscous modulus
G1	Hydrolysate from Glu-C at time 1
G2	Hydrolysate from Glu-C at time 2
G3	Hydrolysate from Glu-C at time 3
Gc	Cluster from Glu-C hydrolysate
GRAVY	Grand average hydropathy
HS	Hydrolysate suspension
LH1	*Lactobacillus helveticus* ITGLH1
LH77	*Lactobacillus helveticus* ITGLH77
MS	Mass spectrometry
MW	Molecular weight
NaCas	Sodium caseinate
NCN	Non casein nitrogen
NPN	Non protein nitrogen
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PC	Principal component
PCA	Principal component analysis
pI	Isoelectric point
RP-HPLC	Reverse phase – high performance liquid chromatography
RUF	Ultrafiltration retentate
SDS	Sodium dodecyl sulfate
STLO	Science et Technologie du Lait et de l’Oeuf
T1	Hydrolysate from Trypsin at time 1
T2	Hydrolysate from Trypsin at time 2
T3	Hydrolysate from Trypsin at time 3
Tc	Cluster from Trypsin hydrolysate
TEM	Transmission electron microscopy
UF	Ultrafiltration

List of Figures and Tables

TABLES

Part III - Chapter 5: Peptides in semi-hard model cheeses

Part IV - General discussion

FIGURES

General introduction, Objective and Strategy

Part I - Bibliographic Review

Part II – Materials and Methodes

Part III - Chapter 1. An *in silico* approach to establish a relationship between a techno-functional property of a dairy-matrix and a peptide profile.

Part III - Chapter 2: Properties of milk peptides in liquid casein matrices

Part III - Chapter 3: Properties of milk peptides in gel casein matrices

Part III - Chapter 4: Structural changes of caseins and peptides under a high concentration

Part III - Chapter 5: Peptides in semi-hard model cheeses

General Introduction, Objective & Strategy

The consumption mode of food products has deeply evolved since decades. It clearly appears an increase in lipid and polysaccharide contents of the food products, a democratization of the ready-to-use dishes, an increase in the out-of-home catering industry and an increasing request in practicality of food preparation.

The changes in food consumption came from our long human history (Figure 1). Since more than 7 million years, during the prehistory, humans acquired their food by picking, hunting or fishing and, since a few millenniums, by the farming and breeding. The agricultural revolution, followed then by the industrial revolution had great consequences on the human food consumption. The consequences of these upheavals were either positive, i.e. development of the biological potential, higher capacity for work, life longevity, better quality of life, or negative i.e. increase of the overweight, and in various diseases as cardiovascular risks, diabetes... In the XVIIth century, the diets was not just devoted to nourish population but it took more into account the gourmet point of view, the food variety and also the esthetics of the meals. These changes were subjected to the hedonic pleasures in terms of taste, flavor, nutritional contribution and health. From 1789, the taste and the pleasure to eat became more democratic. At this time, the texture and flavor of food products became very important for customers. At the end of the 1800s, a healthy aspect was given to the food. The development of the food conservation occurred with the fridge invention and the canning. There is an increase in the food product diversity with the development of new and complex processes of transformation. New functionalities were than looked for many food components, as its reports by Bauer et al. (2010) for milk proteins. In the XXth century, the ready-to-serve dishes and easy-to-use products, such as pizzas or fresh milky desserts, were a continued success due to time-saving and practicality in the meal preparation.

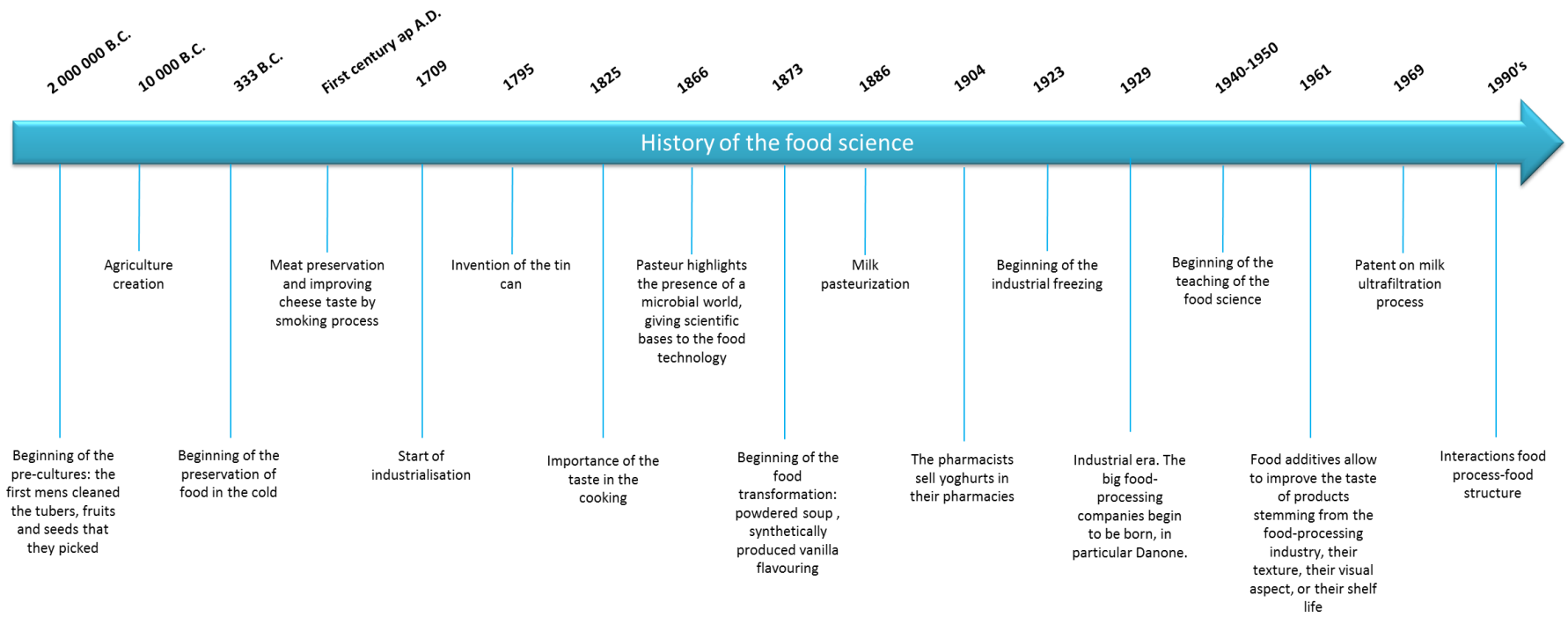

Figure 1. Key steps of the food science history, from prehistory to now (http://www.ania.net/alimevolution)

Consequently, since the 1990s, the households consume more and more ready-to-serve dishes and transformed products such as cheese (Figure 2). A food product is considered as transformed from the moment its raw material is modified, either by a process of manufacture or by chemical or biological modification.

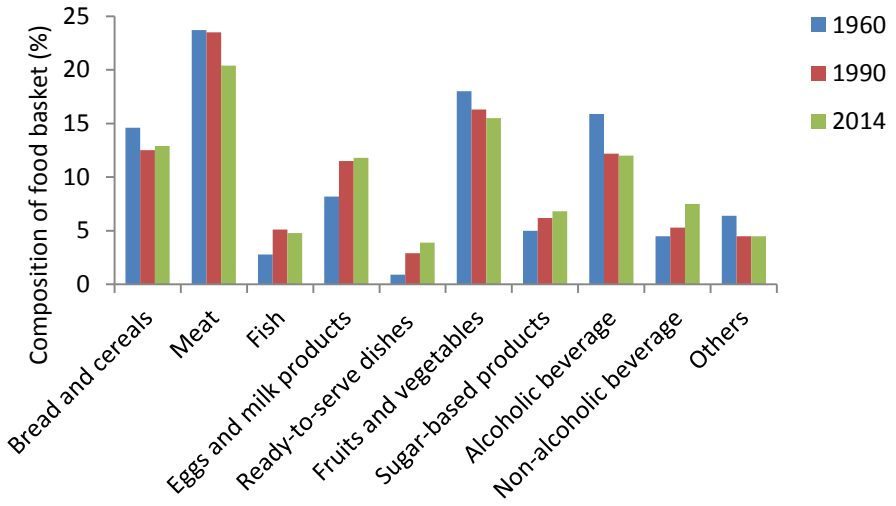

Figure 2. Source : Evolution of the type of food product consommed in France from 1960 to 2014 (Insee 2015, cinquante ans de consommation alimentaire).

Food is a material constantly changing in shape, size and structure depending on the physical and biochemical reactions that take place during the technological operations or during the preservation. Thus, numerous interactions take place between the constituents which deeply modify, in a more or less irreversible way, the texture of these materials. Therefore, it is essential to understand how these changes occur to be able to establish a correlation between food constituent and the changes in the texture of food product. It is also necessary to be able to measure mechanical properties as rheological or textural properties to rely perception in mouth with mechanical and physical behavior that can be predictable and finally controlled.

Milk is one example of choice to understand the relation between constituents and their functionalities. In the 1960s, membrane filtration processes (microfiltration, ultrafiltration) were developed to separate milk component which could have specific functional properties. It was the occasion to distinguish every milk components as new individual entities with specific physico-chemical characteristics. Milk is actually composed by several hundred molecules, including proteins, lipids, carbohydrates, minerals, oligoelements, vitamins…and an overall composition of the main constituents is presented in Table I. It rapidly appeared that among the various constituents proteins and their derived peptides, playing the main role in cheese structure, could be used in other food products to modify their structuration and texturation. Moreover, the whey recovery gave the

opportunity to recycle part of proteins with great functional properties like gelation, stabilization of emulsions or foams for example since the 1980's (Burgess and Kelly, 1979). These scientific insights had thus permitted to develop new formulations of numerous products in bakery, meat preparation, and dairy products with targeted functionalities. From now on, milk is not added anymore as raw milk in many industrial food product formulations but in the form of specific functional molecules that can form various nanostructures and in turns change the texture and techno-functional properties of food products (Barbut, 2007; Crowley et al., 2002).

Table I. General composition of bovine milk (Fox, 2003; Gaucheron, 2005).

Composition	Range ($g.L^{-1}$)
Water	900 – 910
Dry Matter	125-135
Fat	35-45
Lactose	47 – 52
Caseins	24 - 28
- α_{s1}-casein	9 - 11
- β-casein	9 - 11
- κ-casein	3 - 4
- α_{s2}-casein	3 - 4
- γ_1, γ_2, γ_3 caseins (β-casein-derived peptides)	1 – 2
Whey proteins	5 - 7
- β-lactoglobulin	2 - 4
- α-lactalbumin	1 - 1.5
- Bovine serum albumin	0.1 - 0.4
- Immunoglobulins	0.6 - 1
- Protease peptones (casein-derived peptides)	0.6 - 1.8
Non-protein nitrogen (creatin, urea, free amino-acids)	1.5
Minerals	8.5 – 9
- Calcium	1.0 - 1.3
- Magnesium	0.97 – 1.46
- Inorganic phosphate	1.81 – 2.19
- Total phosphore	0.93 – 0.99
- Citrate	1.32 – 2.08
- Sodium	0.39 – 0.64
- Potassium	1.21 – 1.69
- Chloride	0.77 – 1.21

Besides the key role of proteins in the food backbone and their peculiar functional properties, a controlled hydrolysis of these proteins can also increase these functionalities according to the nature

and the quantity of the peptides produced (Betancur-Ancona et al., 2014). Due to their intrinsic physical and chemical characteristics, i.e., sequence, size, charge, structure and their interaction with each other and other molecules, peptides are actually able to form self-assemblies or aggregates and to thus develop new functional properties. The aggregation capacity of proteins and peptides may be either enhanced or impaired when they are present in a heterogeneous and highly concentrated medium such as that of food products, in the presence of other molecules (polysaccharides, lipids and other proteins). One of the current way to control the functional properties of hydrolyzed proteins is to understand the self-assembly mechanisms of proteins and peptides in concentrated products such as foods and to determine which possibilities are available to product these assemblies (Purwanti et al., 2010).

The differences between functional properties of model proteins and peptides were studied and showed that peptides, with amphiphilic characteristics, increased the interfacial properties. This was studied in model milk systems such as β-casein or β-lactoglobulin (Caessens et al., 1999; Haque and Sharma, 1997; Turgeon et al., 1992). The model hydrolysates were characterized most often according to a degree of hydrolysis and their functionalities were tested in model systems, in which the composition and the concentration are far away from concentrated and complex systems like food products. Moreover, some external factors such as temperature, pH or ionic strength added a degree of complexity to understand the role of peptides in the modification of the protein functional properties (Chobert et al., 1987; van der Ven et al., 2002). Then, the relationship between structure of peptides or proteins and their functionalities is still difficult. It is even harder to establish this relationship as peptides can be either produced *in situ* during the transformation process or reincorporated in the food product as functional ingredients.

Cheese appears as an appropriate example of investigation of the peptide impact on the texture characteristics as it is a rare transformed food product for which peptides were clearly involved in the changes of texture and rheological properties (Upadhyay et al., 2004). Some peptides were clearly identified as responsible of the texture changes, especially the α_{s1}-I which is correlated with the cheese softening (Lucey et al., 2003). Recently, a positive correlation between the casein hydrolysis and a stretchability of cheese was shown (Sadat-Mekmene et al., 2013). This study highlighted the importance of (i) the peptide production during the cheese ripening and (ii) the interactions peptides with caseins into the cheese matrix.

The objective of this thesis was to determine how and to what extent casein-derived peptides are able to modify the texture, the rheology and the microstructure of highly-concentrated casein matrices.

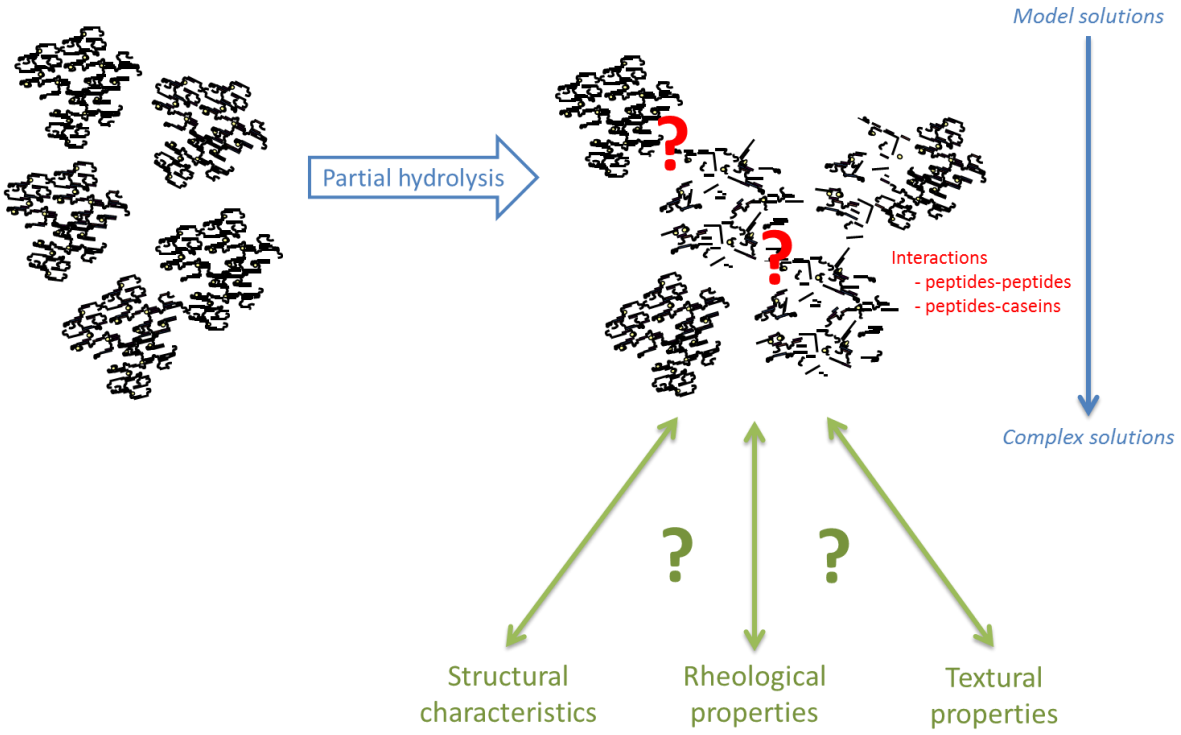

Figure 3. Scheme representing the questions arisen in this thesis.

The aim of this thesis was to establish how defined groups of peptides can induce functional properties of various protein-peptide systems from model system to complex food system in terms of impact on the microstructure, the rheological and the textural properties. In order to focus on the impact of peptides, we chose to reincorporate casein hydrolysates in casein micelle systems of various concentrations in the first parts of the thesis. In the last part, these hydrolysates were incorporated in a model cheese matrix, including other components as fat and other peptides produced by the enzymes already present in milk, added as coagulant or from the microflora.

Three series of questions were addressed throughout this thesis project:

(i) Would it be possible to relate *in silico* the physico-chemical characteristics of peptides and one peculiar techno-functional property of food product? Would it be possible, in turn, to tailor hydrolysate(s) that can be used to modify texture of a complex casein matrix?

(ii) What is the impact of the physico-chemical and structural characteristics of these designed peptides on the rheological and the structural properties of highly concentrated casein matrices in a liquid or gel form?

(iii) Are the same phenomena found from a model solution to a concentrated matrix including food products, as exemplified in a cheese model system?

To answer these questions, a strategy in four steps was developed (Figure 4). First, an *in silico* approach was realized to link a peptide profile to a functional property in a food product. This was done from the dataset obtained from the previous work of Sadat-Mekmene et al., (Sadat-Mekmene et al., 2013), to determine which physico-chemical and structural characteristics of peptides are involved in cheese stretchability. This step has permitted to select two enzymes able to produce defined groups of peptides in the second step. Six hydrolysates of sodium caseinate were produced with the two selected enzymes. This step needed adjustment of the hydrolysis parameters. The third step was to incorporate the peptides produced indifferent casein systems to highlight the role of peptides in the modification of the rheological properties, the textural properties and the microstructure of casein micelles, under three different concentration conditions. The fourth, and final step, was to incorporate these peptides in a model cheese and to confirm how specific hydrolysates can modify on their own the overall texture of cheese. This thesis focused on the techno-functional properties of peptides in a model media which became more and more complex in terms of concentration and types of molecules involved. This thesis project will permit to have insights on the relationship between peptides-peptides and peptides-proteins and *in fine* to have some levers to be able to orientate the hydrolysis of proteins to generate some targeted functionalities.

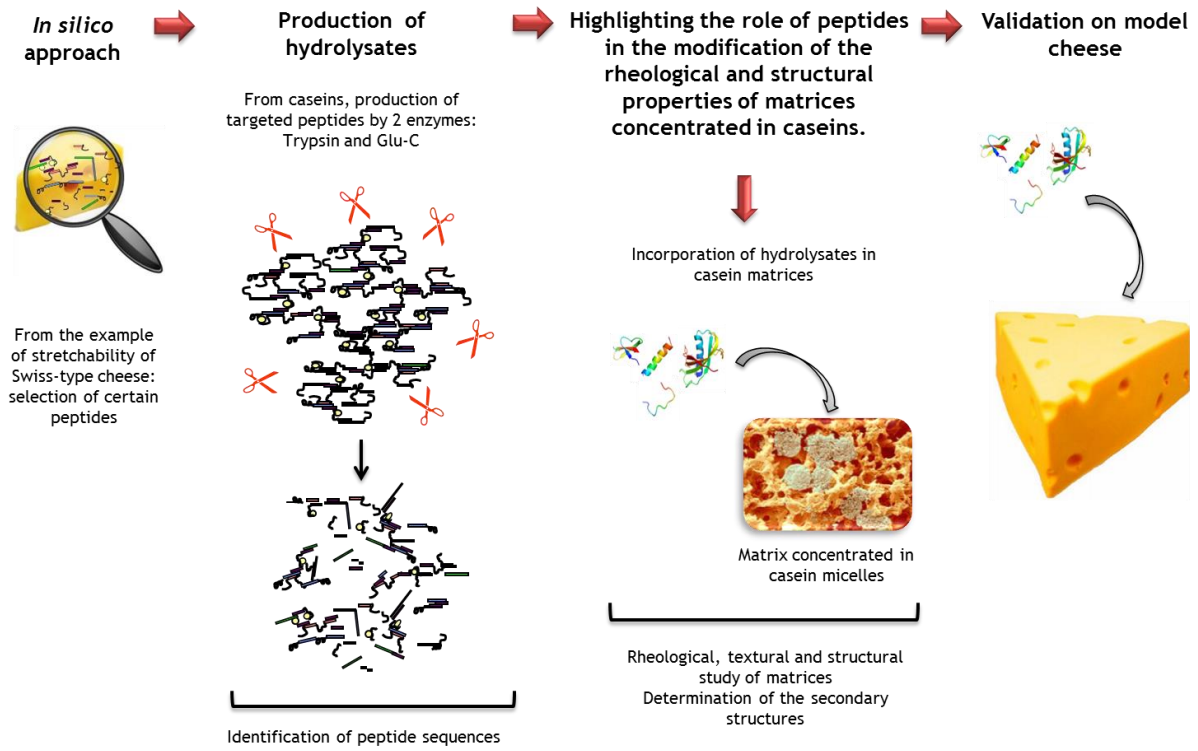

Figure 4. Strategy used in the thesis that is composed of four sequential steps.

The main scientific and technological fallouts of this project are to control (i) the production of peptides *in situ* in terms of quality and quantity and (ii) the interactions between peptides and proteins that are the basis of functional properties by identifying the involved molecular, physico-chemical and structural factors. Concerning the economic fallout, industrial plants have to innovate to be competitive regionally, nationally and internationally. This is particularly a matter of concern of the food stakes of Brittany that are keeping care about manufacturing new food products to increase their added value of products coming from various food sectors (milk, meat, fish products and bakery products). Brittany is the first food-producing region in France. The food-processing companies from Brittany represent 43% of all plants and third party of the industrial jobs. Six sectors are well represented: dairy products, meat, bakery products, fruits and vegetables, fish transformation and ready-to-eat dishes. Among the transformations of food products, milk sector is concentrated in the west part of France, Britany, Pays de la Loire and Normandy with 50% of the French production (Lortal and Boudier, 2011).

This thesis project is a partnership between the joined unit INRA Agrocampus-Ouest, Science et Technologie du Lait et de l'Œuf (STLO, INRA, Rennes) and the Bretagne Biotechnologie Alimentaire association including the main small and medium-sized SMEs and multinational groups as well as research unit centers and technical centers devoted to milk sector. The STLO laboratory has a

research expertise on milk compounds and dairy fermented products. In particular, the “Cheese Microstructure and Bacteria” team focuses on the *in situ* ripening mechanisms at the microscopic scale.

The thesis manuscript includes the following parts:

- In the first part of the thesis, we present a review dealing with the functional properties of peptides, from model solution to food products.
- The second part describes the material and methods used in the different parts of this thesis.
- The third part of thesis describes the results and discussion. It is composed of five chapters.
    a) The first chapter reports on the *in silico* methods used to establish relationships between a techno-functional property of dairy products and a peptide profile.
    b) Chapter 2 gives insights on the rheological properties and the microstructure of milk derived peptides in liquid systems highly concentrated in caseins.
    c) In chapter 3, we investigated how peptide physico-chemical and structural characteristics affects the textural properties and the microstructure of dairy gels.
    d) In chapter 4, the impact of protein and peptide concentration on the texture and appearance of the systems and was evaluated. Which interactions are involved between casein micelles and peptides at concentrations above 200 $g.kg^{-1}$?
    e) In chapter 5, the impact of peptides on the texture of model cheese was investigated.
- The summarized results and concluding remarks are given in the 4^{th} part.
- The valorizations and formations are presented in the last part of this thesis.

Introduction Générale, Objectif & Stratégie

Les modes de consommation des aliments sont en constante évolution depuis des décennies qui a eu pour effet : une forte augmentation du taux de lipides et de sucres dans les produits alimentaires, une démocratisation des plats préparés et une augmentation des repas pris hors domiciles qui requièrent une praticité de préparation des plats.

Les changements de consommation des aliments proviennent de notre histoire (Figure 1). Depuis plus de 7 millions d'années, lors de la préhistoire, les humains acquéraient leur nourriture par la chasse, la pêche et la cueillette. La révolution agricole, suivie par la révolution industrielle, ont eu de grandes conséquences sur la consommation alimentaire humaine. Les conséquences de ces bouleversements ont été soit positives (développement du potentiel biologique, de la capacité de travail, de la longévité de la vie, et de la qualité de la vie d'une manière générale) ou négatives (augmentation du surpoids, obésité, diabètes). Au XVII^{ème} siècle, les régimes alimentaires ne sont plus uniquement dévoués à nourrir la population mais tiennent de plus en plus compte de l'aspect gourmet de la nourriture, de la variété et de l'esthétisme des repas. Ces changements ont été associés à la notion de plaisirs hédoniques en termes de gout, de flaveur, et à l'émergence des qualités nutritionnelles et de santé. A partir de 1789, le goût et les plaisirs de la table se sont démocratisés. A ce moment, la texture et la flaveur des produits alimentaires sont devenus très importants pour les consommateurs. A la fin des années 1980, un fort aspect santé a été donné à l'aliment. Le développement des méthodes de conservation des aliments est venu avec l'invention du réfrigérateur et de l'appertisation. Le développement de nouveaux procédés de transformation alimentaire et l'accroissement de leur complexité a permis de diversifier les types de produits alimentaires. De nouvelles fonctionnalités ont alors été recherchées pour beaucoup de composants alimentaires, comme il est reporté par Bauer et al. (2010) pour les protéines laitières. Au XX^{ème} siècle, les plats préparés et les produits prêts à consommer, tels les pizzas ou les desserts au lait frais, ont connu un grand succès du fait du temps gagné et de la praticité engendrée lors de la préparation des repas.

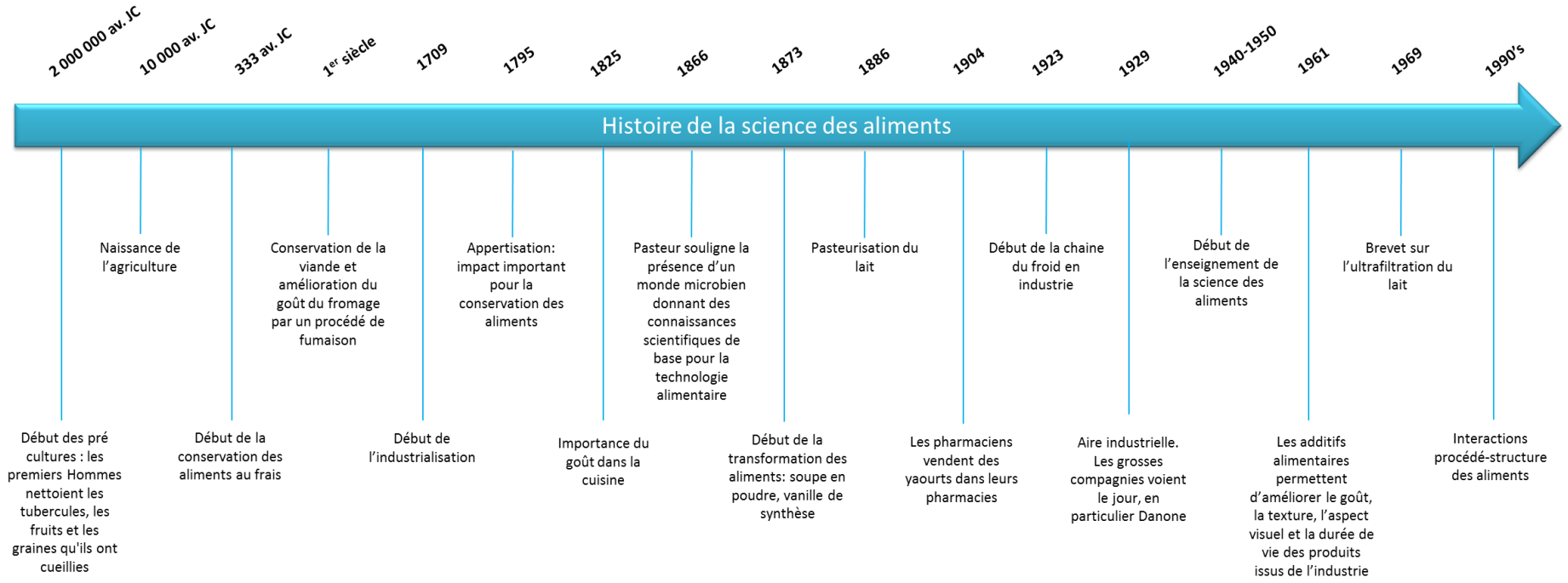

Figure 1. Etapes clés de l'histoire de la science des aliments, de la préhistoire à nos jours (http://www.ania.net/alimevolution).

Par conséquent, depuis les années 1990, les ménages ont consommé de plus en plus de plats préparés et de produits transformés notamment dans le secteur laitier avec comme exemple phare le fromage (Figure 2). Il est ici considéré qu'un produit est transformé à partir du moment une transformation de la matière première a lieu, soit par le procédé de fabrication soit par une modification chimique ou biologique.

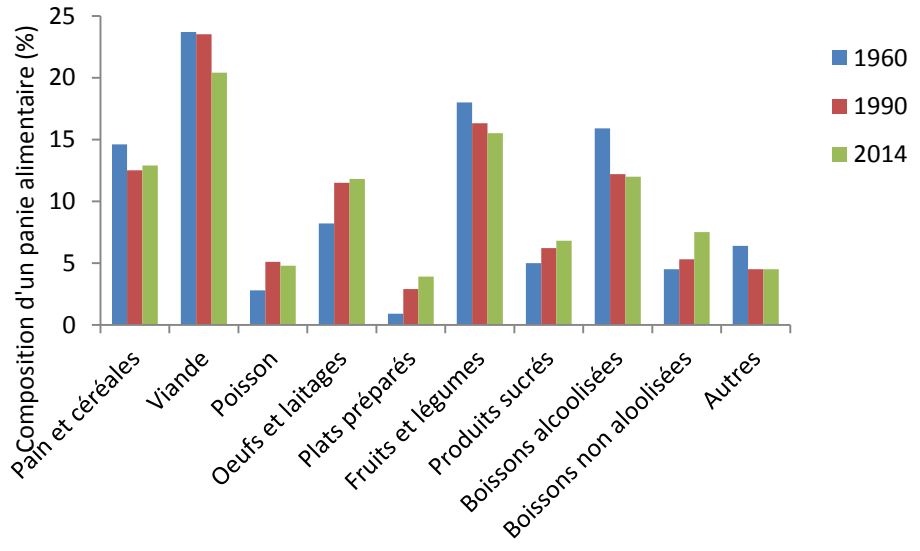

Figure 2. Evolution du type de produit alimentaire consommé en France de 1960 à 2014 (Insee 2015).

L'aliment est une matière subissant des modifications constantes de forme, de taille et de structure, dépendantes des réactions physiques et biochimiques qui ont lieu lors des opérations technologiques ou lors de la conservation. Donc, de nombreuses interactions ont lieu entre les divers constituants, ce qui modifie la texture de ces matières. Toutefois, il est essentiel de comprendre comment ces changements ont lieu afin d'être capable d'établir une corrélation entre les composants de l'aliment et les changements de texture de l'aliment. Le produit alimentaire est aussi une matière sur laquelle il est intéressant de mesurer les propriétés mécaniques telles que rhéologiques ou texturales. Lors de la préparation, lors de la transformation ou lors de la formulation des matières premières, de nombreuses techniques ont été utilisées et de nombreuses interactions ont lieu entre les constituants qui modifient profondément et de manière plus ou moins irréversible la texture de ces matières. La mesure des propriétés rhéologiques des denrées alimentaires permet de planifier leur comportement mécanique lors de diverses étapes de l'élaboration des aliments. Les propriétés rhéologiques sont aussi à l'origine du comportement perçu lors de l'évaluation sensorielle de la texture. Dans le domaine alimentaire, la texture est essentiellement considérée comme une propriété sensorielle incluant, entre autres, la tendreté, la fermeté ou encore le collant en bouche. Certains principes fondamentaux de la rhéologie permettent de mieux comprendre le comportement

mécanique de ces substances, à leur état naturel ou quand elles ont été modifiées par un procédé de texturation. Il y a d'autres possibilités de modification de la texture d'un produit menant au développement de nouvelles formulations alimentaires en utilisant les propriétés fonctionnelles de certains composants tels que la gélification, les propriété épaississantes, l'émulsion ou texturisation.

Le lait est un exemple de choix pour comprendre les relations entre les constituants et leurs fonctionnalités. Dans les années 1960, la filtration membranaire a été développée afin de séparer les composants du lait qui pourraient avoir des fonctionnalités spécifiques. Cela a été l'occasion de découvrir chaque composant laitier en tant qu'entité à part entière. Le lait est composé de plusieurs centaines de molécules, incluant des protéines, des lipides, des glucides, des minéraux, des oligoéléments, des vitamines… (Table I). Il est rapidement apparu que parmi ces nombreux constituants, les protéines et les peptides dérivés de ces protéines jouent un rôle prépondérant dans la structuration et la texturation de l'aliment. Les protéines et les peptides peuvent être réincorporés dans des produits alimentaires pour modifier leur texture et leur structure. De plus, la récupération du lactosérum a permis de recycler une partie des protéines avec de bonnes propriétés fonctionnelles telles que la gélification, la stabilisation d'émulsion ou de mousses depuis les années 1980 (BURGESS and KELLY, 1979). Ces avancées scientifiques ont permis de développer de nouvelles formulations de nombreux produits de boulangerie, de viande ou de produits laitiers avec des fonctionnalités ciblées. Jusqu'à ce jour, le lait est de plus en plus rarement ajouté tel quel comme matière première dans les formulations de produits alimentaires mais sous forme de molécules avec des techno-fonctionnalités spécifiques (Barbut, 2007; Crowley et al., 2002).

Table I. Composition générale du lait de vache (Fox, 2003; Gaucheron, 2005).

Composition	Concentration ($g.L^{-1}$)
Eau	900 - 910
Extrait sec	125-135
Matières grasses	35-45
Lactose	47 - 52
Caséines	24 - 28
- Caséine αs1	9 - 11
- Caséine β	9 - 11
- Caséine κ	3 - 4
- Caséine α_{s2}	3 - 4
- Caséines γ1, γ2, γ3 (peptides dérivés de la caséine β	1 - 2
Protéines sériques	5 - 7
- β-lactoglobuline	2 - 4
- α-lactalbumine	1 - 1.5
- Sérum albumine bovine	0,1 - 0,4
- Immunoglobuline	0,6 - 1
- Protéases de peptone	0,6 - 1,8
Azote non protéique (créatine, urée, acides-aminés)	1,5
Mineraux	8,9 - 9
- Calcium	1,0 – 1,3
- Magnésium	0,97 – 1,46
- Phosphate inorganique	1,81 – 2,19
- Phosphore total	0,93 – 0,99
- Citrate	1,32 – 2,08
- Sodium	0,39 – 0,64
- Postassium	1,21 – 1,69
- Chlorure	0,77 – 1,21

Un contrôle de l'expression des propriétés fonctionnelles des composants alimentaires, depuis le début de leur fabrication, devrait permettre de développer des formulations avec des textures souhaitées. En plus du rôle clé des protéines dans la structure des aliments et de leurs propriétés fonctionnelles particulières, une hydrolyse contrôlée de ces protéines peut aussi augmenter ces fonctionnalités selon la nature et la quantité des peptides produits. En raison de leurs caractéristiques physico-chimiques intrinsèques, telle la séquence, la taille, la charge, la structure et leurs interactions avec d'autres molécules, les peptides peuvent en réalité former des auto-assemblages ou des agrégats et ainsi développer des nouvelles propriétés fonctionnelles. La capacité d'agrégation des protéines et des peptides peut être ou améliorée ou détériorée quand ils sont présents dans un milieu hétérogène et concentré tel un produit alimentaire, en présence d'autres molécules (polysaccharides, lipides et autres protéines). Un des moyens de contrôler les fonctionnalités des protéines hydrolysées est de comprendre les mécanismes d'auto-assemblage des peptides et protéines dans des produits concentrés tels que les aliments et de déterminer quelles sont les possibilités pour produire les assemblages (Purwanti et al., 2010).

Les différences entre les propriétés fonctionnelles des protéines modèles et des peptides ont été étudiées, et ont montrées que les peptides, avec des caractéristiques amphiphiles, augmentaient les propriétés interfaciales des protéines initiales. Les hydrolysats modèles ont été caractérisés le plus souvent selon leur degré d'hydrolyse et leurs fonctionnalités ont été testées dans des systèmes modèles dont la composition et la concentration sont fortement éloignées des systèmes concentrés et complexes que sont les aliments (Caessens et al., 1999; Haque and Sharma, 1997; Turgeon et al., 1992). De plus, certains facteurs externes tels que la température, le pH ou la force ionique en plus du degré d'hydrolyse apportent une complexité supplémentaire à la compréhension du rôle des peptides dans la modification des propriétés fonctionnelles des protéines (Chobert et al., 1987; van der Ven et al., 2002). Par conséquent, la relation entre la structure des peptides ou des protéines et leurs fonctionnalités est toujours difficile à établir. Il est aussi difficile d'établir cette relation puisque les peptides peuvent être soit produits *in situ* lors du procédé de transformation ou isolés et ajoutés plus tard dans l'aliment en tant qu'ingrédient fonctionnel.

Le fromage apparait comme un exemple approprié d'investigations de l'impact des peptides sur la texture puisqu'il est un des rares aliments pour lequel les peptides ont été clairement démontrés comme responsable dans les changements de texture ou de propriétés rhéologiques (Upadhyay et al., 2004). Certains peptides ont été identifiés comme responsables des changements de texture, spécifiquement le peptide α_{s1}-I qui a été corrélé au ramollissement de la pâte du fromage (Lucey et al., 2003). Récemment, une corrélation positive entre l'hydrolyse des caséines et le caractère filant

du fromage a été montrée (Sadat-Mekmene et al., 2013). Cette étude a mis en évidence l'importance de (i) la production de peptides lors de l'affinage du fromage et des (ii) interactions peptides-caséines dans la matrice fromagère.

L'objectif de cette thèse est de déterminer comment les peptides sont capables de modifier la texture, la rhéologie et la microstructure des matrices hautement concentrées en caséines.

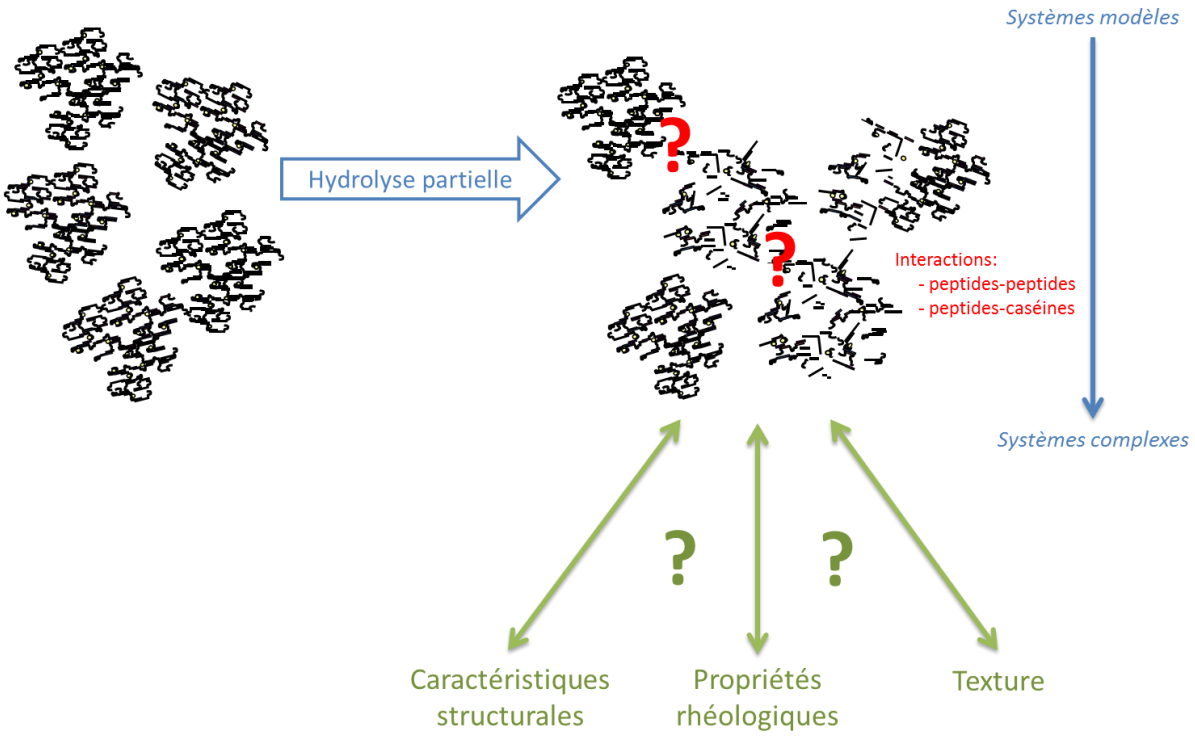

Figure 3. Schéma représentant les questions posées dans cette thèse.

L'objectif de cette thèse a été d'établir comment un groupe défini de peptides peut induire diverses propriétés fonctionnelles, du système modèle au système complexe, en termes d'impacts sur la microstructure, la rhéologie et les propriétés de texture (Figure 3). Dans la première partie de cette thèse, des hydrolysats de caséines ont été incorporés dans des systèmes contenant diverses concentrations de micelles de caséines. Dans la partie finale de cette thèse, ces hydrolysats ont été incorporés dans une matrice fromagère modèle dans laquelle sont présents d'autres composants tels la matière grasse et d'autres peptides produits par les enzymes déjà présentes dans le lait, ajoutés comme coagulant ou à partir de la flore bactérienne.

Trois séries de questions ont été posées à travers ce projet de thèse :

(i) Est-il possible de relier, *in silico,* les caractéristiques physicochimiques de peptides présents dans un aliment à une propriété techno-fonctionnelle particulière de cet aliment? Est-il possible ainsi d'adapter le ou les hydrolysat(s) qui peuvent être utilisés pour modifier la texture d'une matrice de caséines complexe?

(ii) Quel est l'impact des caractéristiques physico-chimiques et structurelles de ces peptides cibles sur la rhéologie et les propriétés structurelles de matrices de caséines hautement concentrées, sous forme liquide ou de gel?

(iii) Les mêmes phénomènes se produisent-ils dans un système modèle, un système concentré en caséines et dans un fromage modèle?

Afin de répondre à ces questions, une stratégie en quatre étapes a été développée (Figure 4). Premièrement, une approche *in silico* a été réalisée afin de relier un profil de peptides à une propriété de texture particulière, le filant d'un fromage. Cette étude a été menée à partir des données obtenues lors de travaux précédents (Sadat-Mekmene et al., 2013) afin de déterminer quelles caractéristiques physico-chimiques et structurelles de peptides sont impliquées dans le filant du fromage. Cette étape a permis de sélectionner deux enzymes afin de produire les hydrolysats spécifiques de la seconde étape. Six hydrolysats ont été produits à partir de trois caséines : la caséine α_{s1}, la caséine β et la caséine α_{s2} grâce à deux enzymes sélectionnées lors de l'étape *in silico*. Cette étape a nécessité un ajustement des paramètres d'hydrolyse selon l'échelle de production des hydrolysats. La troisième étape a été d'incorporer les peptides produits dans des systèmes contenant trois différentes concentrations à deux pH afin de mettre en évidence le rôle des peptides dans la modification des propriétés rhéologique, de texture et sur la microstructure de ces systèmes. La quatrième et dernière étape fut d'incorporer ces hydrolysats dans un fromage modèle et d'observer les modifications de la texture du fromage liées à la présence de ces hydrolysats. Cette thèse s'est focalisée sur les propriétés techno-fonctionnelles des peptides en premier lieu sur un milieu modèle qui est a été complexifié au cours de l'étude en termes de concentration et de type de molécules mises en jeu. Ce projet de thèse va permettre d'obtenir des connaissances sur les relations peptides-peptides et/ou peptides-protéines afin de permettre à terme d'orienter *in fine* l'hydrolyse des protéines pour générer des propriétés fonctionnelles ciblées.

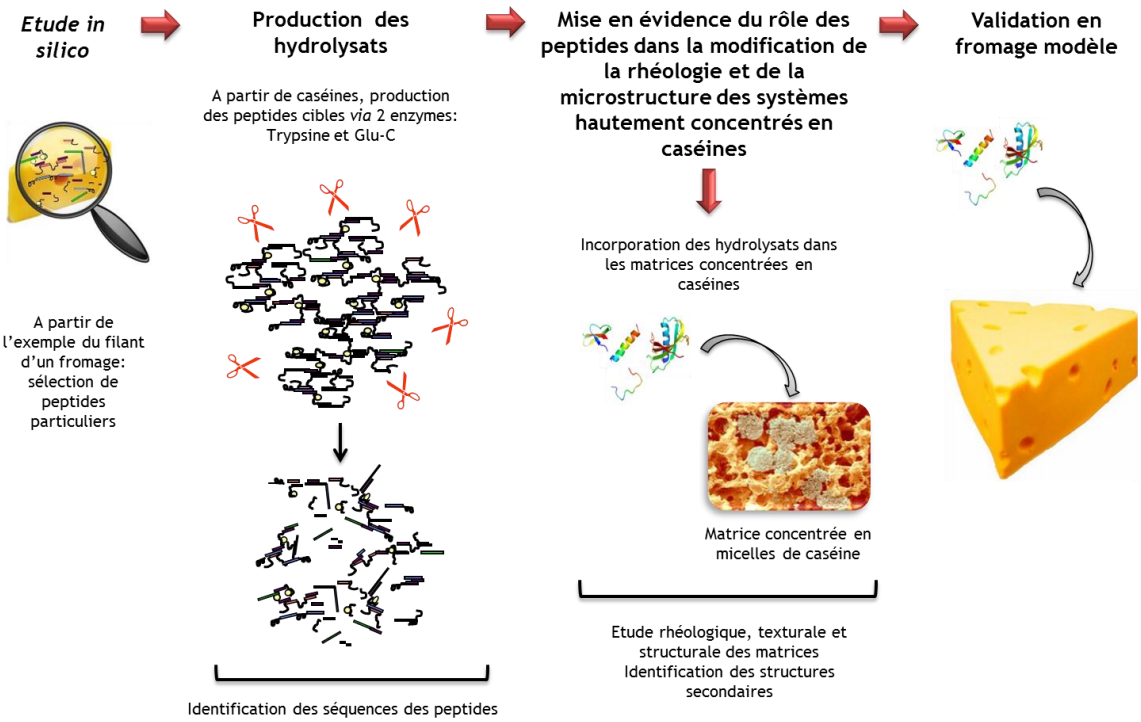

Figure 4. Stratégie de la thèse composée de 4 étapes.

Les principales retombées scientifiques et technologiques de ce projet sont : (i) de contrôler la production des peptides *in situ* en termes de qualité et de quantité et (ii) de générer des connaissances sur les interactions entre les peptides et les protéines, à la base des propriétés fonctionnelles, en identifiant les facteurs moléculaires, physico-chimiques et structuraux impliqués. Concernant les retombées économiques, les entreprises doivent constamment innover pour préserver leur compétitivité. Ceci est une des préoccupations des entreprises agroalimentaires de la Bretagne qui cherchent à fabriquer de nouveaux produits alimentaires afin d'augmenter la valeur ajoutée des produits venant de secteurs alimentaires divers (lait, produits carnés, produits à base de poisson et produits et produits de boulangerie). La Bretagne est la première région agroalimentaire de France. Les entreprises agroalimentaires de Bretagne représentent 43% du total des entreprises et la troisième partie des emplois industriels. Six secteurs sont représentés : produits laitiers, viande, produits de boulangerie, fruits et légumes, transformation du poisson et plats préparés. Parmi les transformations des produits alimentaires, la filière laitière est concentrée dans l'ouest de la France, Bretagne, Pays de la Loire et Normandie avec 50% de la production Française totale (Lortal and Boudier, 2011).

Ce projet de thèse est un partenariat entre le laboratoire de Science et Technologie du Lait et de l'Œuf (STLO, INRA, Rennes) et l'interprofession laitière Bretagne Biotechnologie Alimentaire incluant (SODIAAL, LACTALIS, Bongrain, CF&R, BEL, Coopérative d'Isigny, Laïta, Laiterie de Montaigu, SILL,

Maîtres Laitiers du Cotentin, INRA Sciences et technologie du lait et de l'œuf, INRA Biopolymères interactions assemblages, Laboratoire Universitaire de biodiversité et écologie microbienne, ONIRIS, Actalia Produits laitiers, Institut de physique de Rennes, Polymères colloïdes et interfaces). Le STLO a une expertise en recherche sur les composants du lait et les produits laitiers fermentés. En particulier, l'équipe « Microstructure fromagère et Bactéries » se focalise sur les mécanismes d'affinage *in situ* à l'échelle microscopique.

Ce manuscrit de thèse inclue les parties suivantes :

- Dans la première partie de la thèse, nous présentons les propriétés fonctionnelles des peptides, des systèmes modèles aux produits alimentaires.
- La seconde partie décrit le matériel et méthode utilisé lors de cette thèse.
- La troisième partie de cette thèse décrit les résultats et la discussion. Elle est composée de cinq chapitres.
    a) Le premier chapitre démontre une méthode *in silico* permettant d'établir une relation entre un profil de peptides et une propriété techno-fonctionnelle particulière d'un fromage.
    b) Le chapitre 2 apporte des connaissances sur les propriétés rhéologiques et la microstructure des peptides en systèmes liquides, à diverses concentrations en peptides et caséines.
    c) Le chapitre 3 apporte des connaissances sur la texture et la microstructure des peptides en systèmes gélifiés, à diverses concentrations en peptides et caséines.
    d) Dans le chapitre 4, l'impact de la concentration des protéines et des peptides sur la texture et l'apparence des systèmes a été évalué. Quelles interactions sont impliquées entre les micelles de caséines et les peptides à de très hautes concentrations ?
    e) Dans le chapitre 5, l'impact des peptides sur la texture d'un fromage modèle a été étudié.
- La quatrième partie comprend une discussion générale des résultats.
- La dernière partie inclue les valorisations et les formations réalisées au cours de cette thèse.

Part I – Bibliographic Review

The first part of the manuscript presents the advances made concerning the ability of peptides to bestow particular functional properties on various matrices including foods (Figure 1). We focus on systems ranging from model solutions in which peptides are rationally designed to orient their structure and to form hydrogels to mixtures of peptides in complex food matrices. In the latter case, peptides are an integral part of food formulations due to their production *in situ* or their addition as an ingredient. Examples of complex matrices such as food products, where mixes of peptides are present as hydrolysates with various physico-chemical properties, focus on the ability of peptides to modulate the texture of foods and their functional properties, including solubility, gelation and even emulsifying and foaming properties. Attempts have been made to establish relationships between the physico-chemical and structural characteristics of peptides and their functional properties.

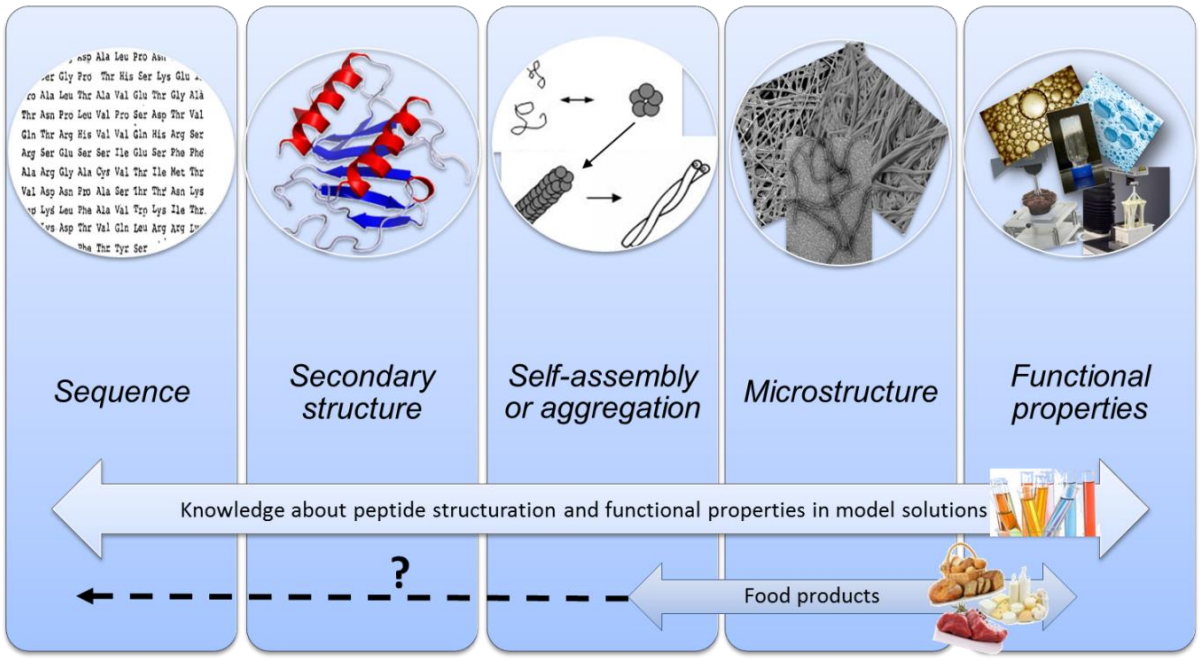

Figure 1. Graphical abstract of the bibliographic review.

This review was accepted in Food Hydrocolloids[1].

[1] Lacou, L., Léonil, J., Gagnaire, V., Functional properties of peptides: from single peptide solutions to a mixture of peptides in food products. Food Hydrocolloids 57 (2016) 187-199.

Partie I – Revue Bibliographique

La première partie de ce manuscrit de thèse présente les avancés scientifiques concernant la capacité des peptides à conférer des propriétés fonctionnelles particulières à diverses matrices, incluant les produits alimentaires (Figure 1). Nous nous sommes concentrés sur des systèmes s'étendant de solutions modèles, dans lesquelles les peptides sont rationnellement conçus pour orienter leur structure et former des hydrogels, aux mélanges de peptides dans des matrices alimentaires plus complexes. Dans le dernier cas, les peptides font partie intégrante des formulations alimentaires en raison de leur production *in situ* ou de leur ajout en tant qu'ingrédient. Nous avons focalisé nos exemples sur la capacité des peptides à moduler la texture des produits alimentaires, où les mélanges de peptides sont présents en tant qu'hydrolysats ayant des propriétés physico-chimiques diverses, et à moduler leurs propriétés fonctionnelles, incluant la solubilité, la gélification ainsi que leur pouvoir émulsionnant et leurs propriétés moussantes. Certaines tentatives d'établissement de relations entre les caractéristiques physico-chimiques et structurelles de peptides et leurs propriétés fonctionnelles ont été réalisées.

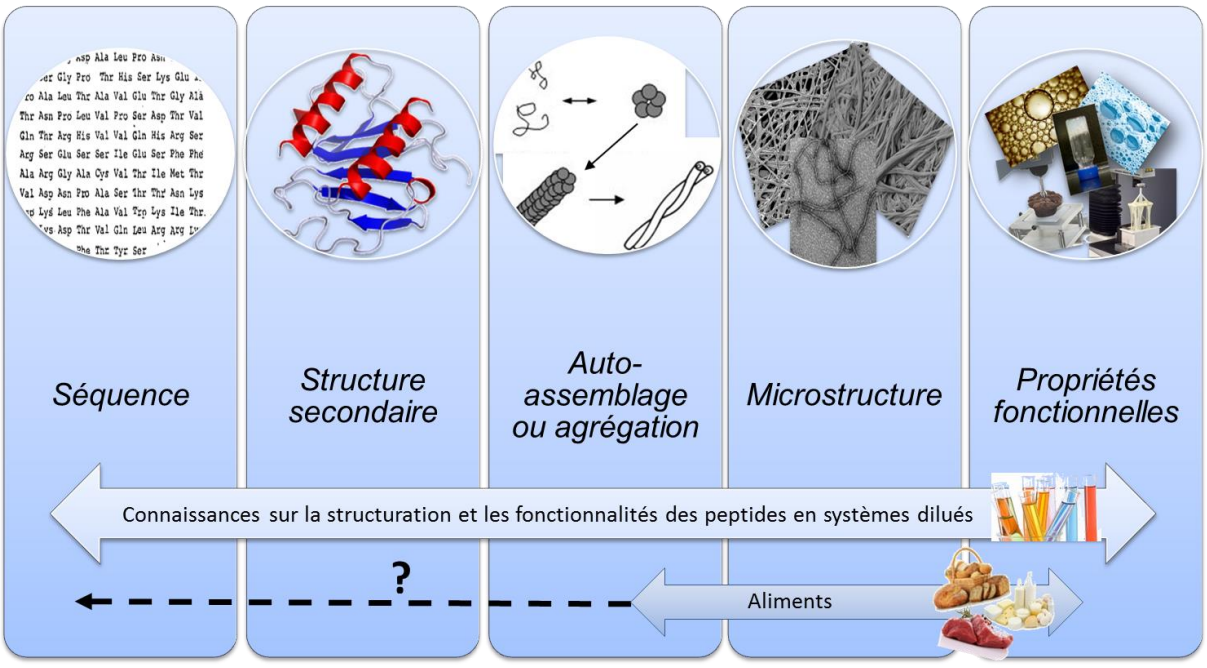

Figure 1. Résumé graphique de la revue bibliographique.

Cette revue est acceptée dans Food Hydrocolloids[2].

[2] Lacou, L., Léonil, J., Gagnaire, V., Functional properties of peptides: from single peptide solutions to a mixture of peptides in food products. Food Hydrocolloids 57 (2016) 187-199.

Food Hydrocolloids 57 (2016) 187–199

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Review

Functional properties of peptides: From single peptide solutions to a mixture of peptides in food products

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ABSTRACT

This review presents the advances made concerning the ability of peptides to bestow particular functional properties on various matrices including foods. We focus on systems ranging from model solutions in which peptides are rationally designed to orient their structure and to form hydrogels to mixtures of peptides in complex food matrices. In the latter case, peptides are an integral part of food formulations due to their production *in situ* or their addition as an ingredient. Examples of complex matrices such as food products, where mixes of peptides are present as hydrolysates with various physico-chemical properties, focus on the ability of peptides to modulate the texture of foods and their functional properties, including solubility, gelation and even emulsifying and foaming properties. Attempts have been made to establish relationships between the physico-chemical and structural characteristics of peptides and their functional properties.

Contents

1. Introduction

Food products have complex structures and variable compositions of macromolecules, including proteins, lipids and carbohydrates, as well as their derived products such as peptides, fatty acids, etc., that change throughout manufacturing and storage. Proteins and peptides largely contribute to the final texture,

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organoleptic properties and health-promoting activities of foods. Peptides can enhance emulsion or foam formation and stabilize them by preventing coalescence or flocculation. They can improve the solubility of proteins, increase the viscosity of a solution, and make gels under appropriate physico-chemical conditions (Caessens, De Jongh, Norde, & Gruppen, 1999; Kilara & Panyam, 2003; Saito, Ogasawara, Chikuni, & Shimizu, 1995). They are also an inherent part of the nutritional value of food through their contribution to the reduction of allergenicity and their various bioactivities, as shown in recent reviews (García, Puchalska, Esteve, & Marina, 2013; Moayedzadeh, Madadlou, & Khosrowshahi asl, 2015; Nongonierma & FitzGerald, 2015).

Due to their intrinsic physical and chemical characteristics, i.e., sequence, size, charge, structure and their interaction with each other and other molecules, peptides are able to form self-assemblies or aggregates and to thus develop new functional properties. Recent advances in this field show the ability of these peptides to form hydrogels, nanotubes and fibers under defined conditions. In these systems, the peptide sequence is tailored to satisfy specific needs (Boyle et al., 2012), the types of interactions involved, the supramolecular structures formed as well as the properties of the solutions or gels, are highly controlled. The aggregation capacity of proteins and peptides may be either enhanced or impaired when they are present in a heterogeneous and highly concentrated medium such as that of food products, in the presence of other molecules (polysaccharides, lipids and other proteins). The concept of molecule confinement, referred to as crowding, has recently been developed in order to understand the modulation of the interactions between peptides and proteins in high concentrations such as those encountered in cell cytosol, which can reach 300–400 g/l, and that can be found in food products.

It clearly appears that such new advances will help us address the question in this review of the extent to which peptides can be an important part of the final functional properties of food products and how this occurs. In this paper, we emphasize: (i) the progress that has been made and existing gaps in our knowledge in order to establish relationships between the structural and functional properties conferred by peptides in various matrices including food products; and (ii) how recent knowledge about the capacity of peptides to self-assemble or aggregate can help us learn more about assembly and functional properties of peptides in complex media such as food.

This review is divided into six sections. Section 1 is devoted to the production of peptides and the method of characterization; Section 2 deals with recent knowledge about peptide self-assembly and their implication in functional properties; Section 3 describes the role of peptides in functional properties when they are mixed with other peptides and proteins and with components other than proteins, i.e., in food products, the subject of Section 4; Section 5 focuses on the concept of crowding that has to be considered as another extrinsic factor capable of exerting deep changes in peptide functional properties under high concentration conditions; and, finally, Section 6 presents the conclusion.

Since much discussion about the definitions of "self-assembly" and "aggregation" exists in the literature, we will consider here that the self-assembly of peptides represents a spontaneous and reversible reaction in contrast to aggregation. In the case of self-assembly, non-covalent bonds such as hydrogen bonds, electrostatic and van der Waals interactions are involved in the peptide assembly (Bouhallab & Croguennec, 2014; Chen, 2005). Concerning aggregation, we will consider that it is an irreversible reaction involving covalent bonds such as disulfide bonds (Bouhallab & Croguennec, 2014; Bouhallab, Riaublanc, & Croguennec, 2011).

2. Production of single molecules or mixtures of peptides and the main methods of characterization

Peptides are chains of two to 100 successive amino acids (Bodanszky, 1988) and of higher amino acid residue numbers for proteins, which are connected to each other via covalent bonds between their amino group and their carboxyl group.

Peptides can be chemically synthesized as a single molecule or produced from the parent proteins by enzymatic hydrolysis as a mixture of peptides, referred to as hydrolysates. In the former case, synthesized peptides have a rationally-designed sequence and are well identified (Kyle, Aggeli, Ingham, & McPherson, 2009). The difficulty is the presence of biohazard products that result from synthesis. In the latter case, peptides can be produced in large amounts at a much lower cost. Hydrolysates are increasingly used in food formulations for their particular functional properties. The composition of the hydrolysate is governed by the enzyme specificity, the enzyme:protein ratio, the extent of hydrolysis and the physico-chemical conditions applied (Gauthier, Paquin, Pouliot, & Turgeon, 1993; Kristinsson & Rasco, 2000), as detailed below.

Hydrolysate composition first depends on the enzyme specificity, which determines the number and the size of the peptides produced (Darewicz, Dziuba, & Dziuba, 2006). Commercial enzymes with well-known specificities are available, the most common of which are trypsin, papain, pronase, pepsin, bromelain, alcalase and chymotrypsin (Kumagai, 2012). The *in silico* method based on the known specificity of enzymes now makes it possible to predict the type and the number of peptides that can be theoretically produced from proteins by taking the possible formation of intermediate and/or end products within the hydrolysate into account. The second key factor essential for hydrolysis concerns the protein used as substrate, its nature, its concentration and the extent of its denaturation. For example, the thermal denaturation of whey proteins improved the extent of their hydrolysis by exposing previously inaccessible amino acids in the native protein to cleavage and, as a result, changing the type and the number of peptides produced compared to the native protein (Tavano, 2013). The third parameter is linked to the conditions in which the hydrolysis is performed, i.e., the enzyme:substrate ratio, the pH, the ionic strength, the temperature and the reaction time that can impact the enzyme activity, the accessibility to substrate and eventually change the final composition of the hydrolysate (Amiza, Kong, & Faazaz, 2012; Neklyudov, Ivankin, & Berdutina, 2000; Panyam & Kilara, 1996; Yin et al., 2010).

Therefore, hydrolysate can contain mixtures of native proteins, peptides and amino acids in various amounts depending on the hydrolysis conditions used, which will have a subsequent impact on the final functional properties (Damrongsakkul, Ratanathammapan, Komolpis, & Tanthapanichakoon, 2008; Liu et al., 2014; Shahidi, Han, & Synowiecki, 1995).

Many different techniques are available to monitor and control the production of peptides and to provide additional information. The most frequently used to qualify a hydrolysate is the degree of hydrolysis of proteins (DH), which refers to the proportion of cleaved peptide bonds per total number of bonds existing in the protein, and which varies from 0 to 100% depending on the intact proteins and the complete conversion of protein into amino acids, respectively (Liceaga-Gesualdo & Li-Chan, 1999; Mahmoud, 1994; Panyam & Kilara, 1996). It is usually determined by monitoring the pH or by quantifying the free NH_2 groups because of the ease with which this can be done (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). Most of the time, the higher the DH is, the smaller and the more soluble the peptides will be (Adler-Nissen, 1976; Ghribi et al., 2015; Jamdar et al., 2010; Quaglia & Orban, 1987). However, it reflects neither the quantity of peptides present in the hydrolysate vs.

residual parent proteins nor the types of peptides present in the hydrolysate.

Several other methods exist and are also used. For example, electrophoresis is used to obtain the profile of the peptides according to their apparent molecular mass by SDS-PAGE, or both their charge and molecular mass by urea-PAGE. It also provides an overall qualitative profile of the residual proteins present in the hydrolysates and of their derived-peptides over 2000 Da, peptides with lower apparent molecular mass are sometimes able to diffuse out of the gel. Another method to separate proteins and peptides according to size is the size exclusion chromatographic method, but separation is not of high resolution. Other chromatographic methods are also used to selectively separate them according to their charges by ion exchange, or according to their hydrophobicity by reverse-phase.

Mass spectrometry (MS) is a tool of choice at this time, increasingly used to elucidate the mass profile and to identify peptides in hydrolysates and give their sequence inside the hydrolysate and the corresponding parent protein they are derived from (Aebersold & Mann, 2003). Depending on the sequence of peptides obtained, it is possible to calculate the physico-chemical and structural characteristics of peptides with several types of software available on ExPAsy.com (Lacou, Lê, Pezennec, & Gagnaire, 2015). Quantitative determination can be made by mass spectrometry using stable isotope tags (Bantscheff, Lemeer, Savitski, & Kuster, 2012). However, this quantification is only relative and based on a comparison of quantity of the same peptide present in two or more experimental conditions or by using an internal standard, but its choice must be compatible with several hundred peptides present within a hydrolysate. Identification of peptides by mass spectrometry is accurate over five amino acids in the sequence, and several chemical derivatization techniques have been developed in order to overcome the difficulty of assigning sequences for di-and tripeptides (Iwasaki et al., 2011; Santa, 2011). Nevertheless, it is not possible to detect all of the peptides since some of them suppress ionization, which makes their detection impossible. In some other cases, interference between some of the composites (oxides and carbides) and peptides makes it difficult to identify them in the hydrolysate.

In order to more finely characterize the peptides in hydrolysates, a combination of the above-mentioned methods must be undertaken, which is what has been done to varying degrees so far.

3. How can rationally-designed peptides that are able to self-assemble or aggregate change the functional properties of model systems?

In this part of the review, we focus on peptide self-assembly and the relationships between peptide structure and their functional properties, preferably in terms of hydrogel formation or their use as thickeners or gelling agents that can be provided by supramolecular structures such as nanotubes, fibrils or aggregates (Dickinson, 2015), rather than in terms of the production of bio-nanomaterials and surfactants used as stabilizers of emulsions and foams already reviewed by Reches and Gazit (2006) and Dexter and Middelberg (2008), respectively.

Peptides can spontaneously assemble or aggregate when the physico-chemical conditions of the solution, i.e., pH, ionic strength and temperature, become suitable. Even short peptides - from tripeptides to hexapeptides - were able to self-assemble and form fibers in a concentration-dependent manner (Hauser et al., 2011). These peptides have a peculiar amphiphilic structure with an aliphatic amino acid tail of decreasing hydrophobicity with aliphatic nonpolar amino acids (N-terminus) and a hydrophilic head group of acidic, neutral or basic nonaromatic polar amino acids (C-terminus). Fiber formation could be accelerated by changing the type of amino acid in the hydrophobic tail and by increasing the temperature, leading to the formation of a solid-like hydrogel (Hauser et al., 2011). Such a change in the sequence would enhance or not the attractive or repulsive interactions between peptides via changes in electrostatic and van der Waals interactions and even hydrogen and disulfide bonds (Loo, Zhang, & Hauser, 2012).

Hauser et al. (2011) hypothesized that the mechanism of fiber formation could be divided into three parts: (i) interaction between two monomers of peptides and the formation of antiparallel pairing with a random coil secondary structure; (ii) transient α-helical conformation of this peptide pair at a threshold peptide concentration (0.9 g L^{-1}), considered to be the critical step; and (iii) peptide pair assembly leading to fibers that are finally condensed into fibrils and form a hydrogel. Once the β-turn structure is formed, fibers remain irreversibly stable (Hauser et al., 2011).

Other examples showed that the way the amino acids are located in the sequence is a key driving force that influences the ability of peptides to self-assemble or to aggregate (Table 1). Hence, the dipeptide Phe–Phe, as well as its analogues with amine and carboxyl modification, are able to form ordered tubular structures at the nanometric scale, whereas diphenylglycine self-assembles into ordered nanospheres (Reches & Gazit, 2006). The tubular ones were shown to have remarkable chemical and mechanical properties (Reches & Gazit, 2006), whereas the nanospheres could be used to encapsulate ligands. Another interesting example is given by the β-lactoglobulin-derived peptide β-Lg(f1–8) for which the change in the amino acid residues present at both the N-terminal and the C-terminal extremities greatly modifies its capacity to self-assemble and to form a hydrogel (Guy & Voyer, 2012). The authors showed that an equilibrium has to be maintained between hydrophobic and charged amino acids in order to improve not only the peptide self-assembly but the gelation rate at neutral and basic pH as well. It actually appeared that β-sheet formation promotes peptide self-assembly (Guy & Voyer, 2012) that is differentially influenced according to the type of amino acid concerned. Thus, the presence of leucine at the N-terminal extremity clearly increased the β-sheet formation over the entire pH range (3–11), while substituting the C-terminal lysine with alanine only favored the β-sheet formation in strongly acidic or basic environments. However, knowledge of the peptide sequence, its secondary structure and the fiber morphologies observed by TEM is not necessarily an indication of the gelation capacity obtained.

Environmental conditions, namely pH, are therefore crucial to favor or not the acquisition of functional properties by peptides. Since peptides are polyelectrolytes, their charges are minimized at their pI, peptide–peptide interactions are favored over peptide-water interactions, and their solubility is generally low at this pH value (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Mahmoud, 1994). At pH values far from the pI, ionizable groups of peptides that are negatively or positively charged either promote electrostatic repulsions between peptides that favor their solubility or promote electrostatic attractions between peptides that favor macromolecular formation. The example of caseinomacropeptide (CMP), either glycated with sialic acid or not, well illustrates the impact of the acidic pH in gel formation (Farías, Martinez, & Pilosof, 2010). Gel formation was enhanced at acidic pH when both CMP and glycated CMP were present because of the low pKa of sialic acid that made the presence of local negative charges possible on the glycated CMP dimers that can interact with the main positive charge of the CMP dimers. The kinetics of the CMP self-assembly was characterized in the pH range of 6.5 to 3. The first stage consisted of self-assembly by hydrophobic interactions to form dimers for both types of CMP, whereas the second stage involved

Table 1
Relationships between the sequence of rationally-designed peptides, their secondary structure and their supramolecular structure.

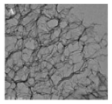
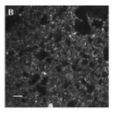
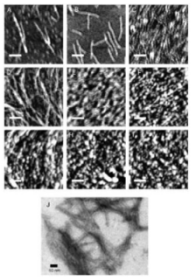
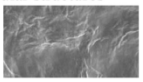
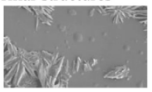
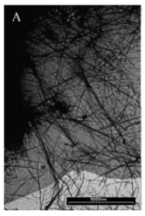
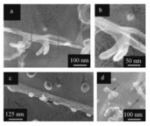
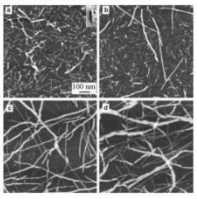
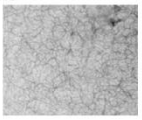
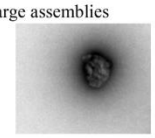
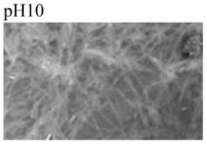
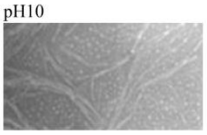
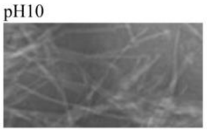
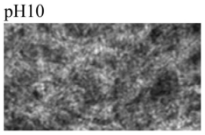
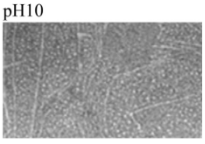
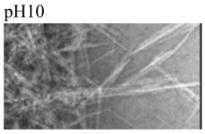
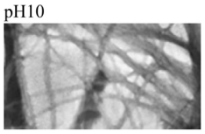
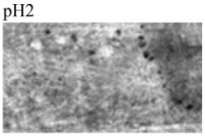
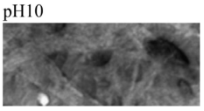
Peptide	Secondary structure	Supramolecular structure	Functional property	References
K170L30	α- helix		Hydrogel	Deming et al., 2005
K160L40	α-helix		Hydrogel	
AEAEAKAKAEAEAKAK			–	Hong et al., 2005
NH_2-((D-1-Nal)-(D-1-Nal)-COOH	Anti-parallel β-sheets + α-helix motifs	Tubular structures 	–	Reches et Gazit, 2006
NH_2-(p-nitro-Phe)-(p-nitro-Phe)-COOH	Anti-parallel β -sheets	Fibrillar structures 	–	
KI**K**ALKYEIAALEQEIAALEQKIAALKQ	α-helical coiled coil structure	Fibers 	–	Gribbon et al., 2008
KI**A**ALKYEIAALEQEIAALEQKIAALKQ	α-helical coiled coil structure	No fiber	–	
KI**E**ALKYEIAALEQEIAALEQKIAALKQ	α-helical coiled coil structure	No fiber	–	
KI**N**ALKYEIAALEQEIAALEQKIAALKQ	α-helical coiled coil structure	No fiber	–	
KIKALK**Q**EIAALEQEIAALEQKIAALKQ	α-helical coiled coil structure	Fiber thinner than KIKALKYEIAALEQEIAALEQKIAALKQ	–	
GGGGGGDD	–	Nanotubes and nanovesicules 	–	Zhao, Pan, & Lu, 2008
Ac-FKFEEKEF-$CONH_2$	Evolution according to the time of solubilization:	Evolution according to the time of solubilization:	–	

Table 1 (*continued*)

Peptide	Secondary structure	Supramolecular structure	Functional property	References
	-a few minutes after the preparation of the peptide solution: β-sheets -in the later stage: anti-parallel β-sheet.	-a few minutes after the preparation of the peptide solution: left side -in the later stage: fibrillar structures and networks 		
$(VGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPG)_9$	–	Gels such as coacervates	–	Kyle, Aggeli, Ingham, & McPherson, 2009
QQRFQWQFEQQ	β-sheet	Fibrils	–	
QQRFEWEFEQQ	β-sheet	Fibrils	–	
RADARADARADARADAE	–	Nanofibres	–	
EVHHQKLVFFAEDVG	–	Amyloid fibrils	–	
AAVVLLLWEE	–	Vesicule	–	
EIKALEQETAALKQKIAWLKQ –– **GN** –– KIKALQEIAALKQEIAYLEQ	Predominantly α-helical Decrease of the helicities of the peptides	Fibers 	–	Boyle et al., 2012
EIKALEQETAALKQKIAWLKQ –**GNGN** – KIKALKQEIAALKQEIAYLEQ		Large assemblies 	–	
LIVTQTMK	β-sheet at basic pH	pH10 	Gel at pH 10	Guy & Voyer, 2012
GIVTQTMK	"Random coil" over a wide range of pH = >self-assembling inhibited	pH10 	No gel over wide pH range	
LLIVTQTMK	β-sheet over wide range of pH	pH10 	Gel at pH < 6	

(*continued on next page*)

Table 1 (*continued*)

Peptide	Secondary structure	Supramolecular structure	Functional property	References
LIVTQTM**A**	β-sheet at low and strong pH	pH10 	Gel over wide pH range	
LIVTQTK**K**	No β-sheet = >electrostatic repulsions	pH10 	No gel over wide pH range	
LLIVTQTK**K**	β-sheet at basic pH	pH10 	Gel at pH 10	
LIVTQTM**R**	β-sheet at pH > 10	pH10 	Gel at pH 10	
LIVTQTM**E**	β-sheet at pH 2	pH2 	Gel at pH < 6	
AcLIVTQTMK	β-sheet over wide range of pH	pH10 	Gel at pH > 6	

electrostatic interactions between the dimers produced in the first stage (Farías et al., 2010).

The aggregation and gelation behavior of peptides can be changed or even reinforced when they are mixed with residual native protein (Kosters, Wierenga, de Vries, & Gruppen, 2013) through their amphipathic nature or their charges. Thus, two β-lactoglobulin-derived peptides, β-Lg(f135–158) and β-Lg(f135–162)-SH, were able to bind with each other and aggregate as well as with the native β-lactoglobulin in a concentration-dependent manner and at an optimum concentration of 5 mg/ml at pH 7 for both, whereas no interaction with peptides occurs below the pI of β-lactoglobulin (del Olmo, Calzada, Gaya, & Nuñez, 2013; Kosters et al., 2013). The peptide sequence also plays a key role in gel strength since the peptide β-Lg(f135–162)-SH with a free sulfhydryl group induced a 10-fold increase in the amount of aggregation of β-lactoglobulin compared to the β-lactoglobulin alone upon heating, and an increase in gel strength in contrast to the peptide β-Lg(f135–158) for which a decrease in gel strength was observed. The question still remains as to which part of the protein both peptides interacted with, but sulfhydryl bonding would be a likely choice. Electrostatic interactions were the basis of hydrogels that spontaneously formed with two undecapeptides designed to have opposite charges and a palindromic sequence (acetyl-KWKAKAKAKWK-amide and acetyl-EWEAEAEAEWE-amide) (Hyland, Taraban, Feng, Hammouda, & Yu, 2012). In order to reinforce electrostatic interactions and in turn the viscoelasticity of the gel, these peptides were mixed with a network of polysaccharides of various sizes and charges composed of chitosan (50–190 kDa, positively charged), alginate (350–450 kDa, negatively charged) and chondroitin (~20 kDa, negatively charged). The resulting hydrogels formed were more resistant to deformation compared to those obtained from the peptides and the polysaccharides alone (Hyland et al., 2012). The peptides changed the morphology of the polysaccharide network, rendering the fibers structurally closer to those formed in pure peptide hydrogel, but with less stiff gels because the mesh size of the mixed hydrogel was greatly increased.

Finally, a complex mixture of peptides is also able to induce gel formation, as in the case of α-lactalbumin-derived peptides (Ipsen & Otte, 2007) through the initial nanotube formation. Thus, a minimum concentration of above 30 g L^{-1} of α-lactalbumin is

required to form nanotubes after hydrolysis by *Bacillus licheniformis* protease. Under these conditions, a mixture of peptides ranging between 11.2 and 11.6 kDa was obtained, providing the major building block of the nanotubes. At concentrations below 30 g L^{-1}, the hydrolysis kinetics leads to the formation of smaller peptides, and only linear fibrils were observed from peptides of 8 kDa produced. Many factors, including the concentration, physico-chemical and structural characteristics of the peptides involved, affect the type of supramolecular structure that can be formed and have an impact on their subsequent functional properties.

Peptides are also a prerequisite to induce formation of heat-induced β-lactoglobulin nanofibrils at pH 2 (Akkermans, Venema, van de Goot, & Gruppen, et al., 2008). As peptides formed under these physico-chemical conditions mainly contained Asp at the N terminal end of the peptides, enzymatic hydrolysis of β-lactoglobulin by AspN endoproteinase was found efficient to produce peptides that were able to form nanofibrils after incubation at pH 2 (Akkermans, Venema, Goot, Boom, & Linden, 2008). This two-step fibrillisation was supposed to be entropy driven and reversible for the first step whereas the second one would render the phenomenon irreversible through collective inter-β sheet formation (van der Linden, 2012). Other proteases trypsin, protease A, pepsin and protease M (Gao, Xu, Ju, & Zhao, 2013) and Corolase N (Mohammadian & Madadlou, 2016) were used to selectively hydrolyze whey proteins. All were able to form nanofibrils at acid pH, having various morphologies. Trypsin hydrolysates led to a faster formation of fibrils that look like those obtained with the native whey proteins compared to the other proteases used (Gao et al., 2013). Capabilities of Corolase N peptides to induce foam overrun and foam stability (Mohammadian & Madadlou, 2016) was lower than that observed with the fibrils produced under heating and pH 2 incubation (Oboroceanu, Wang, Magner, & Auty, 2014).

4. How are mixtures of peptides able to change the functional properties of complex systems?

Through the previous examples, we illustrated that interactions between one or two peptides with each other or with other molecules (proteins, polysaccharides) were able to provide particular functional properties by choosing suitable conditions of pH, ionic strength and temperature. The question then arises as to whether a mixture of peptides produced through enzymatic hydrolysis is still able to maintain these properties or to reinforce or lose them in model systems, including complex systems involving foods. In that sense, the ability to form nanotubes via α-lactalbumin hydrolysate (Ipsen & Otte, 2007) and not via β-lactoglobulin hydrolysate (Ipsen, Otte, & Qvist, 2001) although both systems lead to gel formation, illustrates the complexity that researchers have to face in order to understand how groups of peptides compete with each other or interact together to implement specific interactions and generate functional properties.

4.1. Functional properties of hydrolysates

4.1.1. Solubility and water-holding capacity

Among the functional properties resulting from peptides, solubility is one of the most important. In most cases, the degradation of proteins to smaller peptides leads to more soluble products, notably at a greater extent of hydrolysis over a wide range of pH (Chobert, Bertrand-Harb, & Nicolas, 1988; Gbogouri, Linder, Fanni, & Parmentier, 2004; Klompong et al., 2007; Linder, Fanni, & Parmentier, 1996). However, not all peptides can have good solubility and solvation ability since it depends on the overall hydrophobicity and the charges of the peptides, which is not feasibly predictable in a hydrolysate containing several hundred peptides. For example (Shahidi et al., 1995), showed that Alcalase was more efficient than Neutrase for increasing the solubility of capelin protein. The increase in solubility is due to the exposure of ionizable groups (amino and carboxyl groups) to the solvent revealed by an increase in zeta potential (Liu et al., 2014), which leads to repulsive electrostatic interactions between peptides as well as to hydrogen bond formation between peptides and water molecules (Mahmoud, 1994; Neklyudov et al., 2000; Tavano, 2013). In the above-mentioned examples, only the extent of hydrolysis was used as a criterion to optimize the functional properties. However, the absence of knowledge about the peptide distribution implied in the functional properties makes it impossible to predict the type of interactions required and which extrinsic and intrinsic factors of hydrolysis should be emphasized and, finally, how to intentionally orient the production of interesting peptides.

4.1.2. Emulsifying and foaming properties

Peptides are able to increase foam and emulsion formation and stability compared to native proteins, up to a limited extent of hydrolysis beyond which these capabilities decrease (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007; Hrckova, Rusnakova, & Zemanovic, 2002; Klompong et al., 2007; Kristinsson & Rasco, 2000; Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008). The benefits of peptides vs. proteins lie (i) in their capability to diffuse more rapidly to the interface through their enhanced solubility and, in turn, to adsorb more rapidly at the air/water or lipid/water interface (Davis, Doucet, & Foegeding, 2005); (ii) in the fact that hydrophobic domains initially buried in the proteins are already available in the solution and do not require prior partial denaturation to recover the droplet surface for emulsion or bubbles for foam; and (iii) in the presence of sulfhydryl groups in certain peptides that can form disulfide bonds able to stabilize the interface and to interconnect peptides covalently. Hydrolysates with a low extent of hydrolysis therefore form a more stable emulsion in contrast to hydrolysate with a greater extent of hydrolysis in which peptides are too small to sufficiently reduce the interfacial tension to ensure optimal emulsifying capabilities and to form an interfacial viscoelastic film at the interface to prevent coalescence (Pacheco-Aguilar et al., 2008; van der Ven, Gruppen, de Bont, & Voragen, 2002). Moreover, when environmental conditions change, the surface hydrophobicity of peptides present in the hydrolysate may be modified and may displace other peptides or proteins at the interface, consequently modifying the initial emulsifying or foaming properties like other small surfactant molecules (i.e., tweens, SDS) with proteins (Lam & Nickerson, 2013). The destabilization of emulsion or foam is particularly evident when electrostatic repulsions are reduced, i.e., close to the pI or in the presence of high ionic strength or when the hydrolysate is heat-treated and favors excessive hydrophobic bonding and aggregations that can have dramatic consequences depending on the peptide composition of the hydrolysates in terms of coalescence and creaming of the emulsion (Lam & Nickerson, 2013). This is generally concomitant with a weak solubility of the hydrolysates under the above-mentioned conditions (Klomklao, Benjakul, & Kishimura, 2013; Klompong et al., 2007).

Improved emulsifying properties have been attributed to a particular peptide or group of peptides in the hydrolysate from peptides arising from β-casein and from β-lactoglobulin. Thus, the amphiphilic β-casein-derived peptide β-CN(f114–169) purified from tryptic hydrolysis of β-casein present at the interfacial area of the emulsion droplets showed remarkable surface activity and was able to form elastic film at the triolein-water interface (Girardet et al., 2000). Two peptides identified from β-lactoglobulin hydrolysis by trypsin β-Lg(f21–40) and β-Lg(f41–60) were shown to have good interfacial properties due to the clustering of hydrophilic and

hydrophobic peptides into distinct zones peptides (Turgeon, Gauthier, Mollé, & Léonil, 1992). The peptide β-Lg(f41–60) was identified as also participating in the aggregation process with peptides β-Lg(f 1–8) and β-Lg(f 15–20) (Groleau, Morin, Gauthier, & Pouliot, 2003). Other types of peptides from β-lactoglobulin produced after hydrolysis by *B. licheniformis* protease revealed the ability to aggregate with molecular weights ranging from 1700 to 4000 Da. Four of them were identified: β-Lg(f75–89), β-Lg(f75–108), β-Lg(f135–157) and β-Lg(f135–158), the latter being the most significant one (Otte, Lomholt, Halkier, & Qvist, 2000).

4.1.3. Gelling properties

Another well-studied functionality is the gelation capacity of peptides derived from: (i) whey proteins (Ju, Otte, Madsen, & Qvist, 1995); (ii) meat (Damrongsakkul et al., 2008); (iii) wheat (Anjum et al., 2007); (iv) fish (Amiza, Ow, & Faazaz, 2013); and (v) soy (Hrckova et al., 2002). These experiments demonstrated that only a limited hydrolysis improved the gelling properties of the products, whereas the greater extent of hydrolysis tended to decrease the viscosity of the solution (Diniz & Martin, 1997). This can be attributed to protein–protein interactions that are stronger than both peptide-water and peptide–peptide interactions. Nonetheless, exceptions were observed: (i) when the peptide chains maintained the appropriate size to successfully form a network with enhanced viscosity and further a gel according to specific environmental parameters (Damrongsakkul et al., 2008; Ju et al., 1995); and (ii) when peptides are highly concentrated, as in the case of whey protein isolate at 20% (w/v) hydrolyzed by Alcalase (Doucet, Gauthier, Otter, & Foegeding, 2003). Heat-treatment of hydrolysate can also modify the rheological properties, resulting in the doubling of the viscosity of the solution without leading to the formation of a gel (Kotlar, Ponce, & Roura, 2013).

The presence of calcium in the hydrolysate could also act as a stabilizing agent through the formation of Ca^{2+} bridges between peptides, thus enhancing gelation, which is rarely taken into account. This was shown with the partial hydrolysis of α-lactalbumin by the proteinase of *B. licheniformis* (Ipsen et al., 2001).

pH changes are also crucial for modifying gelling properties that favor the occurrence of various interactions depending on the charges exhibited at the acidic or basic pH scale. At 80 °C and pH 3, the partial hydrolysis of whey proteins by Neutrase at a DH < 3.4% induced a stronger gel than the gel produced by native whey proteins (Ju et al., 1995). At the pI of sodium caseinate, the gel obtained from Protamex hydrolysates heated at 80 °C and with a DH < 5.3% had a higher apparent viscosity than unheated sodium caseinate (Flanagan & FitzGerald, 2002). However, when the production of peptides induced an increase in charge repulsion between peptides and a decrease in protein–protein, protein–peptide and peptide–peptide interactions, the functional properties were negatively affected, resulting in (i) a reduction of gel-forming ability (Lamsal, Jung, & Johnson, 2007), or (ii) a gel weaker than the gel produced by native proteins (Banach, Lin, & Lamsal, 2013). However, the improvement or decrease of the gelation properties could not be attributed to either the peptides or to the interactions between peptides and native proteins alone (Giardina, Pelizzola, Avalli, Iametti, & Cattaneo, 2004).

Regardless of the functional properties studied, it is still difficult and sometimes even impossible to conclude that changes in functional properties are only due to either (i) the nature of the peptides produced and the way they interact together, or (ii) to the presence of both residual native proteins and peptides and to their respective interactions in the hydrolysate. Experiments with a hydrolysate containing only peptides compared to a hydrolysate containing both peptides and native proteins would provide useful information. In that sense, the absence of unhydrolyzed β-lactoglobulin protein in hydrolysates produced after the action of Alcalase, trypsin and pepsin revealed that the foaming and interfacial properties of the hydrolysates were only due to the presence of peptides (Davis et al., 2005). Tsumura et al. (2005) set up an interesting strategy to produce hydrolysate with an equal degree of hydrolysis of soy protein isolate (SPI) in which either the glycinin or the β-conglycinin fractions were selectively hydrolyzed by pepsin and papain, respectively, under definite soy protein denaturation conditions. Comparing various functional properties of both hydrolysates vs. the control SPI, the authors clearly showed that β-conglycinin was implied in the apparent viscosity and the emulsifying property, while glycinin together with the presence of peptides increased the gel-forming ability in the presence of a meat protein. In agreement with the above-mentioned β-lactoglobulin hydrolysate, the foaming properties were greatly increased in both hydrolysates compared to the SPI control, highlighting the role of the peptides more than that of the proteins. On the basis of these two examples that are rare in the literature, the key role of one or the other protein in establishing certain functional properties can be emphasized, as well as the extent to which the peptides play a strong role, especially in acquiring foaming properties.

4.2. Advances achieved to link peptides to their functional properties in food products

Peptides in foods are either directly added to the food product as an ingredient, as in the case of prepared sauces, processed meats, beverages and bakery products, or they are produced *in situ* by hydrolysis of the proteins by various proteolytic enzymes present in fermented products such as cheese, yogurt and dry-cured ham. Two levels of complexity should be taken into account to understand how and to what extent peptides can impact the functional properties of foods. First, food products have a complex structure and distribution of various macromolecules, including proteins, lipids and polysaccharides, as well as their derived products, and other components such as minerals, organic acids, etc. with which peptides can interact and, in turn, adversely affect or not the functional properties. Second, environmental conditions vary throughout the manufacturing process and storage, depending on whether the products are formulated or fermented.

Thanks to the high diversity of hydrolysates that can be produced, it should be easier to rationally adapt the type of hydrolysate in formulated products to obtain the required functional properties. Thus, the hydrolysates containing small and highly soluble peptides that are able to easily diffuse in a matrix are actually suitable for preparing sauces, juices and other beverages. Notably, when the protein hydrolysates are acid-soluble, peptides may thus contribute to the fortification of acidic beverages, including caramelized soft drinks and cola-based beverages (Neklyudov et al., 2000). Hydrolysates with enhanced whippability could be used as egg white substitutes, while those with low viscosity and high gelling ability could be preferable for meat products, as previously shown for soy protein hydrolysates (Tsumura et al., 2005).

To have an acceptable texture for consumers, meat must remain firm during cooking to reduce loss when cooked. Barbut (2007) reported that whey protein hydrolysates made it possible (i) to lower the cooking loss compared to a control meat, (ii) to favor interactions between hydrolysates and meat, and (iii) to increase the water-binding capacity with a specific degree of hydrolysis beyond which adverse effects were observed. The softness and pastiness of hams have been associated with intense proteolysis. It was demonstrated that a specific peptide profile is linked to certain texture properties. At nine months of dry-curing, a ham containing higher concentrations of hydrophobic peptides presented both a weaker shear force and a weaker cutting force than a ham

containing a higher concentration of hydrophilic peptides (del Olmo et al., 2013).

Most studies conducted so far have highlighted the amphiphilic nature of the peptides for most of the functional properties in model hydrolysates, even if the peptides were small and the distinct hydrophobic and hydrophilic zones reduced. Size should be the most important criterion in food in order to make the interactions between peptides and between peptides and other components that are present in food more effective. Wang, Zhao, and Jiang (2007) reported that molecular mass distribution of peptides in gluten hydrolysate with a molecular mass of more than 50 kDa improved the viscoelastic characteristics of wheat dough and decreased the crumb firmness of bread compared to bread produced by gluten protein (Wang et al., 2007). However, the effective capacity of hydrolysates observed under *in vitro* conditions is questionable in food. Crowley et al. (2002) had a disappointing experience with caseinate hydrolysate used as a functional ingredient in doughs and baking applications. Under *in vitro* conditions, one of the casein hydrolysates produced with Neutrase exhibited low drainage values at all of the pH tested, high foam expansion and efficient water-binding activity, compatible with the required functional properties of bread. Nevertheless, none of these properties were transferred to the bread, probably due to unexpected interactions between casein-derived peptides and wheat flour components.

In yogurt, texture is usually fortified by adding milk powder to enrich the final protein content. Depending on the DH of the proteins used and the concentration of the peptides added to yogurt, the addition of hydrolysates can result in various types of textural properties. In some cases, they can decrease both its viscosity and granularity (Sodini, Lucas, Oliveira, Remeuf, & Corrieu, 2002; Sodini, Lucas, Tissier, & Corrieu, 2005). In other cases, they can improve adhesiveness, beneficial to thickness and stability in yogurt, notably due to peptides lower than 1 kDa compared to larger ones present in hydrolysates obtained from lower DH (Zhao, Wang, Zhao, Jiang, & Chun, 2006). They can also have no effect whatsoever (Dave & Shah, 1998) in terms of firmness, elasticity, cohesiveness, chewiness and resilience (Zhao et al., 2006). The differences between these studies can be explained by (i) differences between enzyme specificity used to produce hydrolysates and, consequently, the size and physico-chemical characteristics of the peptides present, (ii) the bacterial strains used, and (iii) differences between the manufacturing conditions of the yogurt. The question thus arises as to whether the lactic acid bacteria strains used in these studies were able or not to produce exopolysaccharides, which could strongly induce changes in both viscosity and firmness in yogurt, regardless of the peptides present. Additionally, peptides could also have indirectly acted on the texture since they can be used as nutrients for bacterial growth, increasing the acidification rate and decreasing the fermentation time of yogurt (Zhao et al., 2006). This consumption of peptides in fermented products (dairy or meat) could therefore impede the overall capability of incorporated peptides to change the functional properties at the same level as the one previously determined under *in vitro* conditions. The initial distribution of peptides would not be the same since the bacteria can selectively import some peptides within the bacterial cells and/or the final peptide concentration would be decreased enough to lose some degree of its initial capability.

Among the food products in which peptides are directly produced, cheeses allow greater insights into the relationship between peptides and functional properties, although this knowledge is not yet complete. This aspect will be dealt with in more detail below. Proteolysis is indeed a continuous process during cheese ripening. The role of peptides has been explored in terms of their textural, organoleptic and health aspects for decades, with considerable progress. Former research primarily dealt with the way that peptides influence textural and techno-functional properties such as melting and stretching when cheeses are heated. Obviously, the final functional properties of a cheese are also dependent on other components such as fat, but we will focus mainly on peptides here. The extent of proteolysis influences the textural properties of cheese during aging because it modifies the nature, the number and the strength of casein interactions and involves proteinases and peptidases from different sources: milk (plasmin), coagulants added during processing (chymosin) and microorganisms (lactic acid bacteria and other ripening flora) (Lucey, Johnson, & Horne, 2003). The composition of peptides in various types of cheeses has been extensively characterized and numerous peptides have been identified by tandem mass spectrometry in Cheddar (Singh, Fox, & Healy, 1995; Singh, Fox, & Healy, 1997), in Grana Padano (Fernández, Singh, & Fox, 1998; Ferranti et al., 1997), in Emmental (Gagnaire, Mollé, Herrouin, & Léonil, 2001), in Ragusano (Gagnaire et al., 2011) and in Serra (Sousa & Malcata, 1998), and quantified for some of them as well. The first step of processing is hydrolytic and has been extensively studied and reviewed (Upadhyay, McSweeney, Magboul, & Fox, 2004). It induces the gelation of caseins present in milk, forming supramolecular structures known as casein micelles. Specific hydrolysis by chymosin of the Phe_{105}-Met_{106} bond of κ-casein, casein mainly located at the micellar surface, removes its hydrophilic C-terminal part (caseinomacropeptide or CMP) that interacts well with water and that protrudes 5–10 nm from the surface of the micelle. The hydrophobic counterpart of the casein micelles is then destabilized and a smooth gel is formed (Dalgleish, 2011). Chymosin can act on the other types of caseins such as α_{s1}-casein, whose proportion is associated with hardness, adhesiveness, fracturability and elasticity in various cheeses and notably in Cheddar (Borsting et al., 2012; Brickley, Auty, Piraino, & McSweeney, 2007; Creamer & Olson, 1982). When the peptide α_{s1}-(f24–199), also referred to as α_{s1}-I, is produced after chymosin action, a softening of the cheese is observed because this hydrophilic peptide can interact with water and trap it (Hynes, Delacroix-Buchet, & Zalazar, 2001). This peptide is used as a marker of softening in various types of cheeses such as soft cheeses (Camembert), semi-hard and hard-type cheeses (Cheddar, Raclette, Emmental). As an added protease, changes in chymosin specificities have been used to vary proteolysis and, in turn, cheese texture. Thus, cheeses produced with bovine chymosin are softer, smoother, and have a more cohesive texture than cheeses made with camel chymosin. Moreover, a smooth coating was observed on cheese produced by bovine chymosin (Bansal et al., 2009). Borsting et al. (2012) confirmed these results and added that α_{s1}-casein hydrolysis and β-casein hydrolysis of Cheddar with bovine chymosin leads to a higher amount of the following peptides: β-CN(f193–209), α_{s1}-CN(f24–199), α_{s1}-CN(f1–9), α_{s1}-CN(f1–13), α_{s1}-CN(f1–14), α_{s1}-CN(f1–17), α_{s1}-CN(f10–16) and α_{s1}-CN(f17–23). The last six peptides were produced through the hydrolysis of the N-terminal part of the α_{s1}-casein by the cell envelope proteases of lactic acid bacteria used as starter. These proteases with large substrate specificities as well as numerous peptidases that are released after the death of bacteria in the cheese during ripening are able to give a specific peptide fingerprint per type of cheese (Sadat-Mekmene et al., 2013). These bacterial proteolytic enzymes are another link in the casein chain hydrolysis throughout ripening that can impact the final functional properties of cheeses. Differences between the proteases of *Lactobacillus delbrueckii* spp. *bulgaricus* and *Lactobacillus helveticus* strains were shown to influence functional properties such as hardness, cohesiveness, melting strength and even stretching quality (Oommen, McMahon, Oberg, Broadbent, & Strickland, 2002). Additionally, soluble peptides derived from the

cleavage of α_{s1}-CN(f1–23) by different strains of *Lactobacilli* positively correlate with meltability and the stretching characteristics of cheeses made with those strains. Cheeses made with *Lactobacilli* were both more hydrolyzed and softer than the control cheese made using *Streptococci* alone (Oommen et al., 2002). Richoux, Aubert, Roset, and Kerjean (2009) and, more recently, Sadat-Mekmene et al. (2013) confirmed these results. They showed that *L. helveticus* led to good stretchability and that *L. helveticus* ITGLH77 (containing prtH3 protease), in particular, was a key factor in imparting stretchability to Swiss-type cheeses. The number of peptides identified by MS/MS containing more than 20 AA was 1.3 times higher in ITGLH77 cheeses, and the peptides containing more than 20 amino acid residues arising from α_{s1}-and α_{s2}-caseins were only present in ITGLH77 cheeses. These experiments made it possible to assume that peptides with more than 20 amino acids, preferably hydrophobic, increased the elasticity of cheeses and improved their viscoelastic properties under cooking conditions. It was therefore assumed that hydrophobic peptides were involved in the formation of fibers via hydrophobic bonds between native caseins and peptides (Richoux et al., 2009). More recently, an *in silico* method to establish relationships between a techno-functionality property of cheese and a peptide profile was published by our team and showed that not only the size of peptides but their physico-chemical and structural characteristics as well are involved in cheese stretchability (Lacou et al., 2015).

The reduction in elasticity and the decrease in stress at fracture observed in many cheeses are mainly due to a decrease in interactions between native caseins (Creamer & Olson, 1982; Richoux et al., 2009). During ripening, the stress at fracture, the strain at fracture and the deformability modulus decrease in cheeses such as Feta. Strain at fracture and the deformability modulus are low when the number of hydrolyzed bonds is high (Borsting et al., 2012; Hou, Hannon, McSweeney, Beresford, & Guinee, 2014; Wium, Kristiansen, & Qvist, 1998). The higher the level of proteolysis is, the higher the cheese meltability will be. However, excessive proteolysis in cheese is generally associated with defects in texture such as splits and cracks of the cheese paste (Brickley et al., 2007; Giardina et al., 2004). Therefore, the challenge is to strike the right balance between a desirable conversion of proteins into peptides and the production of desired peptides, in order to be able to orient peptide production and to enhance functional properties as much as possible (Fig. 1). This would provide the opportunity to adapt cheese production to the required functionality and to provide clues for other fermented products.

5. The concept of crowded media as a new way to approach peptide assembly and functional properties in complex systems

The concept of "crowded solutions" recently emerged to study the effect of confined environments on molecule self-assembly and aggregation. At high concentrations of more than 300 g/kg, macromolecules take up a large proportion of the volume of the solution, which reduces the volume of solvent that is available for other macromolecules. This excluded volume effect increases the effective concentration of macromolecules (increasing their chemical activity), which then alters the way they interact together and the enzymatic reactions that can take place. This concept, initially applied in the area of pharmaceutical research to understand cellular phenomena (Saluja & Kalonia, 2008) could also be extended to food. The crowded environment in food may actually affect protein and peptide stability and aggregation, considering that they are present in a heterogeneous and highly concentrated medium. Such an approach will help provide insights into how proteins and peptides can interact and form supramolecular structures within food products or how peptides, through their increase in concentration, can impede protein interactions and can provoke the opposite reaction between *in vitro* hydrolysates and peptide reincorporation in food, depending on the concentration used.

The crowded environment is experimentally produced in the presence of a crowding agent (dextran 70, PEG, Ficoll), or macromolecules such as proteins or peptides at elevated concentrations (example: > 80 g L^{-1} for bovine serum albumin, Rivas, Fernandez, & Minton, 1999) that are largely encountered in food products such as cheeses, meat or at the interface of bubbles in foam, for example. In such crowded environments, intermolecular interactions between protein molecules and protein rheological behavior are extensively modified (Saluja & Kalonia, 2008) and can lead to ordered polypeptide aggregates such as amyloid fibrils (White, Buell, Knowles, Welland, & Dobson, 2010). Crowding also impacts the solvation of proteins and peptides, which has a considerable impact on the aggregation of peptides as well (Rivera, Straub, & Thirumalai, 2009). Thus, this can be considered as a new extrinsic parameter to be taken into account in food, in addition to temperature, pH and ionic strength. The aggregation of the peptide hIAPP is a good example. The peptide hIAPP is a 37-amino-acid peptide that possesses a hydrophobic core and that is surrounded by polar uncharged residues. This peptide forms a stable β-sheet via interactions between residues of the hydrophobic core (residue F23GAIL27) at low and at high concentrations. Under confined conditions, the peptide hIAPP is more likely to form β-sheet nucleation than the peptides with a polar N-terminus and a nonpolar C-terminus. The crowded environment results in decreasing solvation and increasing peptide aggregation due to the reinforcement of their secondary structure. Hydrophobic interactions are the driving forces involved in the association of the β-sheet to form fibers.

Confinement appears to be sufficient to induce a change in the viscosity of the aqueous solution without changing the composition and the temperature of the solvent (Pavone, Crescenzi, Tancredi, & Temussi, 2002). At high concentrations of proteins or peptides, macromolecular crowding increases the interactions among themselves and changes the physico-chemical properties of the environment such as the local viscosity (Magno, Caflisch, & Pellarin, 2010; Munishkina, Cooper, Uversky, & Fink, 2004; Pastore, Salvadori, & Temussi, 2007). Thus, gelation was observed during extensive hydrolysis of whey protein isolate (WPI) with Alcalase 2.4L at high solid content (20% w/v) (Doucet, Gauthier, & Foegeding, 2001). This phenomenon was unexpected and the types of interactions subsequently studied by Doucet et al. (2003) showed that the gelation was mainly driven by hydrophobic interactions, whereas hydrogen bonding and electrostatic interactions played a minor role. Identification of peptides will help us to understand which peptides mainly contributed to the gelation and how. Such a gelation, which was stable over a wide pH and temperature range, has points in common with another reaction, known as plastein as shown by Doucet et al. (2003), in which an enzymatic synthesis can occur at a concentration of 30–50% total solids to form a high-molecular-weight protein-like substance by transpeptidation and condensation (Watanabe & Arai, 1992; Yamashita, Arai, Kokubo, Aso, & Fujimaki, 1974).

Even short peptides, dictated by their charges, can have strong interactions with proteins under confined environments (Wang & Woodbury, 2014). This can explain why they can have adverse effects in food, as previously shown. Moreover, as a multi-component system, more or less compartmentalized food would be a subject of choice to transfer the concept of crowded media to help us understand the interactions between proteins and peptides.

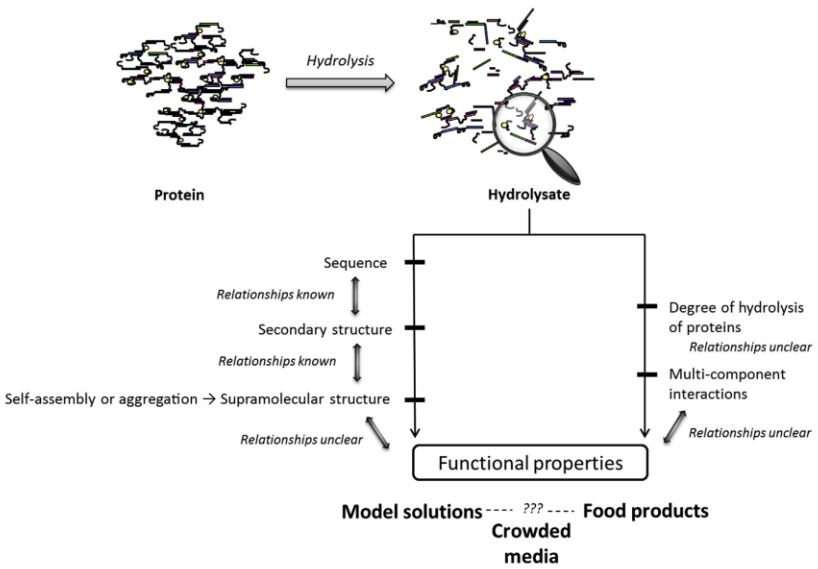


Fig. 1. State of the art of the current knowledge about the relationships between peptides and their functional properties in model solutions and in food products.

6. Concluding remarks

Food texture remains an important determinant of consumer acceptability. Control of the peptide and protein self-assembly mechanism is therefore essential to identify the levers that determine the texture of food products. Enzyme specificity is the key parameter to orient the types of peptides produced with specific sizes and physico-chemical characteristics and, in turn, has an impact on the functional properties of a food product. Even though the use of protein hydrolysates, composed of a mixture of peptides and of native proteins, improves protein functional property mechanisms, the induction of peptide functional properties generated by enzymatic hydrolysis is still only partially understood in food products.

Studies of the relationship between peptide structures and peptide functional properties have been carried out in food products, but often in terms of the global DH of protein. Since hydrolysates contain a mixture of peptides of different sizes and different characteristics as well as a part of the remaining native proteins, the agents responsible for the specific functional properties are defined for only a few of them. Progress in the characterization of peptides by mass spectrometry has led to a new comprehensive perspective on groups of peptides present and which of them are actually involved in the functional properties, either *in vitro* in model hydrolysate or *in situ* in food. Nevertheless, extrapolation of the results obtained from the model to food products is still difficult because unspecific interactions as well as the confinement of peptides and proteins in food impede our actual overall understanding of the acquisition of functional properties. Research on more concentrated systems such as crowded media will help to bridge this gap. The next steps will be to control (i) how the peptides are produced and interact with the other components, and (ii) the quantity of peptides necessary to obtain optimal functional properties.

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Part II – Materials & Methods

Sodium caseinate preparation

Sodium caseinate (NaCas) powder (Armor-Protéines, France) contained 87% (w/w) of proteins, determined by both Kjeldhal method and UV spectrophotometry (UVmc2, Monaco SAFA, France) at λ = 280 nm using extinction coefficient of 0.81ml. mg^{-1}. cm^{-1} (Oliva et al., 2001; Schmidt, 1979), including only minor amount of whey proteins as shown by SDS-PAGE electrophoresis (protocol described in the part "Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis" of this manuscript). This powder was dispersed in milliQ water using motor agitation in order to obtain a solution at 10 g of caseins.kg^{-1}.

α_{s1}-and β-casein purification

Caseins were prepared from raw milk that was skimmed and precipitated to pH 4.6 by adding dropwise 1M HCl in order to remove whey proteins. The pellet was centrifuged at 4 000 × *g* during 15 minutes at ambient temperature. The obtained pellet was suspended in MilliQ water with an ultra-turrax. Three washing steps were realized by increasing pH to 7 with 1M NaOH and decreasing it to pH 4.6.

The first step of purification consisted of remove the κ-casein by precipitation according to the method of Zittle and Cutter (Zittle and Custer, 1963). The freezed pellet, with approximatively 50 g of total caseins, was diluted in 500 ml of 6.6 M urea in order to obtain a protein concentration of 10%. After dissolution, 7N sulfuric acid was added to adjust pH to 1.5. The mix was diluted with MilliQ water to a concentration of 2.2 M urea. After the precipitation, the mix was centrifuged at 5 000 × *g* for 30 minutes at ambient temperature. The pellet was dissolved in 500 ml of 6.6 M urea and the pH was adjusted to 4.6 with 10 M NaOH. The urea was diluted with MilliQ water to attain a concentration of 3.3 M urea. The precipitate was centrifuged at 5 000 × *g* for 30 minutes at ambient temperature.

Purification of α_{s1}-casein: the pellet was dissolved in 200 ml of 6.6 M urea and the pH was adjusted to 7 with NaOH 1M and then dialyzed overnight. The pH of the dialyzed solution was adjusted to pH 7.2 with ammoniac (Brignon et al., 1976). This dialyzed solution was diluted with an equal volume of absolute ethanol and with a solution of 2 M ammonium acetate until the formation of a precipitate. The supernatant was evaporated to remove the ethanol, dialyzed and freeze-dried.

Purification of β-casein: The supernatant obtained was diluted with MilliQ water to 1.7 M urea and the pH was adjusted to 4.9. The mix was heated at 20°C and the precipitate was centrifuged at 3 000

×*g* during 20 minutes. The pellet was dissolved in a minimal volume of MilliQ water and the pH was adjusted to 7 with NaOH 1 M before dialysis and freeze-drying.
This powder was dispersed in MilliQ water using motor agitation in order to obtain a solution which will be hydrolyzed by Trypsin and Glu-C.

Determination of hydrolysis conditions

NaCas, α_{s1}- and β-caseins were hydrolyzed by trypsin and *Staphylococcus aureus* V-8 protease in immobilized form. We chose both enzymes in order to obtain peptides with very different size and sequences and consequently different physico-chemical and structural characteristics (Figure 1).

(i) Tosyl-phenylalanine-chloromethyl-ketone (TPCK) – treated trypsin was cross linked on 4% beaded agarose (Thermo Fisher Scientific, France) This serine protease (EC 3.4.21.4), has a pH optimum ranging between 7.5 and 9 and is highly selective. Trypsin cleaves only peptide bonds in which the carboxyl group is composed by a lysine or an arginine residue, regardless of the length or amino acid sequence of the chain.

(ii) *Staphylococcus aureus* V-8 protease (EC 3.4.21.19), also referred as to endoproteinase Glu-C (Thermo Fisher Scientific, USA) was crosslinked by covalent bonds on 6% beaded agarose.V-8 protease cleaves the protein at glutamic acid residues or cleavage at glutamic and aspartic acid residues (Drapeau et al., 1972). The endoproteinase Glu-C, has two pH optima, one at pH 4.0 and the other at pH 7.8, and it is specific for cleavage at the carboxyl terminal end of glutamic acid and aspartic acid residues. The protease is further specific for only glutamic digestion in buffers which do not contain phosphate at either pH optimum. S. aureus V-8 protease is very stable. The native structure of the protease is stabilized mainly by electrostatic interactions, and not by hydrogen bonding and β-structures.

Immobilized TPCK Trypsin and Glu-C were preferentially used instead of the free form of the enzymes because immobilization, even if it can lead to partial denaturation of the enzymes during cross-linkage on beads minimizes autolysis, allows control of the hydrolysis by removing the enzyme-linked to the beads physically by filtration and avoid further contamination of the sample by the protease. Finally immobilized enzymes are also more stable against heat-induced and physico-chemical denaturation, resulting in keeping activity for a longer period of time and are reusable. For that purpose, enzymes were washed after each hydrolysis period by various washing steps i) with 4M

urea for both enzymes, and with 1% Triton X100 only for Glu-C, ii) Phosphate Buffer Saline 1X and iii) MilliQ water.

Prior to each hydrolysis, the activity of the Trypsin was measured by spectrophotometry at 247 nm using 0.01 M 4-toluene-sulphoyk-arginine-methyl-ester, TAME (Sigma Aldrich, France), according to the method of Worthington Laboratories, using the UV-mc2 spectrophotometer (Monaco-SAFAS, France). The activity of Glu-C was measured by the pH-stat method using sodium caseinate (NaCas) (Armor-Protéines, France) as substrate with a ratio of 100 µl immobilized enzyme / 0.2 g of NaCas. Activity control was made for both enzymes to check that both no enzyme was released from the beads during treatments and activity was the same than the initial.

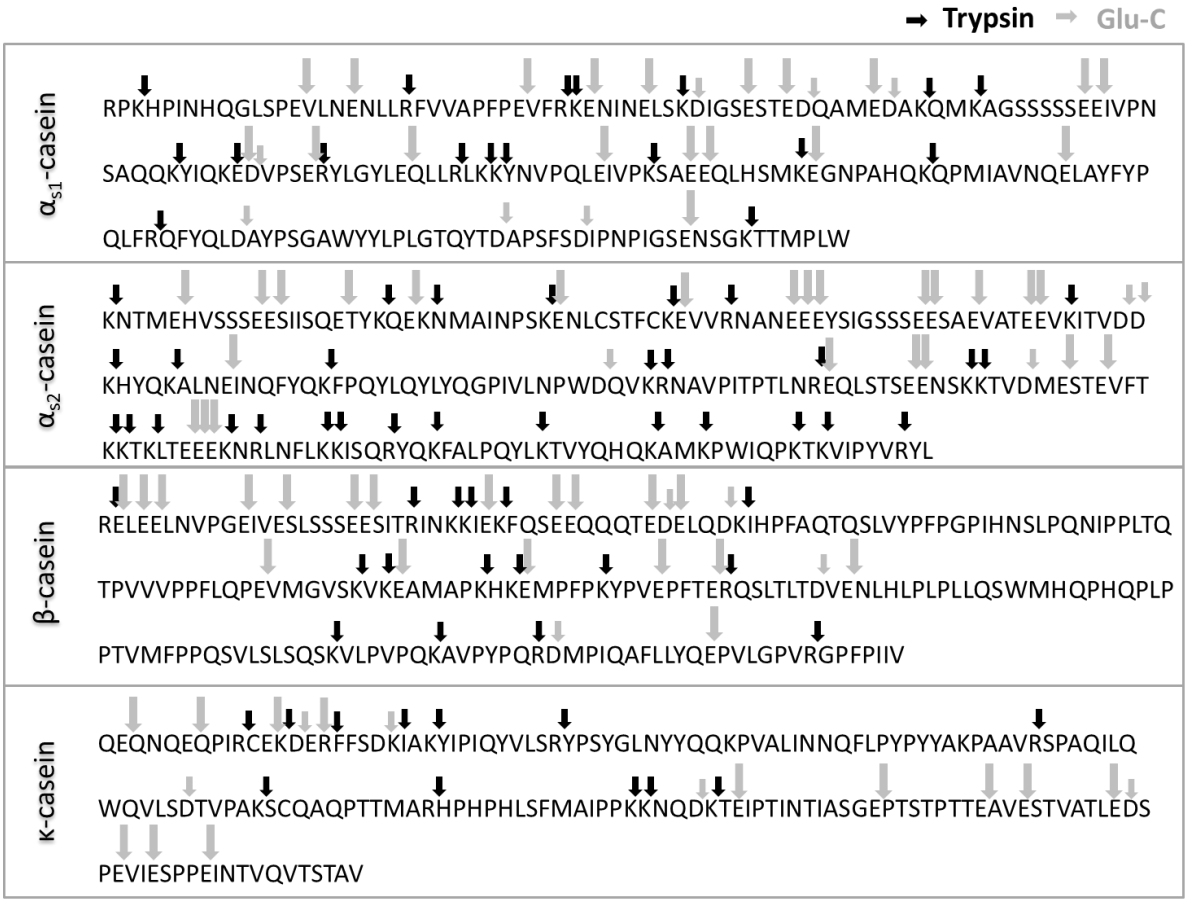


Figure 1. Theoretical cleavage sites of Glu-C and Trypsin on the four caseins. Large arrows represent the sites cleaved more often. Glu-C cleaves peptide bonds C-terminal to aspartic acid residues (D) at a rate 100-300 times faster than at glutamic acid residues (E). Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine (K) or arginine (R), except when one of this amino acid residue is followed by a proline.

In order to determine the conditions of casein hydrolysis, some experimental developments were necessary. We have chosen to hydrolyze separately α_{s1}- and β-caseins to show the impact of peptides derived from these caseins on the rheological properties of high concentrated matrices.

Many enzyme:substrate ratios were tested to select the optimal conditions to hydrolyze α_{s1}-casein, β-casein and NaCas by Trypsin and Glu-C. These tests were realized on 40 ml of protein solution at 5 $g.L^{-1}$. The hydrolysis was followed by the pH-stat method at 37°C, using 0.01 M NaOH as titrating agent to keep pH to 7 throughout hydrolysis time. For Trypsin, the ratio 9.6 U / 0.2 g of caseins was selected whereas for Glu-C it was of 720 µl / 0.2 g of caseins.

SDS-PAGE and Urea-PAGE (see the part "*Protein profile by electrophoresis*") were realized in order to show, respectively, the apparition of peptides and the degradation of caseins according to the hydrolysis time.

The three times of hydrolysis chosen for the hydrolysis of β-casein with Trypsin were (Figure 2):

- T1 (hydrolysate 1 with Trypsin) = 1500 sec : around 70% of caseins and 30 % of peptides
- T2 (hydrolysate 2 with Trypsin) = 2500 sec: around 50% of caseins and 50% of peptides
- T3 (hydrolysate 3 with Trypsin) = 5000 sec: around 20% of caseins and 80% of peptides

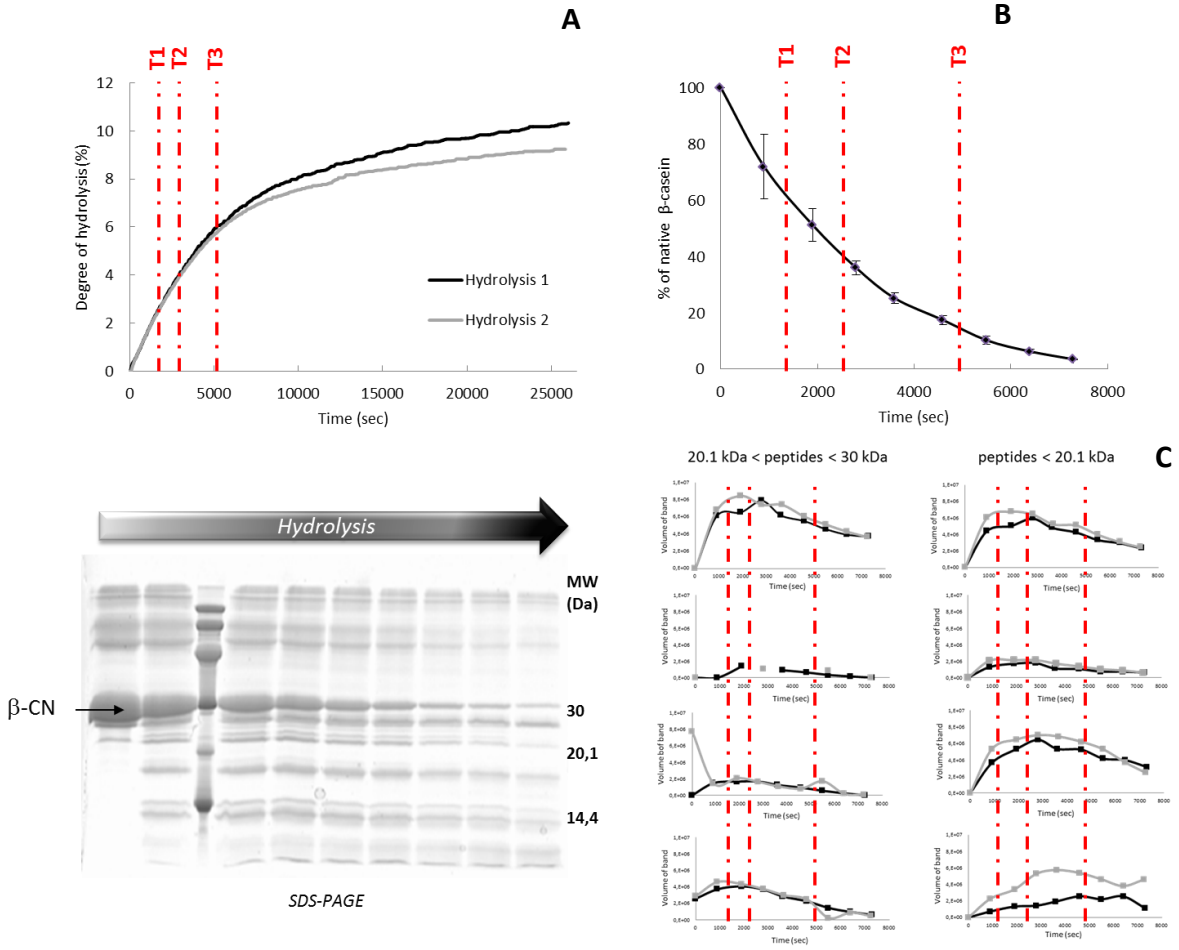


Figure 2. Kinetics of β-casein hydrolysis by Trypsin. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of β-casein according to the time of hydrolysis determined by SDS-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. T1 represents the hydrolysate 1; T2 represents the hydrolysate 2; T3 represents the hydrolysate 3.

The three times of hydrolysis chosen for the hydrolysis of as1-casein with Trypsin are (Figure 3):

- T1 = 1500 sec : around 70% of caseins and 30 % of peptides
- T2 = 2500 sec: around 50% of caseins and 50% of peptides
- T3 = 5000 sec: around 20% of caseins and 80% of peptides

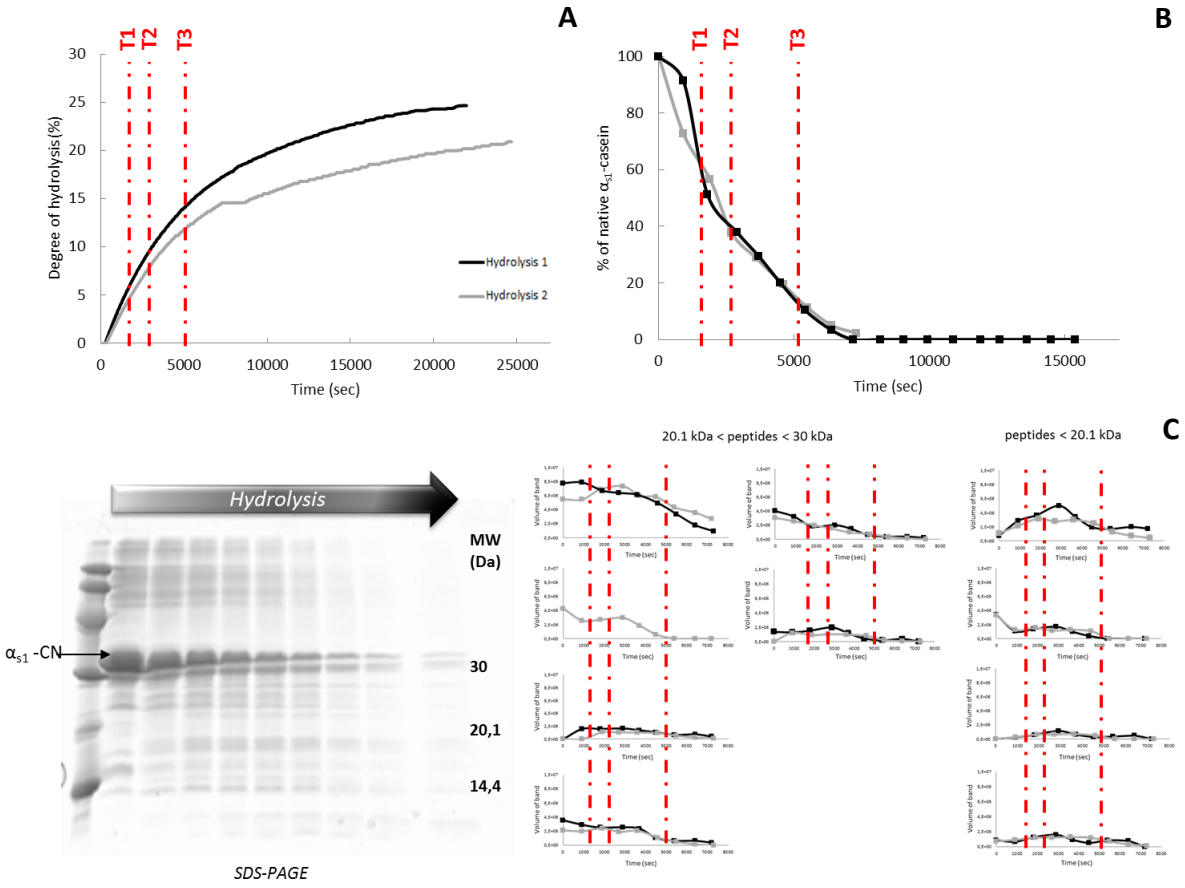
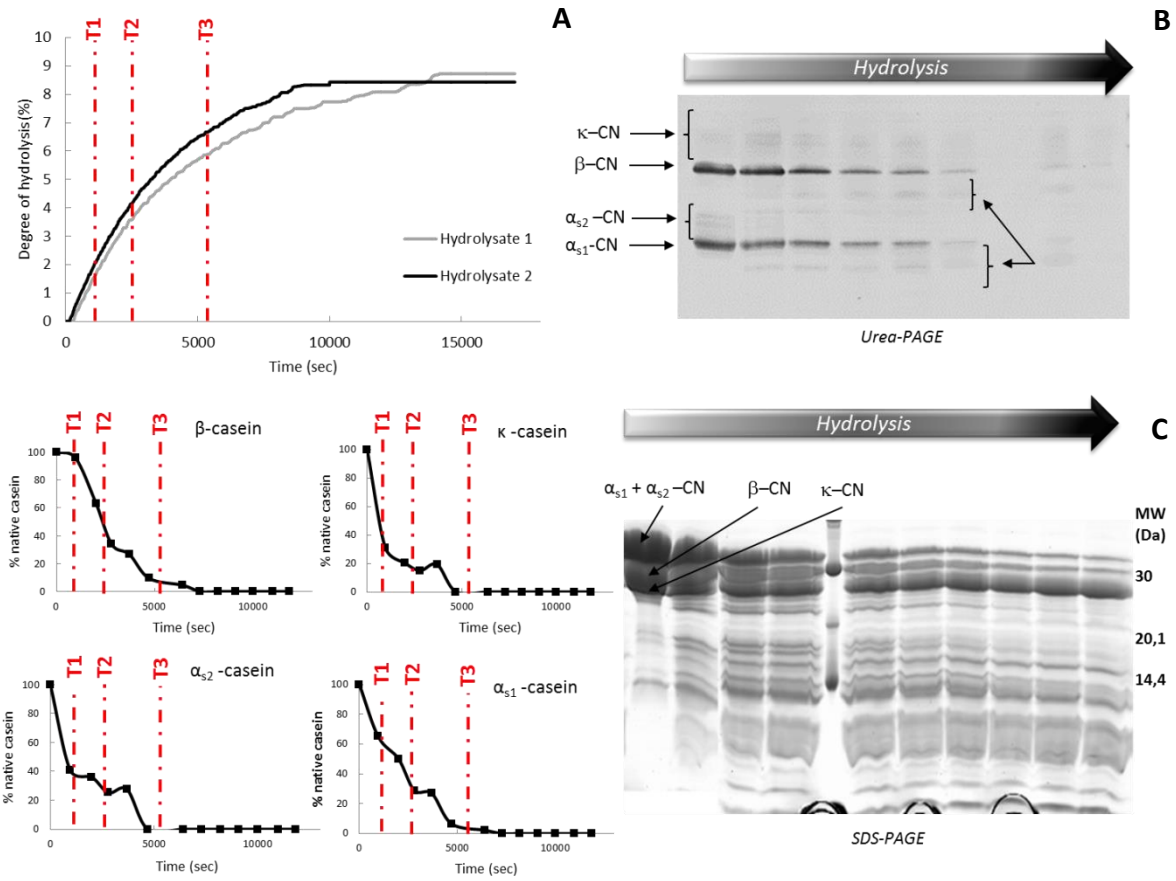


Figure 3. Kinetics of α_{s1}-casein hydrolysis by Trypsin. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of α_{s1}-casein according to the time of hydrolysis determined by SDS-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. T1 represents the hydrolysate 1; T2 represents the hydrolysate 2; T3 represents the hydrolysate 3.

The three times of hydrolysis chosen for the hydrolysis of NaCas with Trypsin are (Figure 4):

- T1 = 1000 sec : around 70% of caseins and 30 % of peptides
- T2 = 2500 sec: around 50% of caseins and 50% of peptides
- T3 = 5000 sec: around 20% of caseins and 80% of peptides



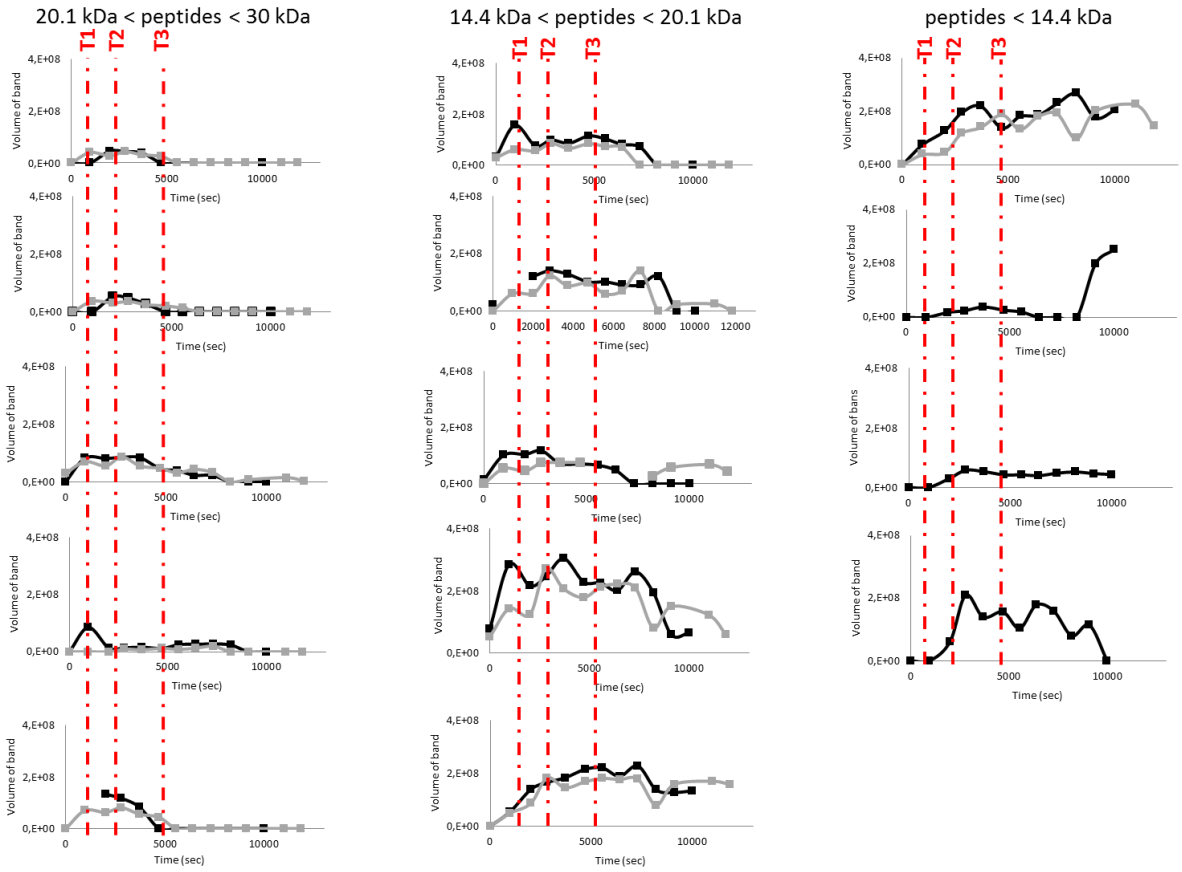


Figure 4. Kinetics of NaCas hydrolysis by Trypsin. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of κ, α_{s1}, α_{s1} and β-caseins according to the time of hydrolysis determined by Urea-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. T1 represents the hydrolysate 1; T2 represents the hydrolysate 2; T3 represents the hydrolysate 3.

The three times of hydrolysis chosen for the hydrolysis of β-casein with Glu-C are (Figure 5):

- G1 (hydrolysate 1 with Glu-C) = 1000 sec : around 70% of caseins and 30 % of peptides
- G2 (hydrolysate 2 with Glu-C) = 9000 sec: around 40% of caseins and 60% of peptides
- G3 (hydrolysate 3 with Glu-C) = 17000 sec: around 30% of caseins and 70% of peptides

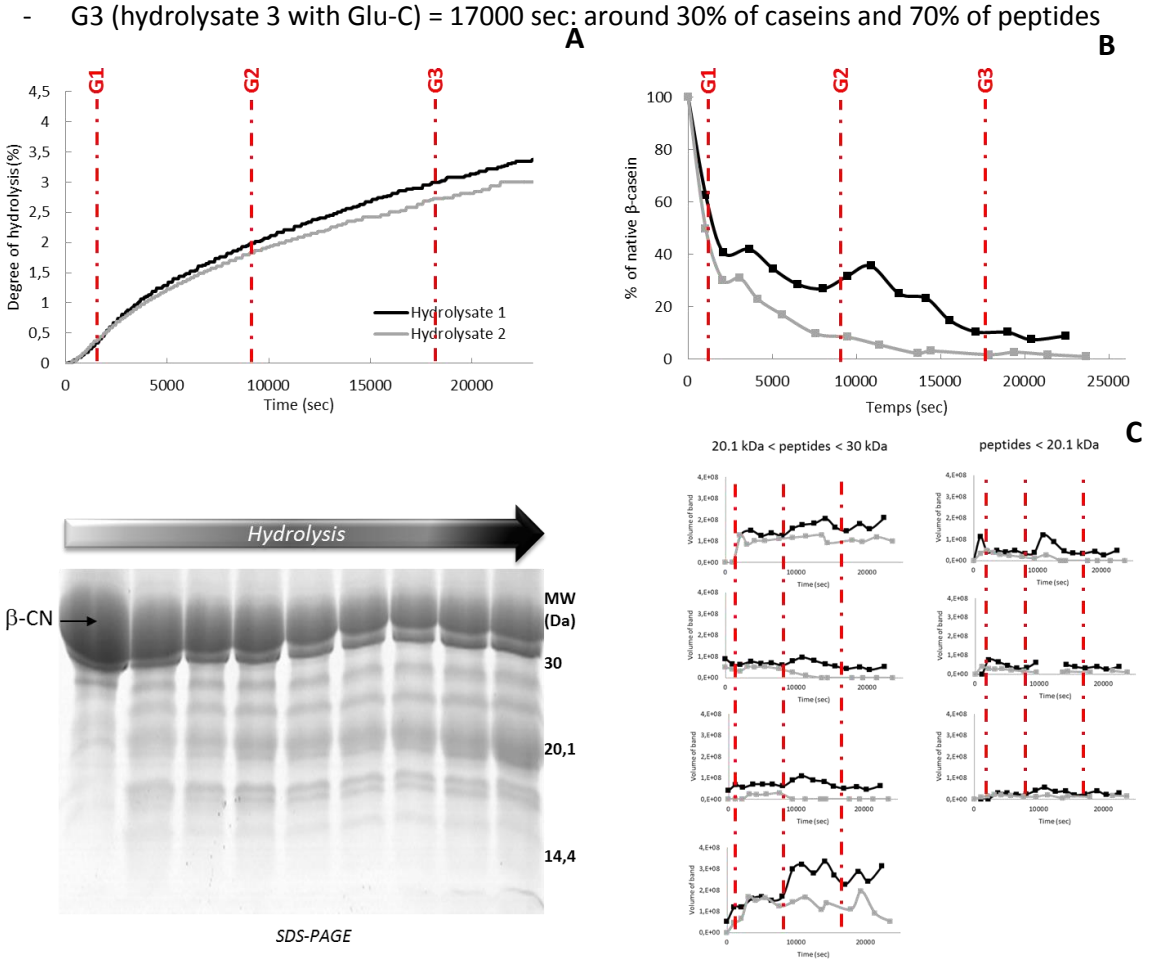


Figure 5. Kinetics of β-casein hydrolysis by Glu-C. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of β-casein according to the time of hydrolysis determined by SDS-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. G1 represents the hydrolysate 1; G2 represents the hydrolysate 2; G3 represents the hydrolysate 3.

The three times of hydrolysis chosen for the hydrolysis of as1-casein with Glu-C are (Figure 6):

- T1 = 1000 sec : around 70% of caseins and 30 % of peptides
- T2 = 5000 sec: around 50% of caseins and 50% of peptides
- T3 = 10000 sec: around 30% of caseins and 70% of peptides

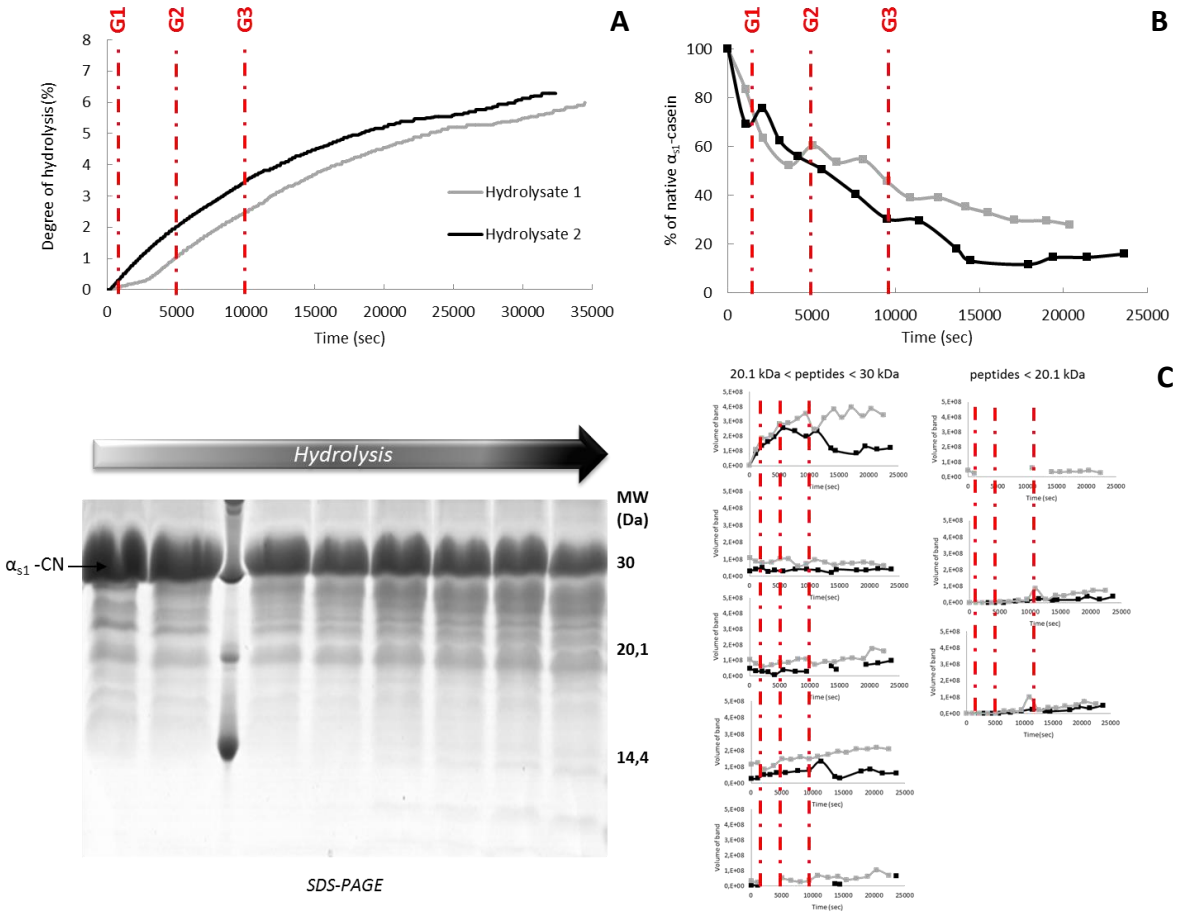


Figure 6. Kinetics of α_{s1}-casein hydrolysis by Glu-C. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of α_{s1}-casein according to the time of hydrolysis determined by SDS-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. G1 represents the hydrolysate 1; G2 represents the hydrolysate 2; G3 represents the hydrolysate 3.

The three times of hydrolysis chosen for the hydrolysis of NaCas with Glu-C are (Figure 7):

- T1 = 1000 sec : around 60% of caseins and 40 % of peptides
- T2 = 5000 sec: around 40% of caseins and 60% of peptides
- T3 = 10000 sec: around 10% of caseins and 90% of peptides

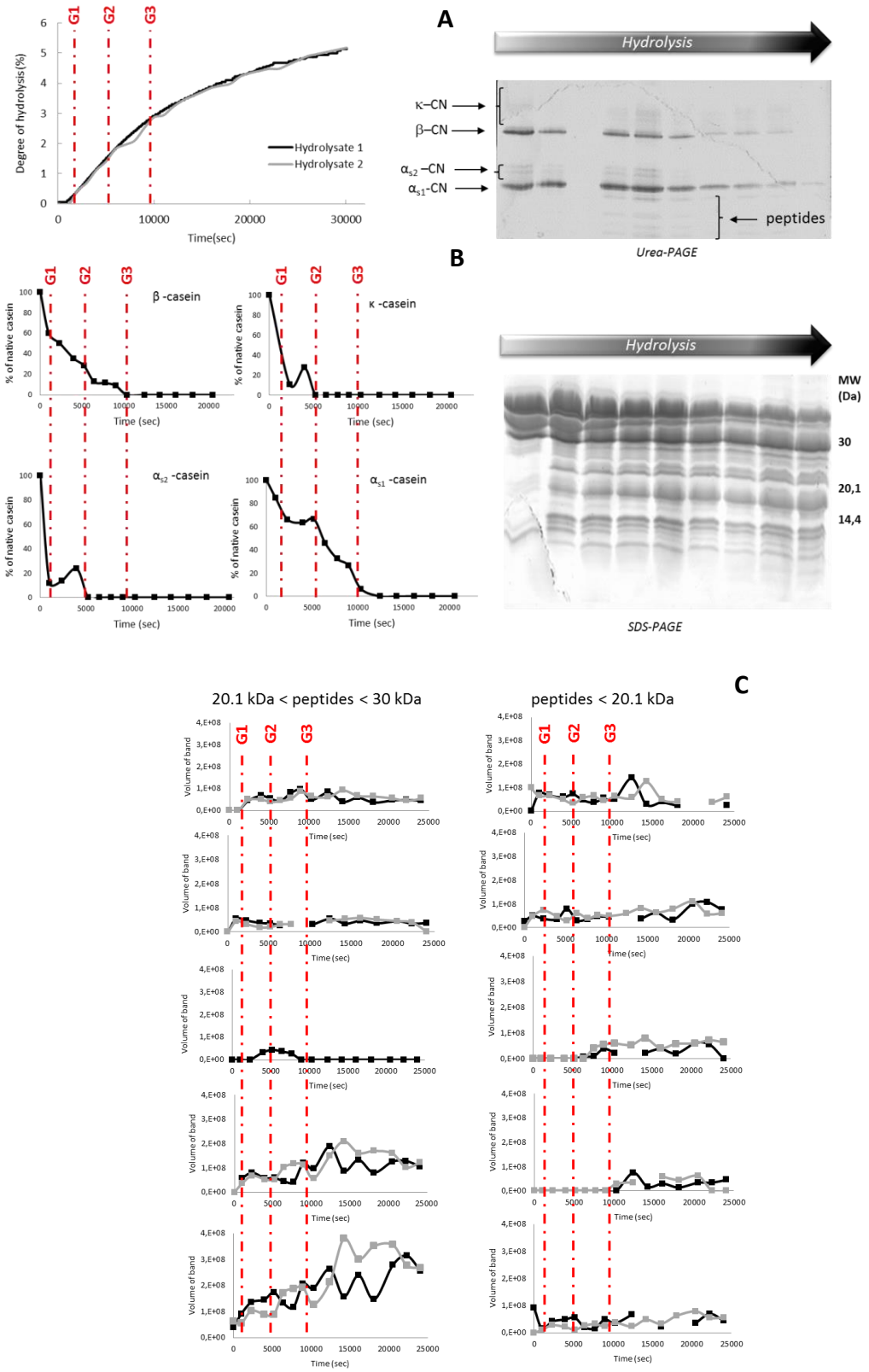


Figure 7. Kinetics of NaCas hydrolysis by Glu-C. (A) The degree of hydrolysis was determined based on pH-stat method (n = 2: hydrolysis 1 and hydrolysis 2). (B) The evolution of degradation of κ, α_{s1}, α_{s1} and β-caseins according to the time of hydrolysis determined by Urea-PAGE. (C) The evolution of peptides production according to the time of hydrolysis determined by SDS-PAGE. G1 represents the hydrolysate 1; G2 represents the hydrolysate 2; G3 represents the hydrolysate 3.

The hydrolysis were stopped by centrifugation 3 minutes at 14 000 g.

When the conditions of hydrolysis were well determined, the conditions of the hydrolysate production in larger volume had to be adjusted. The hydrolysates were produced in liters (2.9 L of NaCas at 10 $g.L^{-1}$ for the hydrolysis with Trypsin and 1.45 L of NaCas at 10 $g.L^{-1}$ for the hydrolysis with Glu-C).

Firstly, the hydrolysis was stopped by centrifugation as in small samples. The centrifugation did not permit an immediate stop of the reaction that was continued during the 10 minutes of centrifugation (Figure 8). Moreover, many beads were lost with this method.

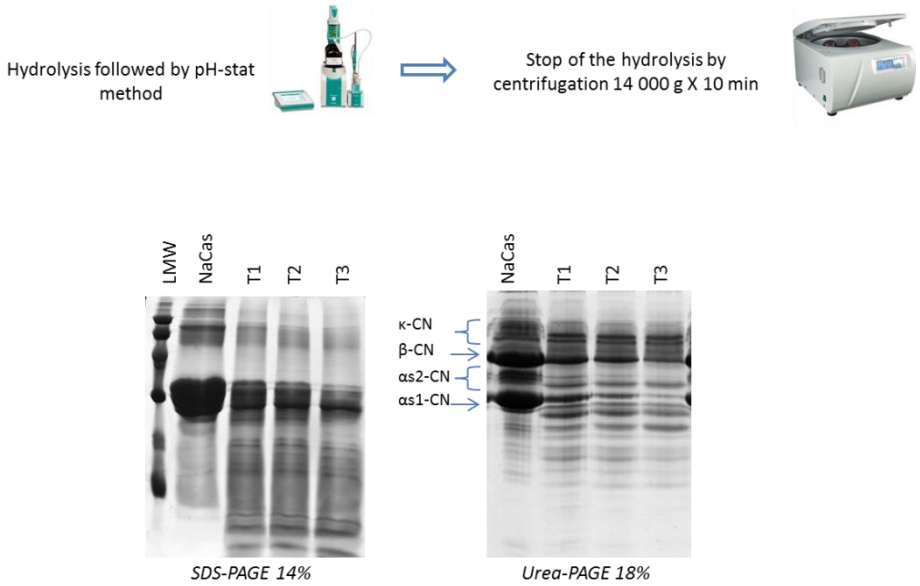


Figure 8. Hydrolysis stopped by centrifugation. SDS-PAGE and Urea-PAGE of the three groups of hydrolysates T1, T2 and T3, produced by hydrolysis of NaCas with Trypsin. T1: hydrolysate 1 produced by Trypsin; T2: hydrolysate 2 produced by Trypsin; T3: hydrolysate 3 produced by Trypsin; LMW: Low Molecular Weight.

To solve this problem, the hydrolysis was stopped by filtration as shown in Figure 9.

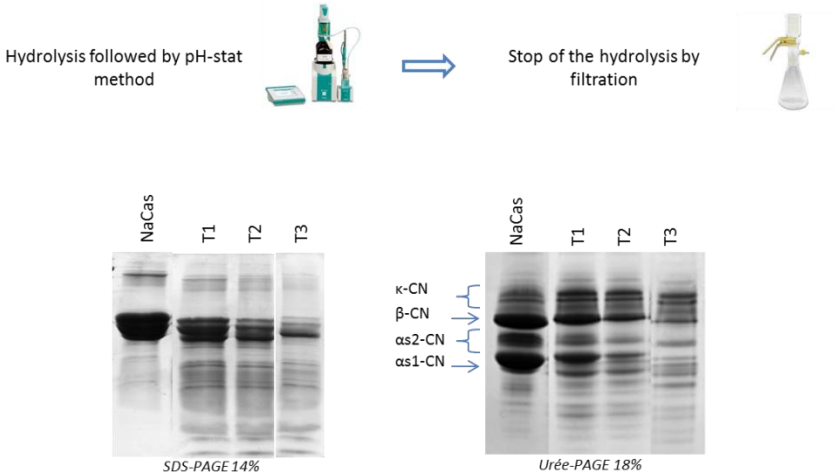


Figure 9. Hydrolysis stopped by filtration. SDS-PAGE and Urea-PAGE of the three groups of hydrolysates T1, T2 and T3, produced by hydrolysis of NaCas with Trypsin. T1: hydrolysate 1 produced by Trypsin; T2: hydrolysate 2 produced by Trypsin; T3: hydrolysate 3 produced by Trypsin; LMW: Low Molecular Weight.

By this way, the scale-up of hydrolysate production was effective (Figure 10).

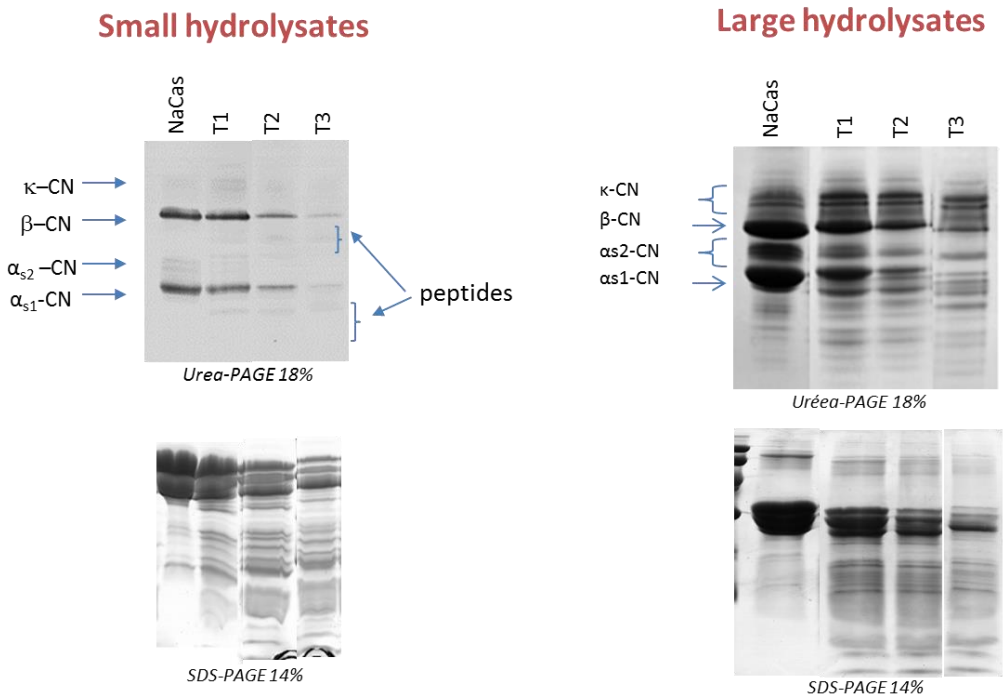


Figure 10. Comparison of peptide profile and casein degradation in small groups and in large hydrolysates, determined respectively by SDS-PAGE and Urea-PAGE. The example is given for the hydrolysis of NaCas by Trypsin.

Production of trypsin and Glu-C hydrolysates at a larger scale

Because of time limitation, only NaCas was hydrolyzed in large volume with Trypsin and Glu-C and the peptides produced were further used to be reincorporated into various casein matrices.

NaCas at 10 g.kg^{-1} was heated to 37°C before the enzyme was added. The ratios used were: 1398 U/29 g of NaCas for Trypsin and 34.8 mL of immobilized Glu-C/14.5 g NaCas (see part "*Determination of hydrolysis conditions*"). The pH was maintained at 7, using the pH-stat (Mettler Toledo, France) and NaOH at 1M as titrating agent.

The degree of hydrolysis (DH) of each hydrolysate was determined according to the pH-stat method (Spellman et al., 2003).

$$DH = B \times Nb \times {}^{1}/_{a} \times {}^{1}/_{mp} \times {}^{1}/_{htot} \times 100$$

$${}^{1}/_{a} = 1 + 10^{pk-pH}$$

$$pk = 7.8 + \frac{298 - T}{298 \times T} \times 2400$$

B= volume of NaOH

Nb= NaOH molarity

mp = protein mass

h_{tot}= 8,2 meq.g^{-1} for casein

T = temperature (Kelvin)

The hydrolysis reaction was stopped by filtration at three defined times corresponding to different compositions both in peptides and in native caseins: (i) around 70% caseins + 25% peptides, (ii) around 50% caseins + 50% peptides and (iii) around 5% caseins + 95% peptides. They were referred respectively as T1, T2 and T3 for hydrolysis with Trypsin and G1, G2, and G3 for hydrolysis with Glu-C.

Freeze-drying

A thin layer of hydrolyzed peptide solution was poured into stainless steel trays and covered with tissue paper to prevent dust contamination. The solution was then freeze at -20°C and lyophilized (S.G.D.-SERAIL type CS 10-0.8, Argenteuil France). The layer of dried sample obtained was crushed in order to have a homogeneous powder, which was then vacuum sealed and stored in darkness, at ambient temperature, until further use.

Preparation of maleate-$CaCl_2$ buffer

A maleate-$CaCl_2$-thimerosal buffer of pH 7.1 at ionic strength of 80 mM, corresponding to that encountered in milk was prepared using 25 mM Maleic acid (Sigma-Aldrich, France), 2 mM calcium

Chloride.2H_2O (AnalaR, France) and MilliQ water, with 0.02% (w/v) of Thimerosal or sodium azide as bacteriostatic agent (Sigma-Aldrich, France). 2 M NaOH was used for pH adjustments. This buffer was used for all hydrolysates, mix and casein resuspension solutions.

Preparation of Hydrolysate Suspension (HS)

The lyophilized peptide powder was then dispersed in maleate buffer to make peptide stock solutions of 50 $g.kg^{-1}$, 100 $g.kg^{-1}$ and 200 $g.kg^{-1}$. The dispersion was then stirred with a magnetic stirrer overnight (12h) at ambient temperature, before being transferred to a 50°C water bath for another 3 h of mixing to allow for complete rehydration.

Preparation of Casein Micelle Suspension (CMS)

Native phosphocaseinate powder (Promilk 825B, Ingredia, France) was dissolved in maleate-$CaCl_2$ buffer at pH 7.1 (80 mM ionic strength corresponding to that of milk aqueous phase) at room temperature at different concentrations (w/w) in presence of 3 mM sodium azide (NaN_3) as bacteriostatic agent during 24 hours with a weak stirring. Then, this solution was heated at 50°C during 3 hours and continues to be mixed during the last 21 hours. To check the complete rehydration of the powder, an analysis of the particle size was determined by dynamic light scattering using a Mastersizer (Malvern Instrument, United-Kingdom) which was equipped with a He/Na laser working at 633 nm and an attenuator that automatically adjusts the laser intensity to the specific range for scattered light detection. Protein samples were diluted in MilliQ water. Intensity of scattering is detected at 173° (backscatter detection) to reduce multiple scattering. The hydrodynamic diameter of the aggregates was calculated using Stockes-Einstein equation, taking the calculated diffusion coefficient from the fit of the correlation curve. The samples were measured in triplicate. The measure of particle size was realized in function of both the number of the particles and the volume of particles in order to detect the presence of aggregates. The solution was kept at 4°C during one week.

We chose to prepare solutions of CMS and HS at 50 $g.kg^{-1}$, 100 $g.kg^{-1}$ and 200 $g.kg^{-1}$ in order to:

- 50 $g.kg^{-1}$: to be higher than the protein concentration in milk and be close to some milk products concentration.
- 100 $g.kg^{-1}$: to double the basic concentration.
- 200 $g.kg^{-1}$: To be close to the concentration of proteins in cheese.

Mixtures NaCas:CMS and hydrolysates:CMS

Casein micelle suspension (CMS) and NaCas and hydrolysates solutions (in maleate buffer pH 7 containing 25 mM Maleic acid (Sigma-Aldrich, France), 2 mM calcium Chloride.$2H_2O$ (AnalaR, France) and 3mM sodium azide as bacteriostatic agent (Sigma-Aldrich, France) in order to reach the 80 mM ionic strength of milk)) were mixed at a mass ratios hydrolysate:CMS of 1:4 (25 % of hydrolysate), 1:1 (50 % hydrolysate), 3:4 (75 % hydrolysate) and 4:0 (100% hydrolysate) at 20°C. The same ratios were used for the control NaCas:CMS suspensions. All suspensions were prepared by gently rotating the tube to mix well, without introducing too much air bubbles. The mixtures were conserved at 20°C during maximum 48h.

The Figure 11 summarized the experiments realized.

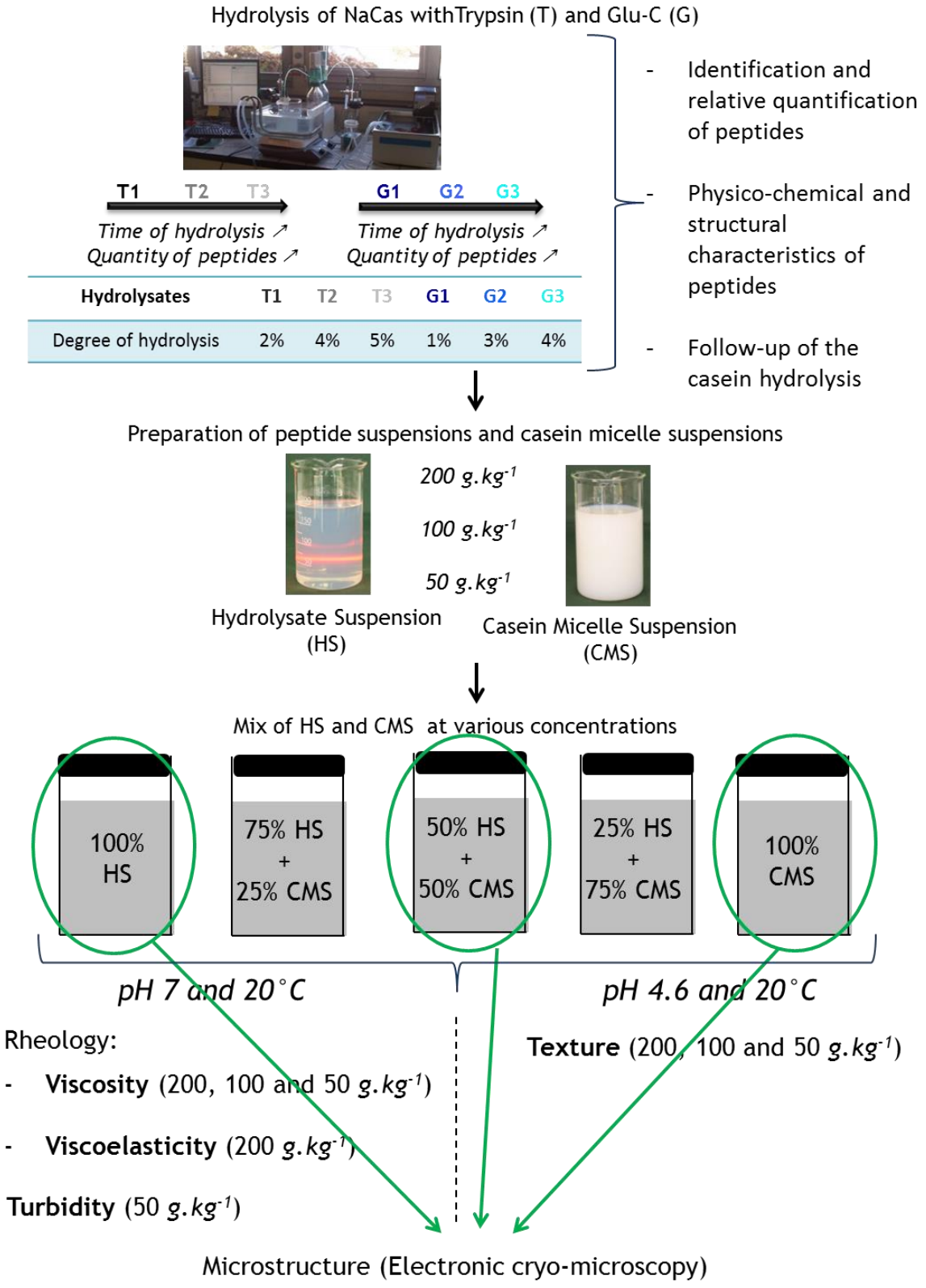


Figure 11. Experimental design from the production of hydrolysates to the rheological and microscopic study. CMS, Casein Micelle Suspension, HS, Hydrolysate Suspension.

Concentration by osmotic stress technique

Prior to the equilibration process, equal masses of casein micelle suspension and the peptide solutions were weighed into a plastic tube, and this 1:1 mixture was prepared by gently rotating the tube to mix well, without introducing too much air bubbles.

From previous trials, it has been determined that peptide samples and casein samples of concentrations over 200 $g.kg^{-1}$ will not be achievable from the dispersion of powder into a solvent. The strategy to obtain highly concentrated samples was therefore through the use of the osmotic stress technique, as described by (Bouchoux et al., 2009), where water is forced out of the sample, through a semi-permeable membrane into a solution of higher osmotic pressure, π.

The stock solution of 50 $g.kg^{-1}$ peptide or mixture or casein suspension was placed in a dialysis bag, which was then submerged in a reservoir containing solutions of polyethylene glycol (PEG) that fixed the osmotic pressure of the solution at concentrations that correspond to the required osmotic pressure. Over time, the chemical potential of water across the membrane reaches equilibrium and thus the osmotic pressure of the sample equals the osmotic pressure of the polymer solution.

Determination of protein concentration

The samples of CMS were centrifuged at 10 000 $\times g$ for 15 min. The supernatants were kept and the pellets were removed on. The final protein concentration of the supernatants was measured by a UV-visible spectrophotometer (UVmc2, Monaco SAFAS, France). The protein concentration ($g.L^{-1}$) was determined from the absorbance of the solution measured at 280 nm (A280nm) in cuves with a width L, using an extinction molecular coefficient of 0.81 $ml.mg^{-1}.cm^{-1}$ (Oliva et al., 2001):

$$C = \frac{A_{280nm}}{\varepsilon \times L}$$

Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis

The extent of casein degradation was evaluated by Urea-PAGE from the insoluble pH 4.6 nitrogen fractions according to Collin et al. (1987). The peptide pattern was analyzed by SDS-PAGE/Tris-Tricine buffer as described by Schägger and von Jagow (Schägger and von Jagow, 1987) using Protean II system (16 × 16 × 0.1 cm; BioRad, Marnes-la-Coquette, France) with SDS/Tris/Tricine buffer and a concentration gradient from 12 to 18% of acrylamide used according to Sadat-Mekmene et al. (Sadat-Mekmene et al., 2011).

SDS-PAGE was performed using a Mini Protean II system (Bio-Rad Laboratories, A Technologies, Dublin, Ireland) as described by Laemmli (Laemmli, 1970) using 14% acrylamide separating gels and 4% concentration gels. Protein samples were diluted 10 folds with the denaturing buffer (77.975% 0.08M Tris-HCl pH 6.8; 20% glycerol; 2% SDS; 0.025% bromophenol blue) under reducing conditions (with DTT and 5 min at 100°C). Samples were loaded in the sample slots and were separated at 150V for around 90 min. Gels were stained with Coomassie Brillant Blue G250. A low molecular weight marker kit (LMW 14.4-94 kg/mol, GE Healthcare, France) was used for molecular weight (MW) calibration. The gels were scanned by LabScan (GE Healthcare, France) and analyzed by ImageQuant software (GE Healthcare), band intensities were estimated by the net volume parameter.

Analysis of peptides by nano-RPLC coupled on-line to tandem mass spectrometry (ESI/MS/MS)

Mass spectrometry (MS) experiments were performed using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, USA) equipped with a nanoelectrospray ion source. A preliminary sample concentration step was performed on a nanotrap PepMap 100 (C18, 3 µm Inner Diameter 5ID) x 20 mm Length (L)) (Dionex, Netherlands). Separation was performed on a reverse-phase column PepMap RSLC C18 3 µm, 100 Å (75 µm ID, 150 mm L) (Dionex, Netherlands) at 35°C, using solvent A (2 % v/v acetonitrile, 0.08 % v/v formic acid and 0.01 % TFA in deionized water) and solvent B (95 % v/v acetonitrile, 0.08 % v/v formic acid and 0.01 % v/v TFA in deionized water). 5 – 35% of solvent B in 67 min and 35 – 8 % in 2 min was applied as separation gradient at a flow rate of 0.3 µL.min-1. 3 µL were injected onto the column corresponding approximately to 60 ng of peptides. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode and a voltage of 2 kV with the help of a Proxeon Nanospray Flex ion source (Thermo Scientific, USA). Spectra were recorded in full MS mode and selected in a mass rage 250-2000 m/z for MS spectra with a resolution of 70.000 at m/z 200. For each scan, the ten more intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17.500 at m/z 200 and the parent ion was subsequently excluded of the analysis dring15 sec. The instrument was externally calibrated according to the supplier's procedure. To identify peptides, all data (MS and MS/MS) were submitted to X! Tandem using the X! Tandem pipeline developped by PAPPSO (Plateforme d'Analyse Protéomique de Paris Sud-Ouest, INRA-Jouy-en-Josas, France, htp://pappso.inra.fr). The search was performed against a database composed of a homemade database named STLO containing milk and egg proteins and cRAP database allowing excluding list of proteins such a human keratin for example. Database search parameters were specified as follows: unknown enzyme cleavage was used with one missed cleavage and the peptide mass tolerance was

set to 10 ppm for MS and 0.05Da fo MS/MS. As caseins are phosphorylated, phosphorylation (corresponding o mass adduct of 79.96633 Da) were chosen as variable modification as well as oxidation of methionine (corresponding to mass adduct of 15.99494 Da). For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation. Only peptides containing more than 5 amino-acid residues and less than 5 500 Da can be identified by this methodology.

Physicochemical characteristics of the identified peptides

A number of physico-chemical characteristics of the quantified peptides were considered, including: the number of positive and negative charges at pH 7, the size, the isoelectric point (pI) and the hydrophobic index (GRAVY). The isoelectric point was predicted using ExPASy “Compute pI/Mw” tool (Artimo et al., 2012). The number of positive and negative charges was calculated with Python software from EMBOSS software suite (http://emboss.sourceforge.net). The GRAVY was calculated according to the method of Kyte and Doolittle (Kyte and Doolittle, 1982).

Size exclusion chromatography

Tryptic and GluC peptides were suspended at 5 g.L-1 in 50 mM phosphate buffer and 150 mM sodium chloride, pH 7.2 and 50 µl samples were injected onto a Superdex Peptide 10/300 GL column (GE healthcare, VWR, France). Isocratic elution was performed at 0.5 ml/min in the same buffer at room temperature and detection was performed at 214 nm. For the molecular mass calibration, □-casein (23 960 Da, 5 mg.mL-1 phosphate buffer pH7.2) was used to determine the exclusion volume of the column and synthetic peptides were used as standards: lysine (146.2 Da) , bradykinin (1060.2 Da), Ala-Pro-Gly (243.3 Da), Gly-Leu-Leu-Gly (406.48 Da), polypeptides standard (BioRad, France) containing triose phosphate isomerase (26 626 Da), myoglobin (16 950 Da), □-lactalbumin (14 437 Da), aprotinin (6512 Da), insulin B chain oxidized (3496 Da) and bacitracin (1423 Da).

Turbidity

The turbidity of the mix at 50 $g.kg^{-1}$ was analyzed by spectrophotometry (UVmc2, Monaco SAFA, France) at λ = 685 nm. The measurements were expressed as optic density. Series of dilutions were realized until that samples have a maximal absorbance of 1.

The relationship between absorbance and turbidity (τ) is expressed as:

$$T = \frac{It}{Io}$$

T = transmittance

I_0 = intensity of the incident light

I_t = intensity of the transmitted light

$$Abs = log\frac{It}{Io} = -\log T$$

$$\tau = \ln(10) \times \frac{Abs}{l}$$

l = length of the cell

τ = turbidity

Secondary and tertiary structures: Circular dichroism & Fourier Transform Infrared Spectroscopy

The overall tertiary and secondary structures of hydrolysates were examined using far- (190 – 250) and near-UV circular dichroism (CD) spectroscopy (250 – 350 nm) with a Jasco J-810 automatic recording circular dichroism spectropolarimeter fitted with a xenon lamp (Jasco, France) using casein solutions prepared to reach a maximum of absorbance of 0,8 at 280 nm. Spectra were recorded in the wavelength interval 185-260 nm (optical pathway of 2 mm) and 250-360 nm (optical pathway of 10 mm), respectively, using a quartz cuvette STARNA (Starna Scientific, England) of path length 2 mm and 10 mm, respectively. The scan parameters were: 100 nm/min scan rate, 1 nm bandwidth and 2 s averaging time. Spectra shown are averages of 3 accumulations for each sample, corrected by subtracting the baseline scan of the appropriate buffer and subjected to noise reduction.

Fourier Transform Infrared spectroscopy (FITR) was used to elucidate the conformational changes in protein and peptide structure upon the different hydrolysate:CMS ratios. Infrared light absorbance spectrum of each protein/peptide sample was measured in the attenuated total reflection (ATR) mode between 4 000 cm-1 and 850 cm-1, with a resolution of 4 cm-1, using the Tensor 27 spectrophotometer (Bruker, France), equipped with a Germanium ATR crystal (mono-reflection) and a mercury-cadmium-telluride detector cooled with liquid nitrogen, and controlled by the OPUS software. Background spectra were recorded for the maleate buffer used to prepare protein solutions. Each spectrum was reported form the average result of 256 scans. The protein solutions were at 30 g of caseins.kg-1. Before implementing the multivariate statistical analysis, spectra were corrected in automatic way contributions of the atmospheric steam and by some liquid water. To

focus on conformational changes, the differences in overall infrared absorbance due to differences in concentration between samples needed to be compensated. In addition, a major drawback of using IR spectroscopy on aqueous solution is that H – O – H bending vibration of liquid water absorbs strongly around 1643 cm-1, which interferes with the amide I band (Güler et al., 2011). Since the background spectra were recorded using buffer, differences in sample protein concentration resulted in differences in contribution of this liquid water absorption, which also needed to be compensated. The Extended Multiplicative Scatter Correction (EMSC) was used for these automatic compensations (Martens and Stark, 1991). EMSC consists in calculating a mean spectrum and in expressing each sample spectrum as a linear combination of the mean spectrum and of the liquid water "interference" spectrum. The coefficients estimated b linear regression then allow the water contribution to be subtracted and the overall absorbance to be normalized. The spectral variability among corrected spectra was then analyzed using principal component analysis (PCA). Spectral corrections and statistical computations were performed using the R software (Team, 2005).

Gelation

The lyophilized hydrolysates were resuspended in maleate buffer pH 7 containing 25 mM Maleic acid (Sigma-Aldrich, France), 2 mM calcium Chloride.2H2O (AnalaR, France) and 3mM sodium azide as bacteriostatic agent (Sigma-Aldrich, France) in order to reach the 80 mM ionic strength of milk. The solution was stirred during 12h at 20°C. Then, this peptide solution was heated at 50°C for 3 hours in a water bath. The casein micelle suspension (CMS) was added at several concentrations (w/w) to the hydrolysate solution (HS) and the mix was stirred during 12h. To reach a pH of 4.6, GDL (Merck, France) was then added to the mix. On addition of GDL to native phosphocaseinate solutions, the mixture was stirred vigorously for approximatively 2 minutes before to put in a water bath at 20°C during around 24h to reach pH 4.6. After acidification, gels were conserved at 4°C until rheological measurements.

Ultracentrifugation of gels

Gels at 200 g.kg-1 including 50% or 100% of hydrolysates were ultracentrifuged at 100 000 g for 1h using a Sorvall Discovery 90 Ultracentrifuge (Hitachi Koki, France). Under these conditions, all undissociated caseins were pelleted. The peptide pattern of the supernatants was analyzed by SDS-PAGE.

Rheological properties

Flow measurements were performed with a Low Shear 400 (Lamy Rheology, France) for dispersions with low viscosity (< 1000 Pa.s). This instrument was operated with Couette geometry using the system MS-LS 2T-2T Titan with inner and outer radii of 5.5 and 6 mm respectively. The measurements were realized at 20°C, by using a shear rate gradient from 1 to 100 s^{-1}. Measurements were repeated twice or three times showing very good reproducibility (error rate of 5%).

Flow measurements were performed with the MCR301 rheometer (Anton-Paar France S.A.S., France) for dispersions with viscosity > 1 000 Pa.s. These measurements were operated with cone plate geometry. The measurements were realized at 20°C with a shear rate gradient from 0.01 s-1 to 1 000 s-1. Measurements were repeated twice or three times showing very good reproducibility (error rate of 2%).

Oscillatory shear measurements were performed using the MCR301 rheometer (Anton-Paar France S.A.S., France). A cone-plate geometry was used. The mix hydrolysate:CMS was prepared and then quickly transferred onto the plate, and the upper plate was gently lowered until the sample filled the gap of a constant normal force was reached (about 0.7 N). The storage modulus (G') and loss modulus (G'') were first measured as function of time at a frequency of 1Hz. The frequency-dependent response was then measured from 0.001 Hz to 100 Hz at a strain of 0.01, % within the region of linear response. All rheological experiments were performed at a fixed temperature of 20°C. The measuring system was carefully regulated to the measuring temperature before each measurement. The solution was also kept for 10 minutes in the rheometer prior to performing the measurements. To prevent evaporation from the sample, a closed cover setup was used and the surface of the sample was covered with low-viscosity paraffin oil. Measurements were repeated two or three times, showing very good reproducibility (error rate of 3%).

Microstructure

The microstructure of the fourteen dairy system studied (NaCas:CMS, 6 hydrolysates:CMS × 2 pH) was characterized by transmission electron microscopy (TEM) (Figure 12).

The prepared concentrated casein suspensions and gels were frozen using a Leica EM PACT2 high-pressure freezer (Leica Microsystems, Austria). The sample carriers, cellulose microcapillary tubes or flat gold-plated specimen carriers, were pre-coated with 1% phosphatidylcholine (Sigma Aldrich Ltd., France) diluted in chloroform to avoid sample sticking. No cryo-protecting agent was added to the samples. For the freeze-substitution step, Leica EM AFS2 freeze substitution machine (Leica Microsystems, Austria) was used. The frozen samples were transferred in liquid nitrogen to a processing container equipped with a flat spacer (Leica Microsystems, Austria). The frozen samples

were freeze-substituted in 2% osmium tetroxide diluted in anhydrous acetone. After an initial incubation of 8 h at 90 C the temperature was gradually (5 C/h) rose to 30°C and samples were then left for another 8 h at this temperature. The solvent fixative solution was replaced with pre-chilled mix of ethanol 3:1 resin (epon-araldite mix; Sigma Aldrich Ltd). The temperature was then gradually brought up to room temperature and further ethanol: resin substitutions were done following the resin manufacturer instructions. Thin sections (90 nm) of embedded samples were cut with a diamond knife using a Reichert ultramicrotome. The sections were contrasted with 4% aqueous solution of uranyl acetate and observed using a JEM-1400 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) operated at 120 kV accelerating voltage. Digital images were acquired using the Gatan SC1000 Orius® CCD camera (4008 x 2672), set up with the imaging software Gatan DigitalMicrograph™ (Gatan, USA).

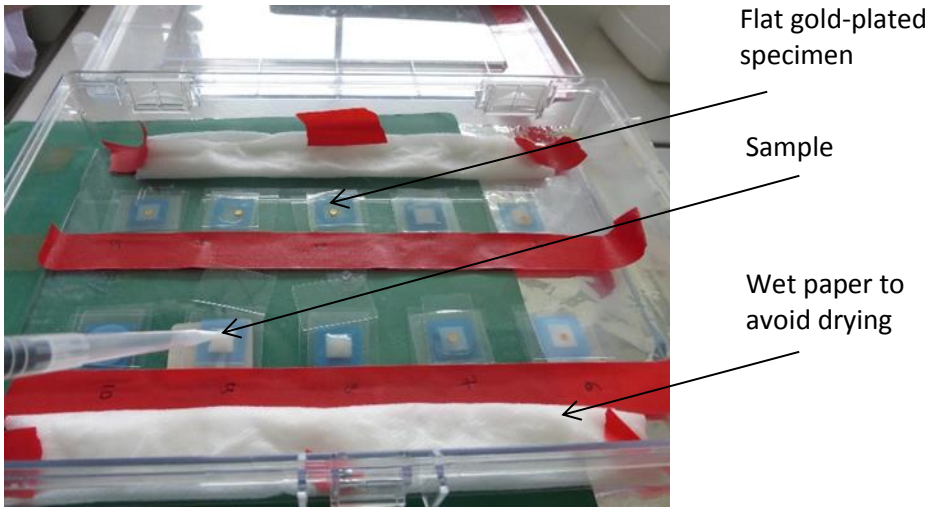


Figure 12. Preparation of samples for the Cryo-MET.

The microstructure of the seven samples of 100% hydrolysates studied (native NaCas, T1, T2, T3, G1, G2, G3) was characterized by transmission electron microscopy (TEM) by negative coloration. Drops of 0.005 mM protein suspensions were deposited onto glow-discharged carbon-coated microscopy grids. The liquid in excess was blotted with filter paper and a drop of distilled water was deposited on the preparation in order to rinse out the residual glucose and buffer salts. The water in excess was blotted and, prior to drying, the preparations were negatively stained with 2% (w/v) uranyl acetate.
The samples were observed using a JEOL JEM 1400 microscope operating at 120 kV. Images were recorded on camera Gatan Orius SC 1000 at Microscopy Imaging Center platform (MRic), situated in Rennes, France. The microstructure of the twelve (6 hydrolysates x 2 pH) dairy systems studied was observed by transmission electron microscopy (TEM). The prepared concentrated hydrolysate suspensions and gels were frozen using a Leica EM PACT2 high-pressure freezer (Leica Microsystems,

Austria). The sample carriers, cellulose microcapillary tubes or flat gold-plated specimen carriers, were pre-coated with 1% phosphatidylcholine (Sigma Aldrich Ltd, France) diluted in chloroform to avoid sample sticking. No cryo-protecting agent was added to the samples. For the freeze-substitution step, Leica EM AFS2 freeze substitution machine (Leica Microsystems, Austria) was used. The frozen samples were transferred in liquid nitrogen to a processing container equipped with a flat spacer (Leica Microsystems, Austria). The frozen samples were freeze-substituted in 2% osmium tetroxide diluted in anhydrous acetone. After an initial incubation of 8h at −90 °C the temperature was gradually (5 °C.h-1) rose to −30 °C and samples were then left for another 8 h at this temperature. The solvent-fixative solution was replaced with pre-chilled mix of ethanol 3:1 resin (epon-araldite mix; Sigma Aldrich Ltd, France). The temperature was then gradually brought up to room temperature and further ethanol:resin substitutions were done following the resin manufacturer instructions. Thin sections (90 nm) of embedded samples were cut with a diamond knife using a Reichert ultramicrotome. The sections were contrasted with 4% aqueous solution of uranyl acetate and observed using a JEM-1400 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) operated at 120 kV accelerating voltage. Digital images were acquired using the Gatan SC1000 Orius® CCD camera (4008 x 2672), set up with the imaging software GatanDigitalMicrograph™ (Gatan, USA).

Several TEM micrographs were taken on different parts of two independent samples of each of the twelve dairy systems. The resulting images showed the microstructure of the samples at a magnification of ×20000. Images were saved as 32-bit grayscale .dm3 images of 4008 x 2670 pixels (where 1 pixel = 2.5 nm).

Cheese making

Two cheese-making experiments were carried out following the MMV process (Maubois et al., 1969) (Figure 13). The raw milk was pasteurized at 72°C during 20 secondes, skimmed and microfiltered on a membrane with pore size of 1.4 µm. The fat:protein (1:1) content was standardized by adding heat-treated cream (120°C for 20 s).The standardized pasteurized skim milk was than ultrafiltrated on a mineral membrane with pore size of 0.02 µm, a surface of 1.8 m² (Membralox, Pall Exekia, France) at 50°C during 2.5 hours (1 hour up to a concentration factor of 3 and diafiltered with 1 diavolume during 1.5 hours up to a concentration factor of 6). The protein content of the milk retentate was concentrated six times compared to the original milk protein content and the lactose was partly removed by diafiltration. The lactose was removed in order to have a cell lysis of Lactococcus lactis to liberate the internal enzymes and to mimic the conditions of real cheeses. In this case, only the peptides which were eat and internalized by the bacterial cells did not participate to the modification

of cheese texture. Then, 1.5% of NaCl was added or not to the milk retentate at 50°C. 15% of hydrolysate or NaCas (w/w), suspended in ultrafiltrate permeate, were added to this retentate kept at 50°C. After, the mix was cooled at 30°C and both Lactococcus lactis starter (MM100, Danisco®, France) and chymosin (CHY-MAX Plus, Chr. Hansen A/S, Denmark) were added to the mix. This mix was incubated at 30°C until both coagulation and lactic fermentation were complete (around 20 hours). Cheeses were vacuum-packed and were ripened at 12°C and withdrawn for further analyses at 3 days (D3), 13 days (D13) and 23 days (D23).

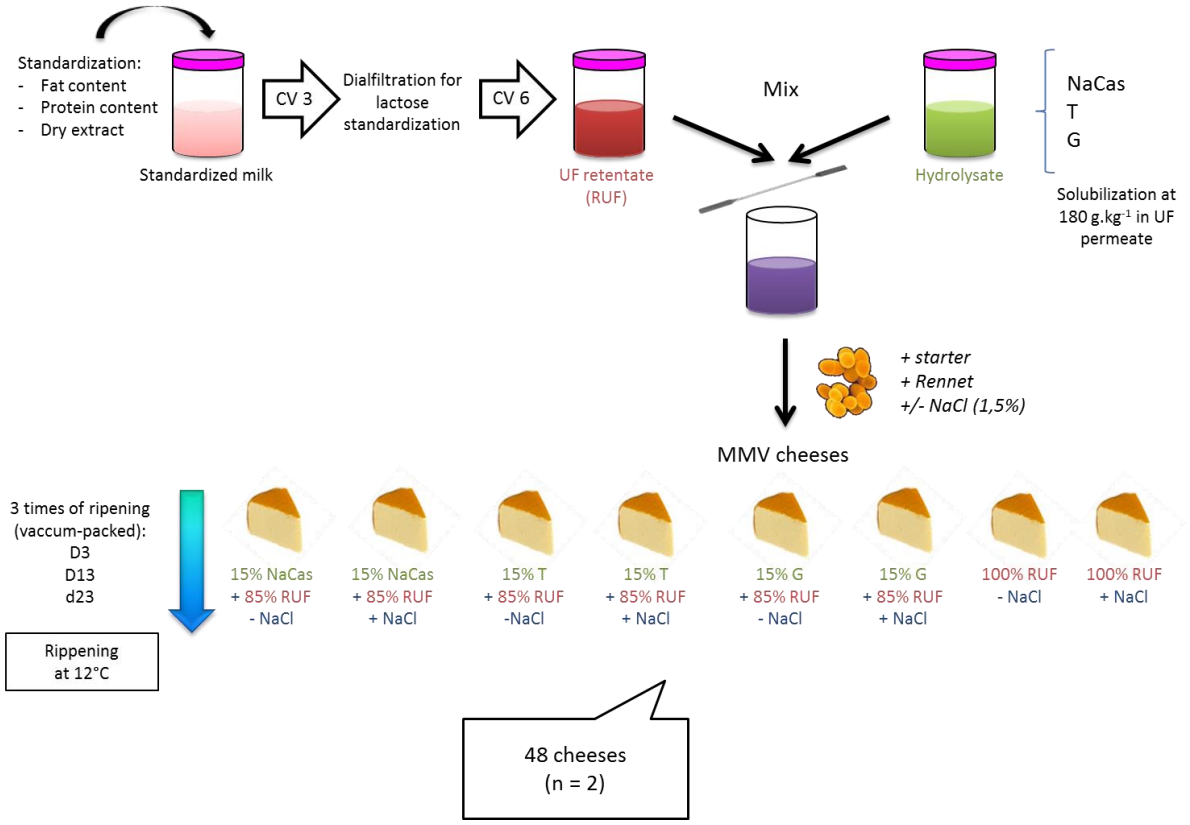


Figure 13. Manufacture of MMV cheeses with addition of hydrolysates (Tryptic or Glu-C hydrolysates) or sodium caseinate (NaCas).

Hereafter, the cheeses manufactured using only retentate, or with addition of caseinate, tryptic hydrolysate and Glu-C hydrolysate are designated R, C, T, and G cheeses respectively. Moreover the salting of the cheeses are noted + with NaCl and – without.

Cheese Analysis

pH was measured directly in the middle of cheeses using a pH meter (WTW pH 197i, Geotech, USA). MMV cheeses were analyzed for moisture (IDF ISO 5537:2004, 2004), fat content (Heiss, 1961) and dry matter (DM) (ISO 2920:2004 IDF 58: 2004).

Lactococcus lactis enumeration

Under a sterile atmosphere, one gram of grated MMV cheese at D3, D13 and D23 was dispersed in10 mL of sterile sodium citrate 20g.L^{-1} in a bag and crushed in a pendular blender Mixwel^{+} (Awel, France) for 120 s. From this suspension, the enumeration of the *Lactococcus Lactis* flora cell counts was made extemporaneously, by successive series of dilution in tryptone water in microwells according the method of Baron et al. (2006) and plate count on M17 medium (Terzaghi and Sandine, 1975) at 30°C for 48h . The citrate suspension was then frozen and conserved at -20°C until used.

o-phthaldialdehyde (OPA)

Proteolysis was measured in triplicates using the OPA method of Church et al. (Church et al., 1983) with methionine as a standard and adapted to microplate by Darrouzet-Nardi et al. (Darrouzet-Nardi et al., 2013). Citrate suspension of cheeses were thawed in a water bath at 30°C for 15 min, precipitated with HCl 1N at pH 4.6 or with trichloroacetic acid 12 % (w/v) final concentration. Then, samples were centrifuged at 3500 *g* for 10 min at 25°C before the supernatants were diluted at a ratio 1:5 with sodium tetraborate 0.1 M pH 9.5 for each sampling except initial time samples (ratio 1:2). The results were expressed as mmol methionine equivalent. L^{-1}.

Texture Profile Analysis (TPA)

Textures of gels and cheeses were analysed by Texture Profile Analysis.

Gels and cheeses were equilibrated at 20°C for at least 1 hour prior to testing. MMV cheeses were cut into cylindrical samples (20 mm thick and 20 mm dia.) using a cookie cutter and a wire cutter. A two-bite compression test was performed using the texturometer Lloyd instrument (Ametek, France) with a 5 kN (for gels) and a 100 kN (for cheeses) load cell. A 50% compression was used and crosshead speed was 10 mm.min^{-1} (for gels) or 60 mm.min^{-1} (for cheeses).

Data collection was done using the Nexigen Plus software and TPA parameters (hardness 1 for the first compression, hardness 2 for the second compression, cohesiveness, adhesiveness, stickiness, adhesive force, elasticity and gumminess) were determined in duplicate or triplicate from the texture profile curve as described by Bourne (1978).

Cheese Stretchability

Cheese stretchability was assayed by a method involving vertical traction of the cheese melted at 82°C according to Richoux et al. (2001). The length (mm) of strands of heated cheese was measured at the breaking point of the stretched strand.

Chapter 1: An *in silico* approach to establish a relationship between a techno-functional property of a dairy-matrix and a peptide profile.

Food products are complex matrices. They contain molecules from several families, proteins, lipids, carbohydrates that interact with each other to generate specific structures and new functionalities. Among these molecules, proteins were shown to have a key role in the structuration of the network that composed the food products (Foegeding and Davis, 2011).

Peptides resulting from protein hydrolysis can also be an integrated part of the structure and functional properties as do the proteins (Lacou et al., 2014). However, it is still difficult to establish a clear relationship between a mix of peptides and their techno-functionalities such as emulsifying, foaming, gelling or even texture properties within a complex matrix. Nevertheless, advances in mass spectrometry methods and their application to food products characterization have made possible the identification of peptides present in food products and namely in cheeses.

To have crucial information on the type of peptides present may help understand how they can be implied in the physical characteristics of food. Thus, Sadat-Mekmene et al., (2013) showed that one typical techno-functionality criterion of Swiss-type cheese, *i.e.* stretchability, was correlated with peculiar groups of peptides, which at a first glance were hydrophobic and composed of 20 amino acid residues. The production of the peptides implied in the stretchability was also dependent of the proteolytic enzymes present in the cheese and notably the proteases arising from the lactic acid bacteria. As the access to the peptide sequence can give information on the size, charge, hydropathy index and isoelectric point of the peptide, as well as on their potential secondary structure, it would be possible to try to correlate physico-chemical and structural characteristics of the identified peptides and the techno-functional properties of the cheese observed using the workflow shown in the Figure 1.

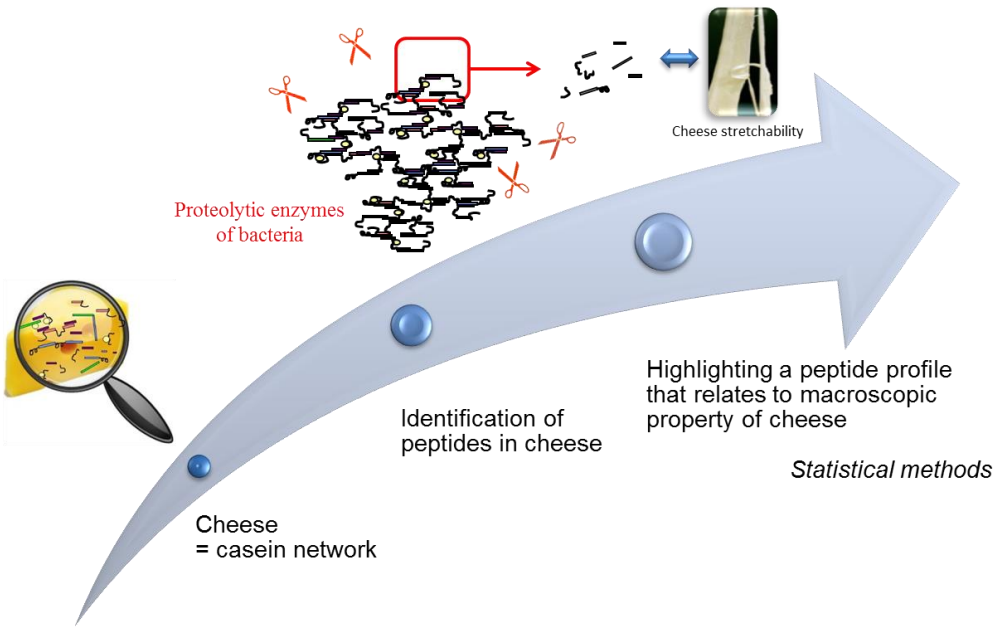


Figure 1. Workflow to determine the relationships between a peptide profile and the cheese stretchability.

Therefore, **we aimed**, from the dataset obtained from the previous work of Sadat-Mekmene et al., (2013), **to determine which physico-chemical and structural characteristics of peptides are involved in the cheese stretchability.** The methodology used was based on Principal Component Analysis and Correspondence Analysis.

The results are presented in this chapter under the form of an article, which has been published in 2015 in *Colloids and Surfaces A: Physicochemical and Engineering Aspects*[3]. This article showed that the peptides predominantly involved in cheese stretchability were a mixture of both hydrophobic and hydrophilic peptides and that they are large enough to interact with each other and with native proteins. This approach could be further applied to better understand the impact of peptides on various food matrices and food techno-functionalities.

From this study, we were able to distinguish peptides that were potentially implied in the techno-functional properties of a food product. They were produced from the various proteolytic enzymes that are active during cheese manufacture and ripening. In order to demonstrate the implication of such peptides on the microstructure, rheology and texture of simplified milk matrices and as it was

[3] Lacou, L. et al. (2015), An *in silico* approach to highlight relationships between a techno-functional property of a dairy matrix and a peptide profile. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 475, 44-54, http://dx.doi.org/10.1016/j.colsurfa.2014.10.052.

not possible to extract them from cheeses and to reincorporate them into various matrices, we chose to produce peptides with proteolytic enzymes of known specificity and to design hydrolysate with defined physico-chemical characteristics that were likely close to those encountered in cheese. This approach is described in the second part of the chapter

As a summary, the main aims of this chapter are to:

- **Link a peptide profile to a macroscopic property of a food product,**
- **Select enzymes with known specificities to produce peptides with defined characteristics.**

Chapitre 1 : Approche *in silico* afin d'établir une relation entre une propriété techno-fonctionnelle d'une matrice laitière et un profil de peptides.

L'aliment est une matrice complexe. Il contient des molécules provenant de différentes familles telles les protéines, les polysaccharides ou encore les lipides qui interagissent entre elles pour générer des structures particulières et de nouvelles fonctionnalités. Parmi ces molécules, les protéines ont été démontrées pour avoir un rôle prépondérant dans la structuration du réseau qui compose le produit alimentaire (Foegeding and Davis, 2011).

Les peptides résultant de l'hydrolyse des protéines peuvent aussi faire partie des éléments clés de l'aliment et contribuer au développement de nouvelles fonctionnalités (Lacou et al., 2016). Cependant, il est encore difficile d'établir un lien clair entre un ensemble de peptides et leurs techno-fonctionnalités comme les propriétés émulsifiantes, moussantes, de gélification ou encore de texture dans un système complexe. Néanmoins, des avancées dans les méthodes d'analyse par spectrométrie de masse et leur application a à la caractérisation des produits alimentaires rend désormais possible l'identification des peptides présent dans les produits alimentaires et notamment dans les fromages.

Avoir des informations sur le type de peptides présent peut aider de manière cruciale à comprendre comment les peptides sont impliqués dans les caractéristiques physiques des aliments. Sadat-Mekmene et al. (2013) ont montré qu'un des critères typiques du fromage type Emmental, le filant, était corrélé avec un groupe particulier de peptides, qui à première vue étaient hydrophobes et composés de 20 acides aminés. La production des peptides impliqués dans le filant du fromage était aussi dépendante du type d'enzyme présente dans le fromage et notamment des protéases des bactéries lactiques. Comme l'accès à la séquence d'un peptide donne des informations sur sa taille, sa charge, son hydrophobicité, sur son pH isoélectrique aussi bien que sur sa structure secondaire potentielle, il serait possible de tenter de corréler les caractéristiques physico-chimiques et structurelles des peptides identifiés aux propriétés techno-fonctionnelles du fromage observées en utilisant la stratégie montrée dans la Figure 1.

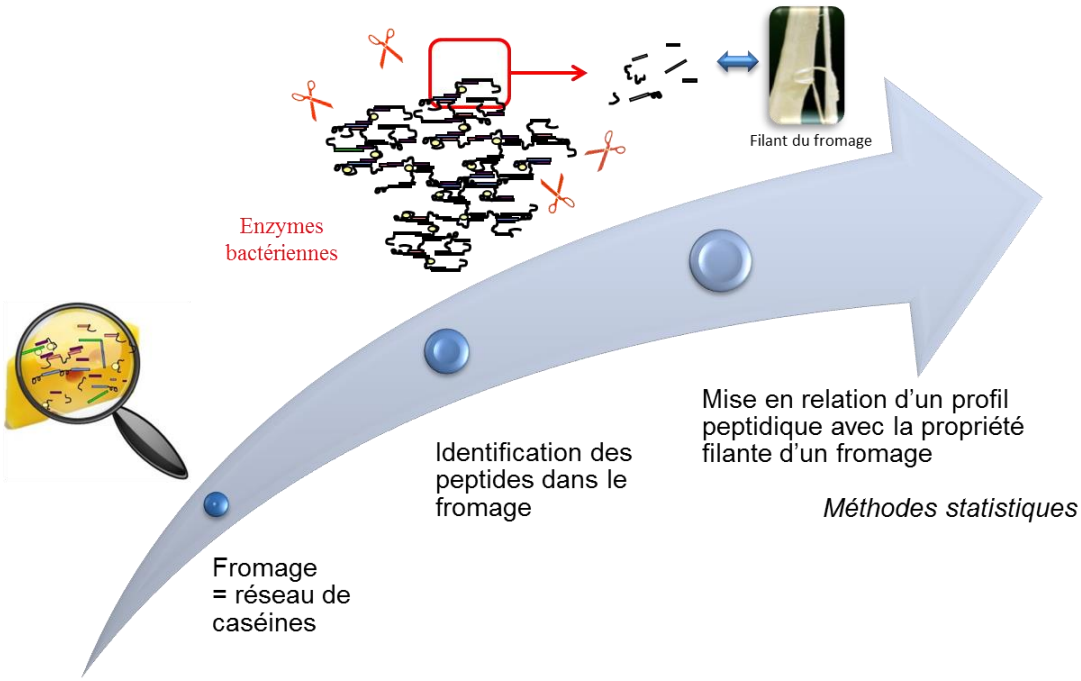


Figure 1. Stratégie pour déterminer les relations entre un profil de peptide et le filant du fromage.

Notre objectif a été, à partir des données obtenues lors du travail de Sadat-Mekmene et al. (2013), de déterminer quelles caractéristiques physico-chimiques et structurelles de peptides sont impliquées dans le filant du fromage. La méthodologie utilisée est basée sur une analyse en composantes principales et une analyse des correspondances.

Les résultats sont présentés dans ce chapitre sous forme d'article publié en 2015 dans le journal *Colloids and Surfaces A: Physicochemical and Engineering Aspects*[4]. Cet article a montré que les peptides majoritairement impliqués dans le filant du fromage sont un mixte de peptides hydrophobes et de peptides hydrophiles et qui sont suffisamment larges pour interagir les uns avec les autres ou avec les protéines natives. Cette approche peut être ensuite utilisée pour une meilleure compréhension de l'impact des peptides sur diverses techno-fonctionnalités de matrices alimentaires.

[4] Lacou, L. et al. (2015), An *in silico* approach to highlight relationships between a techno-functional property of a dairy matrix and a peptide profile. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 475, 44-54, http://dx.doi.org/10.1016/j.colsurfa.2014.10.052.

A partir de cette étude, nous sommes capables de distinguer les peptides qui sont potentiellement impliqués dans les propriétés techno-fonctionnelles d'un aliment. Ils ont été produits *via* des enzymes protéolytiques diverses actives pendant la fabrication de fromage et son affinage. Afin de démontrer l'implication de tels peptides sur la texture et la rhéologie de matrices alimentaires simplifiées et puisqu'il n'était pas possible d'extraire les peptides de fromages et de les réincorporer dans d'autres matrices, nous avons produit des peptides avec les enzymes protéolytiques de spécificité connue et conçu des hydrolysats avec les caractéristiques physico-chimiques définies, proches de celles rencontrées dans le fromage. Cette approche est décrite dans la deuxième partie du chapitre.

Pour résumer, les principaux objectifs de ce chapitre sont de :

- **Lier un profil de peptides à une propriété macroscopique d'un aliment,**
- **Sélectionner des enzymes avec des spécificités connues pour produire des peptides avec des caractéristiques définies.**

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An *in silico* approach to highlight relationships between a techno-functional property of a dairy matrix and a peptide profile

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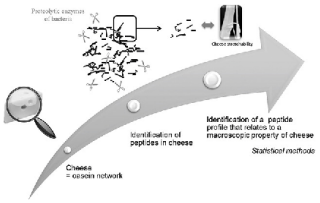
HIGHLIGHTS

- Proteolysis is strongly correlated with the stretchability of Emmental cheese.
- The type of protease affects the cheese stretchability.
- Peptide identification explains the modulation of the cheese stretchability.
- A peptide profile is related to a macroscopic property.

GRAPHICAL ABSTRACT



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ABSTRACT

Food products are complex matrices. They contain molecules from several families that interact with each other to generate specific structures and functionalities. These molecules may include proteins and peptides resulting from protein hydrolysis, *e.g.* in cheese. It is still difficult to establish a clear relationship between a pool of peptides and their techno-functionalities such as emulsifying, foaming, gelling or even texture properties within a complex matrix. Among the data usually known, there is the degree of hydrolysis that provides information about the average size of the peptides formed. A high degree of hydrolysis induces the production of a pool of small-size peptides (less than 20 amino acids). Advances in identification methods have made the identification of peptides present in food products possible. The size, charge, hydropathy index and isoelectric point of the peptide, as well as its secondary structure, can be calculated on the basis of the peptide sequence. The aim of this study was to determine which physicochemical and structural characteristics of peptides are involved in the cheese stretchability. This work is a re-evaluation, using multivariate exploratory analysis, of the experimental data obtained from a former study (Sadat-Mekmene et al., 2013) in which one typical functionality criterion of Swiss-type cheese (stretchability) was measured and peptides were identified. This methodology, based on Principal Component Analysis and Correspondence Analysis, is one way to establish a relationship between peptide characteristics and their techno-functional property within a complex dairy matrix. This statistical approach showed that the peptides predominantly involved in cheese stretchability were a mixture of both hydrophobic and hydrophilic peptides and that they are large enough to interact with each other and with native proteins. This approach could be applied to better understand the impact of peptides on various food matrices and food techno-functionalities.

Abbreviations: TN, total nitrogen; NCN, non-casein nitrogen; NPN, non-protein nitrogen.

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0927-7757/

1. Introduction

Food products have a complex composition that includes various types of molecules such as proteins, lipids and carbohydrates as well as different types of minerals. Over time and depending to the process used, this composition may change, giving a specific final quality to a product in terms of texture and flavour. Among the main food components, proteins play an essential role in the structuring of food products and other types of matrices [1,2]. In some cases, the hydrolysis of proteins enhances their functional properties, *e.g.* solubility, foam or emulsion stability, gelling capacity and even texture properties [3–5]. Until now, relationships have mainly been established between the degree of hydrolysis (DH) of proteins and the enhancement of their functional properties in the final food product [6,7]. The DH includes not only the peptides produced but the residual native proteins as well. In relation to foam or emulsion capacity and similar interfacial related properties, many other parameters should also be taken into account, including peptide composition, which was determined in order to link the peptide composition to the emulsion and foam properties in dilute solutions [8,9]. Since peptides are poorly identified in food products, it is still difficult to establish a clear relationship between a peptide profile and the enhancement of a defined functional property or to know what types of peptides lead to a specific functionality, especially in complex matrices.

Stretchability, a parameter of texture quality increasingly demanded by the consumer, is one of the techno-functionalities of dairy products. Stretchability concerns cheeses such as Emmental, Mozzarella and Cheddar. The cheese stretchability depends on its gross composition, pH, mineralization and proteolysis. Stretchability varies between cheeses depending on cheese-making processes, cheese composition and the ripening conditions used [10].

In silico methods are tools that can be used to analyze protein structure–functional property relationships, among other things. They involve computer simulations that provide knowledge about how peptide or protein structures impact their functionalities. Such a computer simulation is a way to analyze some experimental results without carrying out additional experiments. Moreover, the functional property of one precise component in complex matrix can be studied in more depth and specifically extracted using certain software [11]. *In silico* methods can be used for preliminary studies and/or as a validation tool. As far as we know, the relationships between the resulting fragments of protein hydrolysis and the structural and mechanical properties of food systems have not yet been explored to the same extent.

This work is a re-examination, using multivariate statistical methods, of the experimental data obtained from a former study [12] and some other parameters calculated for the peptides. In a previous study, stretchable cheeses were manufactured and peptides were identified in the aqueous extract of each cheese. The aim of the present study was to understand which physicochemical and structural characteristics of peptides could be involved in cheese stretchability. To fulfil this purpose, a methodology based on statistical methods was developed to establish the relationships between the physicochemical and structural characteristics of peptides produced during processing, and cheese stretchability. The characteristics of peptides identified in the cheese aqueous extract were then calculated. A combination of multivariate exploratory analyses such as Principal Component Analysis (PCA) and Correspondence Analysis (CA) was then used in order to link the typology of the peptides with the cheese composition and stretchability.

2. Methodology

2.1. Cheese making and cheese analyses

The data used here were collected from a former study [12] and are summarized in Fig. 1. Some proteolysis measurements were made on cheeses based on standard protocols used in the dairy sector. They include the percentage of residual caseins and nitrogen fractions that give a general idea of the size of the peptides: (i) the amount of total nitrogen (TN), which includes the amount of protein nitrogen and the amount of non-protein nitrogen; (ii) the non-casein nitrogen/total nitrogen ratio (NCN) in the cheese, which represents the percentage of soluble nitrogen [13]; (iii) the non-protein nitrogen/total nitrogen ratio (NPN) which represents the small soluble peptides and the amino acids [14] and (iv) the percentage of non-protein nitrogen that is soluble ((NCN-NPN)/NCN), which represents the small peptides and soluble amino acids.

2.2. Cheese stretchability

The stretchability of each cheese was measured at various periods of ripening. Cheese stretchability was assayed by a method involving vertical traction of the cheese melted at 82 °C, according to Richoux et al. [15]. The length of strands of heated cheese was measured at the breaking point of the stretched strand.

2.3. Peptide extraction and peptide identification

Peptides were extracted from cheeses with water at day 1 (D1), day 13 (D13), day 27 (D27) and day 41 (D41). The peptides present in the cheeses were separated by reversed-phase HPLC for both the first (F1) and the second (F2) cheese batches. Three fractions were collected as a function of the elution time. Peptides were eluted from the most hydrophilic to the most hydrophobic: (i) fraction A was collected from 2 to 22 min and contains peptides from 9 to 20 amino acids with a mean of 13 amino acids; (ii) fraction B was collected from 22 to 42 min and contains peptides from 6 to 45 amino acids with a mean of 15 amino-acids; (iii) fraction C was collected from 42 to 65 min and contains peptides from 6 to 44 amino acids with a mean of 19 amino-acids. These fractions were quantified according to their respective peak areas on the chromatographic graph.

For F1, peptides from the three fractions were identified by MS/MS at three crucial points of cheese ripening: D1 (at the beginning of the cheese ripening), D13 (when the stretchability is minimal) and D41 (when stretchability is the greatest). All together, 30 peptides were identified in the fraction A, 406 peptides in the fraction B and 139 peptides in the fraction C.

Throughout the text, LH1 refers to *Lactobacillus helveticus* ITGLH1 and LH77 refers to *L. helveticus* ITGLH77. The cheeses are designated according to the following combination: "name of the starter strain – day of peptide quantification and/or peptide identification": LH1-D1, LH1-D13, LH1-D27, LH1-D41, LH77-D1, LH77-D13, LH77-D27 and LH77-D41.

2.4. Physicochemical and structural characteristics of peptides

Peptide characteristics such as size, hydropathy index, isoelectric point (pI) and number of positive and negative charges at neutral pH were calculated on the basis of the peptide sequence using the ProtParam method of Gasteiger et al. [16], available from the ExPASy website [17].

The isoelectric point represents the pH at which the peptide net charge is null. The number of both positive charges and negative charges was calculated at pH 7. The hydropathy index of the peptide is the sum of the hydropathy index of each amino acid

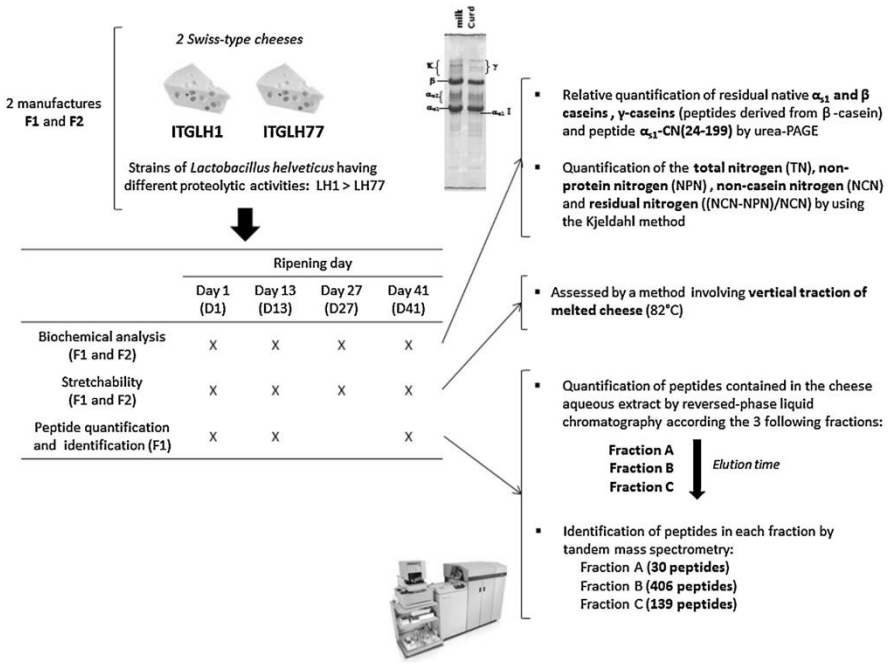

Fig. 1. Schematic representation of the manufacture of Swiss-type cheeses and analyses performed in the study of Sadat et al. [12].

constituting the peptide, divided by the total number of amino acids in the sequence. All these parameters describe the physicochemical characteristics of the peptide. The peptide secondary structure was predicted using the GORIV method [18] from the peptide sequence.

2.5. Statistical analyses

To link cheese stretchability to a specific pool of peptides, a methodology based on multivariate exploratory analyses, such as Principal Component Analysis and Correspondence Analysis, was performed using FactoMineR, an R package [19]. None of these analyses requires a validity condition.

The aim of PCA is to synthesize the information involved in a large number of quantitative variables. To do so, variables are linearly combined into what is usually referred to as "principal components" (PC). These PC are uncorrelated and are such that the variance of the statistical individuals projected on each PC is as high as possible. In other words, the first PC has the largest variance and each successive PC, in turn, has the next highest variance. These PCs are extracted in order to best represent the variables, on the one hand, and the individuals, on the other. For this representation, two variables are close if they are linearly correlated; two individuals are close if they take the same values regarding the variables in the dataset.

Two PCAs were successively performed to distribute the cheeses according to: (i) their biochemical analyses and (ii) both their biochemical analyses and the peptide chromatographic fractions. For these two PCAs, the first two PCs were retained as the totality of the inertia explained by both of them, which represents a large part of the total inertia. For both PCAs, the stretchability was projected as supplementary information, in order to have a better understanding of the results.

PCA and CA are multivariate statistical technique whose aim is to summarize information through orthogonal components. Whereas PCA deals with quantitative variables, CA analyses the relationship between two qualitative variables and is applied on a contingency table, with rows representing the categories of one of the two qualitative variables, and columns representing the categories of the other one. CA defines a specific distance adapted to qualitative variables, referred to as the "chi-2" distance. In our study, CA was used to observe the distribution of cheeses according to their peptide composition. The data were placed in a contingency table that summarizes the peptides in the cheeses. Rows represent cheeses and columns represent the different peptides.

Since the highest stretchability is obtained at D41 for both cheese manufactured with LH1 and cheese manufactured with LH77, several histograms of peptide characteristics were carried out to determine which physicochemical and structural characteristics of the peptides that could belong to both LH1-D41 and LH77-D41 in fractions B and C are predominant on the CA factor map.

3. Results and discussion

Among the lactic acid bacteria used in dairy products, *L. helveticus* is one of the species with the highest proteolytic activity and that hydrolyses caseins during cheese ripening. The cheeses produced had different levels of stretchability depending on the strains used and the level of casein proteolysis obtained, generating different types of peptides within the cheese. A statistical approach was developed in this study to establish the relationships between the proteolysis parameters, either quantitatively for biochemical analyses or qualitatively for the type of peptides

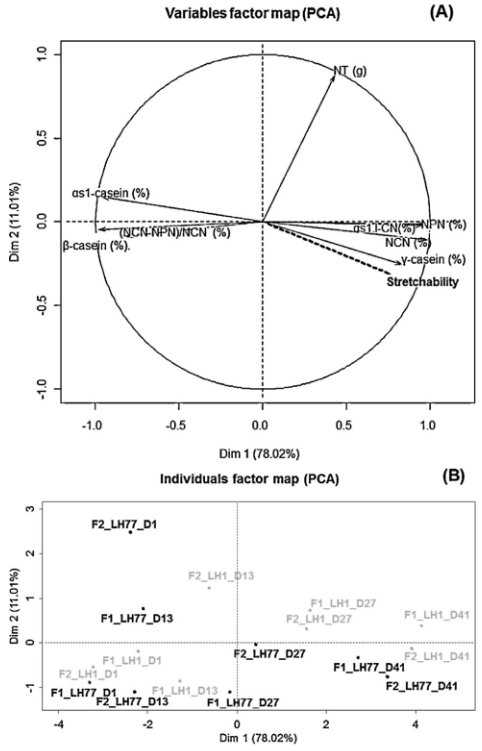

Fig. 2. (A) Biochemical characteristics of cheeses, for both strains LH1 and LH77 at D1, D13, D27 and D41, represented on a principal component analysis variables factor map. α_{s1}- and β-caseins are quantitative variables that represent the percentage of residual native caseins. α_{s1}-I casein: relative percentage of peptide α_{s1}-CN(24-199) derived from the hydrolysis of α_{s1}-casein by chymosin; γ-casein: relative percentage of peptides γ-casein derived from the hydrolysis of β-casein by plasmin; NPN: percentage of non-protein nitrogen; NT: amount of total nitrogen; (NCN-NPN)/NCN: percentage of residual native proteins. Stretchability is not included as a variable but is projected as a supplementary data on the PCA. (B) Distribution of cheeses according to their biochemical characteristics on a Principal Component Analysis individual factors map. F1: cheese manufacture 1; F2: cheese manufacture 2; LH1: *Lactobacillus helveticus* ITGLH1; LH77: *Lactobacillus helveticus* ITGLH77; D1: day 1; D13: day 13; D27: day 27; D41: day 41. In grey: the LH1 cheeses; in black: the LH77 cheeses.

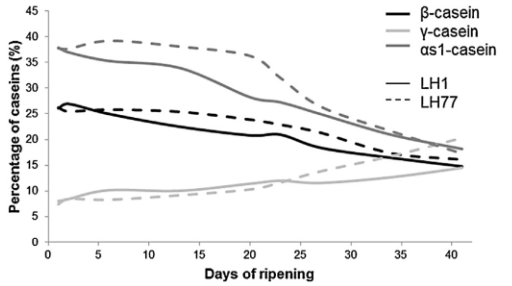

Fig. 3. Evolution of the hydrolysis of α_{s1}-casein, and β-casein and production of γ-caseins according to the day of ripening. The percentages of native caseins represent the mean of the data for F1 and F2.

produced, in addition to cheese stretchability. Firstly, the biochemical characteristics of cheeses were explored by PCA in order to extract the links between the cheese stretchability and some proteolysis parameters. Secondly, cheese distribution was studied using a CA on which the peptide composition was projected for each cheese. This shows which pools of peptides are linked to the highest cheese stretchability. Finally, the physicochemical and structural characteristics of peptides, *i.e.*, their isoelectric point, their size, charge and hydropathy index, included in these pools were explored, in order to establish which peptide characteristics were related to the cheese stretchability.

3.1. The strand length of cheese depends on the peptide profile

3.1.1. Most of the cheese variability is related to proteolysis

As shown in Fig. 2, the first two components resulting from PCA account for 89.03% of the total variability. The biochemical characteristics of cheeses, such as the amount of: (i) residual native α_{s1}-and β- caseins; (ii) both γ-caseins and α_{s1}-CN(24-199) (also referred to as αs1-I casein); (iii) total nitrogen (TN); (iv) the percentage of non-protein nitrogen (NPN); (v) the percentage of non-casein nitrogen (NCN) and (vi) the percentage of non-protein nitrogen that is soluble (NCN-NPN)/NCN, at days 1, 13, 27 and 41 were represented on the so-called correlation circle of the variables (Fig. 2a). The quantities of whey proteins were not represented because they are not quite hydrolysed in cheese due to their globular structure [20]. The first component explains most of the variation (78.02%) and is related to the proteolysis (Fig. 2a). As shown in Fig. 2b, the young cheeses are located on the negative side of the first component. The combined interpretation of Fig. 2a and b allows us to deduce that young cheeses are generally characterized by a high amount of native caseins, whereas old cheeses are instead characterized by a low amount of native caseins. Fig. 3 confirms that the native α_{s1}- and β-caseins were degraded according to the ripening time. At the end of ripening for both bacterial strains, more than half of the α_{s1}-casein was hydrolyzed; α_{s2}-casein was completely degraded [12] whereas around 40% of the β-casein was hydrolyzed. At the end of ripening time, LH77 cheeses were associated with the highest amount of γ-caseins. When stretching values are projected as supplementary information, a positive correlation is observed between stretching and γ-caseins in agreement with the composition of LH77 cheeses. However, the stretchability was inversely correlated with both the residual β- and α_{s1}-caseins (Fig. 2a). Moreover, the stretchability was correlated with the presence of the peptide α_{s1}-CN(24-199) that is known to have particular functional properties such as softening properties and that could be involved in stretchability as well [21–23].

The evolution of stretchability was the same for both cheeses according to the ripening time [12]. As of D20, the stretchability of both cheeses increased and was higher for the cheese produced from *L. helveticus* ITGLH77 than for the cheese produced from *L. helveticus* ITGLH1. The highest stretchability was obtained on D41 for both cheeses. The highest stretchability of cheese manufactured using *L. helveticus* ITGLH77, compared to the cheese manufactured using *L. helveticus* ITGLH1, was attributed to the least overall proteolytic activity of this bacterial strain [12]. *L. helveticus* ITGLH1 is more proteolytic than *L. helveticus* ITGLH77 since LH1 has four proteases whereas *L. helveticus* ITHGLH77 has only one [24]. Moreover, LH1 lyses more rapidly than LH77 and, consequently, releases internal peptidases that contribute to the overall proteolysis in the cheese. Proteinases of both bacterial strains have some common and some different splitting sites. Consequently, the degradation profiles of α_{s1}- and β-caseins by *L. helveticus* ITGLH1 and *L. helveticus* ITGLH77 are different [25], as are the peptide profiles. The representation of the cheese analyses on the PCA variable factor

map confirms that cheese stretchability was strongly correlated with the proteolysis parameters [26]. However, a moderated proteolysis promotes cheese stretchability. Since both cheeses were manufactured using the same milk, the only difference found between them concerned their composition in peptides. It is therefore crucial to determine the peptides that are closely related to stretchability from among all the peptides produced in cheese in order to be able to further orient proteolysis. These peptides could actually be the key factors in maintaining the structure of the cheese matrix with the residual native caseins and could consequently modulate the stretchability of these cheeses.

According to the specificity and activity of the proteolytic enzymes found in cheese arising from *L. helveticus* as well as from the other microflora or endogenous enzymes from milk, the peptide profiles in cheese were modified on the shorter or longer term during ripening time. For both LH1 and LH77 cheeses, some peptides were significantly different between D1, D13 and D41 (Table 1). Since proteolysis is a continuous process, one peptide can be present in cheese during various period of ripening. However, this depends on whether this peptide is a primary product of hydrolysis of the caseins, which can be further degraded, or a final one. Therefore, such a complex mix of peptides makes it difficult to find a simple way to establish the relationship between a pool of peptides and the cheese stretchability.

3.1.2. *The chromatographic peptide fraction C is strongly correlated with the stretchability*

Chromatography analysis is the main way to quantify peptides, as already shown in dairy products [20,27], in which peptides were identified. The area intensities of three fractions, A, B and C, collected for peptide identification, were measured using the reversed-phase chromatographic profiles. These variables were added to the previous data set and were submitted to PCA in order to show the impact of the peptide identification on the cheese distribution. For this second PCA, the first component (PC1) explains most of the variation (77.96%) whereas the second component (PC2) explains 9.39% of the variability. The overall PCA analysis was very similar to the previous one (Fig. 2) showing that PC1 was again related to the proteolysis whereas PC2 may reflect the difference between the type of bacterial proteinases and consequently the change in the peptide composition (Fig. 4a). The main change compared to Fig. 2 is that LH77 cheeses were associated not only with the highest content of both γ-caseins, but also with fractions B and C at the end of cheese ripening, whereas LH1 cheeses were related to fraction A (Fig. 4b). When stretching values were projected as a supplementary data, a positive correlation was observed between this data and both γ-caseins and fractions B and C, in agreement with the composition of LH77 cheeses (Fig. 4a). Fraction C is strongly correlated with stretchability, but it is possible that part of the peptides present in fraction B contributes to cheese stretchability as well. Since fraction B contains numerous peptides there is certainly only one part of them that is involved in cheese stretchability.

The addition of the chromatographic fractions in the PCA also induced changes in cheese distribution on the individual factor map (Fig. 4b). Thus, as of D 27, the distance between the individual cheeses was enlarged on the PCA map, according to the bacterial strain. This means that peptides present in the aqueous extract of cheese represent valuable information linked to cheese stretchability and especially those peptides present in fractions B and C. This PCA also suggested that both the type of proteases from each lactobacilli strain and the overall proteolysis rate affect cheese stretchability. Consequently, the type and the number of proteases, as well as the capability of the lactobacilli strains to die and to release other intracellular enzymes [12] clearly affect the

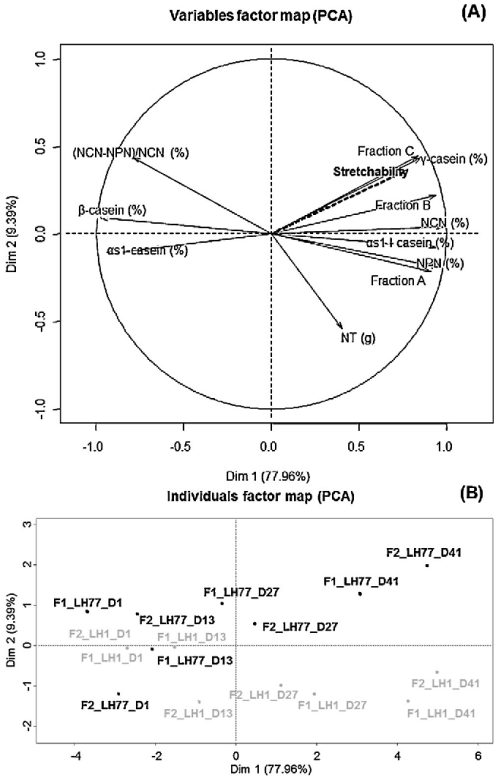

Fig. 4. (A) Biochemical characteristics of cheeses, for both strains LH1 and LH77 at D1, D13, D27 and D41, and the chromatographic peptide fractions represented on a principal component analysis variables factor map. α_{s1}- and β-caseins are quantitative variables that represent the amount of residual native caseins. α_{s1}-I casein: relative percentage of peptide α_{s1}-(f24-199) derived from the hydrolysis of α_{s1}-casein by chymosin; γ-casein: relative percentage of peptides γ-casein derived from the hydrolysis of β-casein by plasmin; NPN: percentage of non-protein nitrogen; NT: amount of total nitrogen; (NCN-NPN)/NCN: percentage of residual large peptides; Fraction A: total area of the first fraction of the HPLC chromatography; Fraction B: total area of the second fraction of the HPLC chromatography; Fraction C: total area of the third fraction of the HPLC chromatography. The stretchability is not included as a variable but is projected as a supplementary data on the PCA. (B) Distribution of cheeses according to their biochemical characteristics and their chromatographic peptide fractions on a Principal Component Analysis individual factors map. F1: cheese manufacture 1; F2: cheese manufacture 2; LH1: *Lactobacillus helveticus* ITGLH1; LH77: *Lactobacillus helveticus* ITGLH77; D1: day 1; D13: day 13; D27: day 27; D41: day 41; in grey: the LH1 cheeses; in black: the LH77 cheeses.

distribution of cheeses on the PCA individual factor map, thus impacting stretchability.

3.2. *A specific peptide profile is related to each cheese*

Numerous peptides were identified (30 peptides in fraction A, 406 peptides in fraction B, and 139 peptides in fraction C) in the cheeses using chromatographic fractions. To determine those that are really implicated in stretchability, a correspondence analysis representing the distribution of cheeses according to their peptide composition was used (Fig. 5). Whey proteins are not quite hydrolysed in cheese due to their globular structure [20], which is the reason why they were not identified and cannot be included in the

Table 1
List of peptides present or absent at each time of ripening.

	Day of ripening					
	Day 1		Day 13		Day 41	
Names of peptides	β-CN(194–208)	1	β-CN(80–90)	1	β-CN(74–88)	1
	β-CN(185–192)	1	β-CN(47–52)	1	β-CN(74–81)	1
	β-CN(84–93)	0	β-CN(32–58)	1	β-CN(30–72)	1
	β-CN(74–82)	0	β-CN(195–207)	1	β-CN(191–206)	1
	β-CN(53–72)	0	β-CN(195–202)	1	β-CN(190–209)	1
	β-CN(35–52)	0	β-CN(194–207)	1	β-CN(107–119)	1
	β-CN(32–52)	0	β-CN(194–206)	1	β-CN(129–141)	0
	β-CN(30–52)	0	β-CN(167–176)	1	α_{s2}-CN(197–203)	1
	β-CN(29–52)	0	β-CN(135–141)	1	α_{s2}-CN(176–182)	1
	β-CN(195–206)	0	β-CN(126–133)	1	α_{s2}-CN(153–163)	1
	β-CN(111–119)	0	α_{s2}-CN(101–115)	1	α_{s2}-CN(151–161)	1
	β-CN(103–113)	0	α_{s1}-CN(181–189)	1	α_{s2}-CN(150–161)	1
	α_{s2}-CN(101–114)	0	α_{s1}-CN(10–23)	0	α_{s2}-CN(105–114)	1
	α_{s1}-CN(24–30)	0			α_{s2}-CN(100–115)	1
	α_{s1}-CN(17–23)	0			α_{s1}-CN(9–23)	1
					α_{s1}-CN(9–22)	1
					α_{s1}-CN(9–16)	1
					α_{s1}-CN(8–23)	1
					α_{s1}-CN(8–21)	1
					α_{s1}-CN(8–17)	1
					α_{s1}-CN(8–16)	1
					α_{s1}-CN(27–34)	1
					α_{s1}-CN(25–36)	1
					α_{s1}-CN(25–34)	1
					α_{s1}-CN(24–37)	1
					α_{s1}-CN(24–34)	1
					α_{s1}-CN(16–23)	1
					α_{s1}-CN(15–23)	1
					α_{s1}-CN(14–22)	1
					α_{s1}-CN(13–23)	1
					α_{s1}-CN(11–23)	1
					α_{s1}-CN(1–13)	0

1: presence only at this time; 0: absence only at this time.

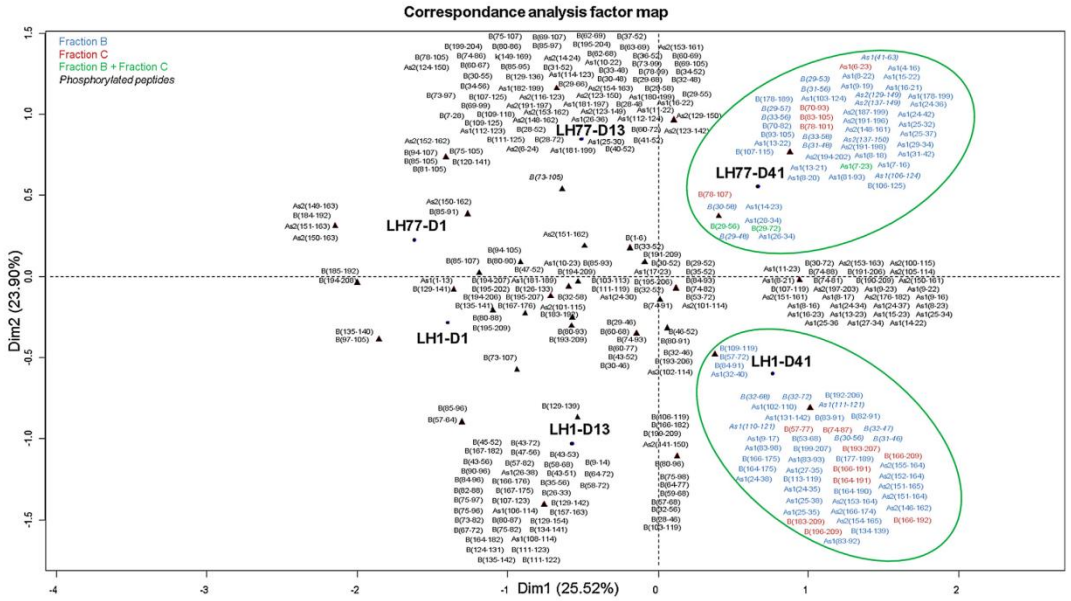

Fig. 5. Distribution of cheeses according to their peptide composition on a Correspondence Analysis factor map. LH1: *Lactobacillus helveticus* ITGLH1; LH77: *Lactobacillus helveticus* ITGLH77; D1: day 1; D13: day 13; D27: day 27; D41: day 41. As1: peptides derived from α_{s1}-casein; As2: peptides derived from α_{s2}-casein; B: peptides derived from β-casein; peptides in black: peptides present in the cheeses; peptides in blue: peptides in fraction B; peptides in red: peptides in fraction C, which is strongly correlated with stretchability; peptides in green: peptides of both fraction B and C. Peptides surrounded by a green circle are peptides that are found mainly for each cheese LH77–D41 and LH1–D41.

list of peptides related to stretchability. Moreover, it is not very probable that the native whey proteins that are entrapped in the cheese interact with peptides since whey protein-casein interactions are not favoured because of their globular form.

The CA summarizes the link between two qualitative variables through a representation of the categories associated with each qualitative variable. In our case, CA provides a representation of the cheeses and the peptides as function of the presence/absence of each peptide in each cheese. In Fig. 5, the two dimensions account together for 49.42% of the total variability. The first axis explains 25.52% of the total variability and is related to the proteolysis; the second axis represents 23.9% of the total variability and is related to the difference between the two types of bacterial proteases (Fig. 5). Cheeses were distributed according to their degree of proteolysis on the first axis, on which cheeses at D1 and D13 were negatively scored and cheeses at D41 were positively scored. Cheeses were also distributed according to the bacterial strains used, on the second axis, on which the LH1 cheeses were negatively scored and the LH77 cheeses were positively scored. Fig. 5 reveals that a pool of peptides was representative of each cheese.

Peptides belonging to the LH1-D41 cheese and LH77-D41 cheese on this CA factor map had heterogeneous physicochemical and structural characteristics, both in the same cheese and between both cheeses (Fig. A and Table A in the supplementary data). Some of them were derived from α_{s2}-casein. The peptides derived from α_{s2}-casein and present in LH77-D41 cheese are larger than peptides derived from α_{s2}-casein that are present in LH1-D41 cheese. Since α_{s2}-casein is able to form specific supramolecular structures, such as amyloid fibrils under defined conditions [28,29], it could be assumed that peptides derived from this casein are able to interact with each other or with native residual casein to give specific functionalities to the matrix, such as stretchability. Moreover, this CA factor map showed that LH77-D41 cheese had more peptides derived from α_{s1}-casein than LH1-D41 (Fig. A and Table A in the supplementary data). For both cheeses, most of the peptides have more than ten amino acid residues, in agreement with the fact that large peptides (in most cases, with more than twenty amino acids) have been shown to enhance the functional properties of proteins [20,26,27,30–35].

3.3. When they are large enough, both hydrophilic and hydrophobic peptides are predominantly involved in the stretchability of cheeses at D41

Since stretchability was the highest at D41 for both cheeses manufactured using *L. helveticus* ITGLH1 and *L. helveticus* ITGLH7, the characteristics of the peptides identified only at this ripening time were studied in more depth in terms of: (i) size; (ii) pI; (iii) hydropathy index; (iv) number of positive charges; (v) number of negative charges (Fig. 6a) and (vi) their secondary structures (Fig. 6b). Actually, the amino acid nature [36], the size of the sequence [35–40], the charge and the periodicity of the hydrophobic/hydrophilic amino acids in the sequence [38,41–43] determine the secondary structure of the peptides. According to their secondary structures and both the peptide concentration and the environmental conditions, peptides interact with each other *via* electrostatic and van der Waals interactions and even hydrogen and disulphide bonds to form supramolecular structures [44]. It has been demonstrated that even short peptides (3–6 amino acids) have a secondary structure and are able to self-assemble [39].

In this study, for both cheeses, peptides only present at D41 (Table 1) had similar physicochemical and structural characteristics (Fig. 6a), except for the peptide β-CN(30-72) found in both LH1-D41 cheese and LH77-D41 cheese. They were small (less than twenty amino acids), had few charges (less than four) and had a pI close to 5 close to that of the native caseins. Most of them were hydrophilic. Fig. 6b shows that these peptides predominantly formed α-helix or random-coil structures. These peptides certainly play a key role in cheese stretchability and may be able to interact with each other and with the native caseins to form macroscopic strands during cheese stretching.

When the peptides present in fraction C of LH1-D41 cheese and LH77-D41 cheese were analyzed more deeply (Fig. 7), peptides derived from α_{s1} - casein were found in smaller amounts in fraction C of the aqueous extract than peptides derived from β-casein. Generally speaking, α_{s1}- and α_{s2}-caseins were less hydrolysed than β-caseins by both bacterial strains. This was in agreement with a lower accessibility of the proteases to the caseins in dairy matrices compared to pure caseins in solution [12,25]. The physicochemical and structural characteristics of peptides were very different, both between peptides present in the same cheese and between peptides present in the two cheeses (Figs. 7a and b). Both LH1-D41 cheese and LH77-D41 cheese had a high stretchability. Since the two cheeses were manufactured using two bacterial strains from the same species with different proteolytic activities, the peptides produced may be more or less common between cheeses. Hence, their physicochemical characteristics appear to be similar to induce cheese stretchability. Nevertheless, the predicted secondary structure was quite different between both pools of peptides (Fig. 7b). Peptides in LH1-D41 cheese had more random coils than other structures whereas peptides in LH77-D41 had as many α-helixes as random coils. This supports the hypothesis that peptides with particular structures self-assemble to form supramolecular structure such as fibrils [28,45–47] and lead to specific functionalities.

While fraction C was strongly correlated with stretchability fraction B was also correlated with this macroscopic property of cheese and it is therefore assumed that part of peptides included in this fraction are also involved in the stretchability. The peptides of fraction B, which are located close to the points LH77-D41 and LH1-D41 in Fig. 5, revealed varied characteristics (Fig. B in the supplementary data). On average, peptides produced by both bacterial strains are weakly charged, are large enough to interact with proteins (more than ten amino acids), are hydrophilic and form more random coils than α-helices or β-strands. Moreover, three peptides found in LH77-D41 cheese are commons to both fractions B and C: α_{s1}-CN(7-23), β-CN(29-56) and β-CN(29-72). They are large (17, 28 and 34 amino acids, respectively) and are hydrophilic. Moreover, peptides β-CN(29-56) and β-CN(29-72) are monophosphorylated. Since caseins are phosphorylated proteins, their hydrolysis produces several phosphorylated peptides (Table C in the supplementary data). Some secondary structures could be involved for some functionalities, implying mineral interactions that could unfortunately not be taken into account by ProtParam software. Consequently, the phosphoryl residues should confer particular physicochemical properties to peptides especially in terms of their cation binding ability. The different ionization states of peptides lead to different affinities towards cations [48]. For example, the higher the number of phoshoseryl residues is, the stronger the interaction strength with colloidal calcium phosphate will be [49]. Consequently, in a mineralized medium such as cheese, the phosphorylated peptides can interact more easily and could induce particular functional properties. Thirteen phosphorylated peptides were identified close to the LH77-D41 point in Fig. 5, including two peptides derived from α_{s1}-casein hydrolysis, three peptides derived from α_{s2}-casein hydrolysis and eight peptides derived from β-casein hydrolysis. There are fewer peptides with phosphorylations close to the LH1-D41 point in Fig. 5, than close to the LH77-D41 point. Two of them are derived from α_{s1}-casein hydrolysis and four of them are derived from β-casein hydrolysis. Since phosphorylated peptides are more numerous in LH77-D41 than in LH1-D41, this could mean that phosphorylations

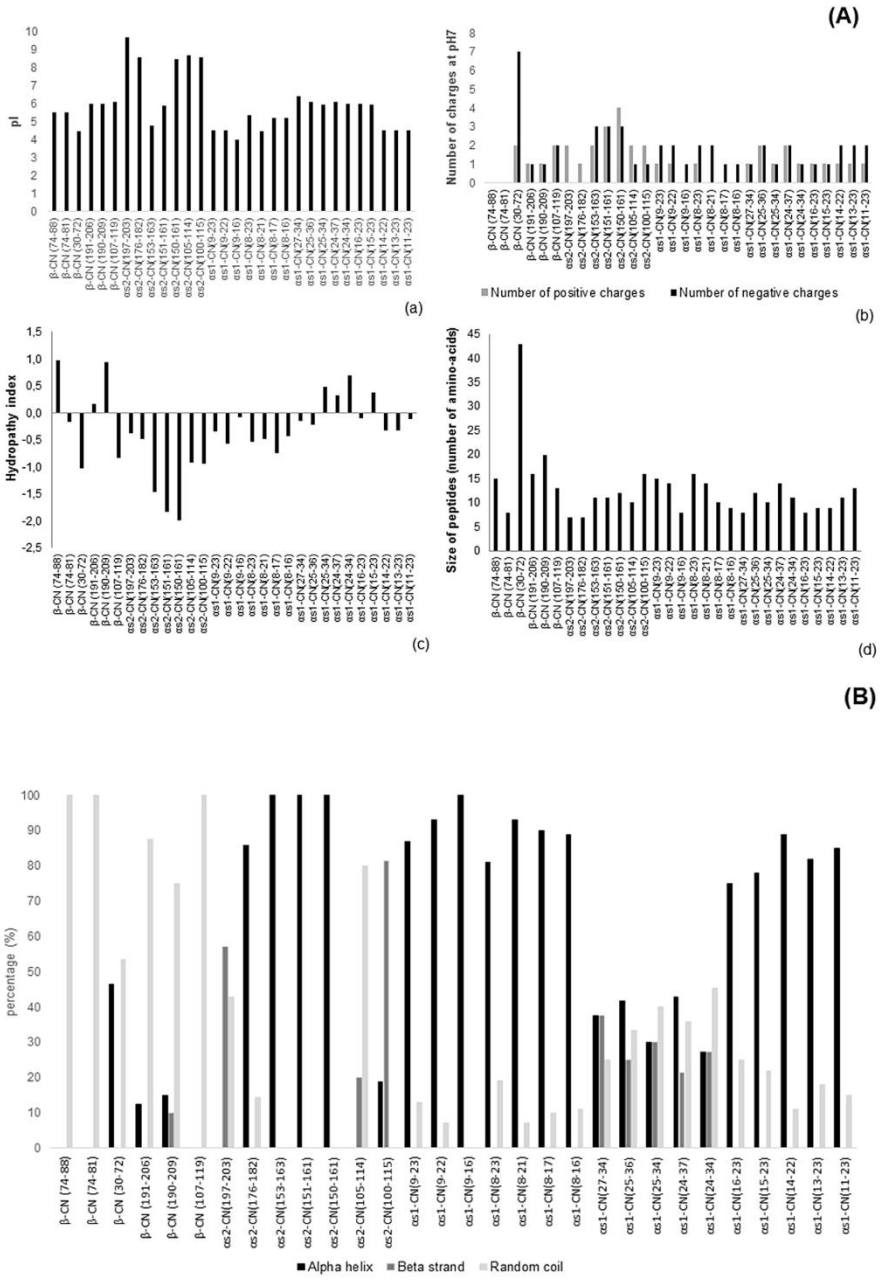

Fig. 6. (A) Physicochemical characteristics of peptides only present at day 41 in Table 1, including all cheeses: (a) isoectric point (pI); (b) number of both positive (in grey) and negative (in black) charges calculated at pH7; (c) hydropathy index and (d) size. (B) Structural characteristics of peptides only present at day 41 in Table 1, including all cheeses. In black: the α-helix; in dark grey: the β-strand; in light grey: the random coil.

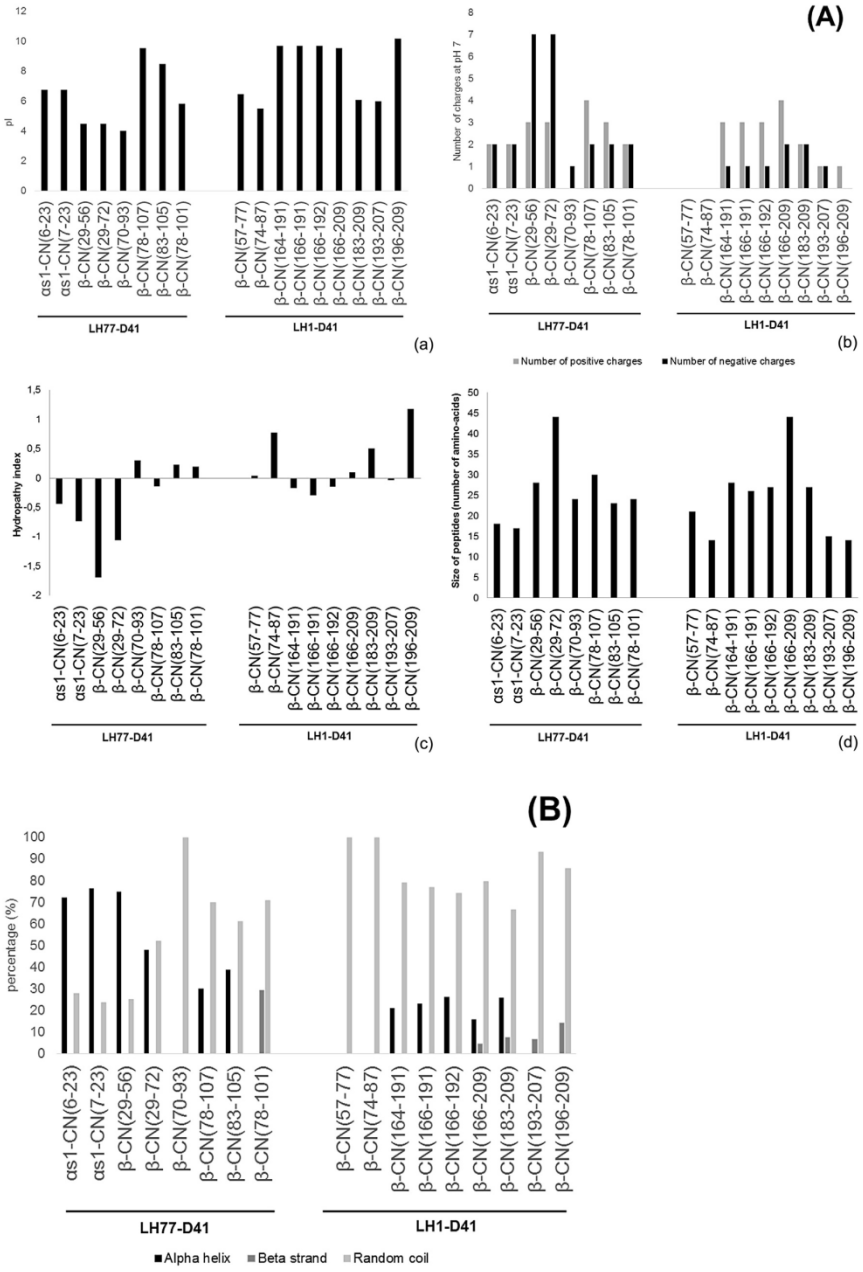

Fig. 7. (A) Physicochemical characteristics of peptides found in both LH1-D41 cheese and LH77-D41 cheese on the CA factor map (Fig. 5) and in the chromatographic fraction C. (a) The isoelectric point (pI) of peptides; (b) the number of both positive (in grey) and negative (in black) charges calculated at pH7 of peptides; (c) the hydropathy index of peptides; (d) the size of peptides. On the left, peptides from LH77 cheese at day 41; on the right, peptides form LH1 cheese at day 41. (B) Structural characteristics of peptides found in both LH1-D41 cheese and LH77-D41 cheeses on the CA factor map (Fig. 5) and in the chromatographic fraction C. In black: the α-helix; in dark grey: the β-strand; in light grey: the random coil.

could play a key role in the increase of stretchability at the end of cheese ripening.

In this case, to induce cheese stretchability, peptides are assumed to be large enough (more than fifteen amino acids) to interact with native residual caseins and to maintain a structure in the cheese thread. Peptides could also have phosphorylation, could be weakly charged at neutral pH, and some could be hydrophilic, whereas others could be hydrophobic. Nevertheless, no information is available about the charge distribution that could have an impact on peptide-peptide interactions. It is also not excluded that some peptides such as phosphorylated ones have charges that are more present in one domain than in another. The presence of such charged peptides could lead to electrostatic interactions, and the presence of both hydrophilic and hydrophobic peptides could tend to form α-helices, which was shown in LH77 cheeses, with greater stretchability properties. Moreover, specific secondary structures, such as random coil structures could favour the flexibility of the peptide chain and increase the probability of interactions with other peptides or proteins to form the strands.

4. Conclusion and perspectives

On the basis of the quantification of proteolysis parameters such as casein degradation, the percentage of appearance of both γ-casein and α_{s1}-I-casein, and the intensity of each peptide's chromatographic fractions, the relationships between a pool of peptides, their characteristics and the techno-functionality of the food matrix could be established.

A more complete view of the peptide profile that may be involved in a techno-functional property of a food product, such as the stretchability of cheese shown in this study, was obtained by a combination of multivariate analysis on both the biochemical analysis of cheese and the peptide physicochemical and structural characteristics of the peptide. This method showed that a profile of peptides is linked to a macroscopic property rather than to one particular peptide. The pool of peptides that is predominantly involved in cheese stretchability has peptides with heterogeneous physicochemical and structural characteristics. Nonetheless, certain characteristics are common to these peptides: they all have more than fifteen amino acids, are either hydrophilic or hydrophobic and form α-helix and random coil structures.

The implication of these peptides, with such physicochemical and structural characteristics, has to be confirmed *in situ*. This will be the next step, given that there are also other parameters involved in stretchability, such as the amount of residual caseins or the cheese physico-chemical environmental conditions. It should be noted that, whereas a clear correlation between two factors is established, it cannot assure that there is a cause and effect relationship between the two.

This method can be applied to other types of peptides and other food techno-functionalities in other food matrices.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfa.2014.10.052.

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Appendix A. Supplementary data

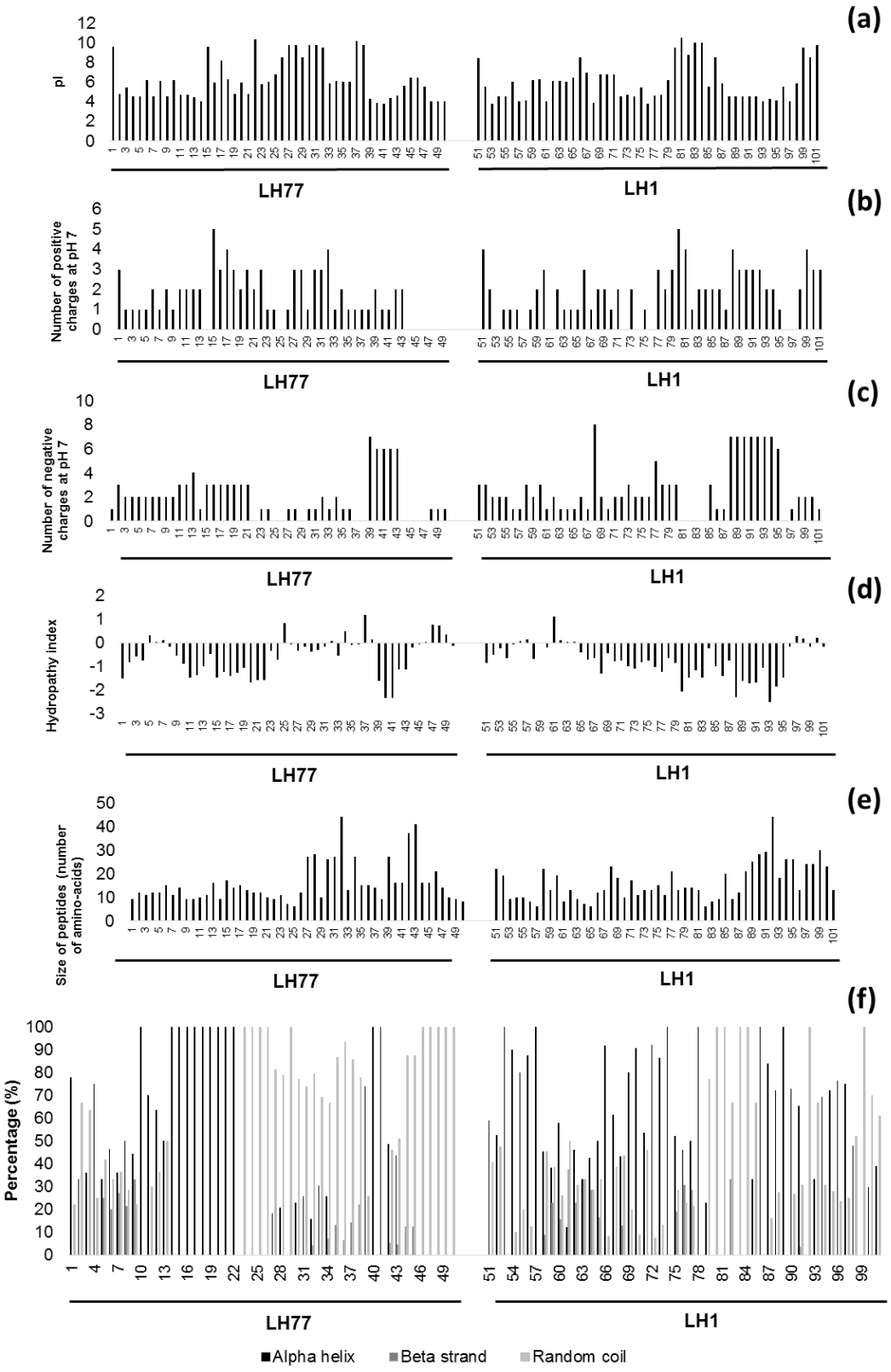


Fig. A: Physico-chemical characteristics of peptides belonging to LH1-D41 cheese and LH77-D41 cheese on the CA factor map (Fig. 4). (a) the isoelectric point (pI) of peptides; (b) the number of positive charges of peptides calculated at pH7; (c) the number of negative charges of peptides calculated at pH7; (d) the hydropathy index of peptides; (e) the size of peptides; (f) the secondary structure of peptides: in black: the α-helix; in dark grey: the β-strand; in clear grey: the random coil. One peptide is represented by one number as in Table A of the supplementary data.

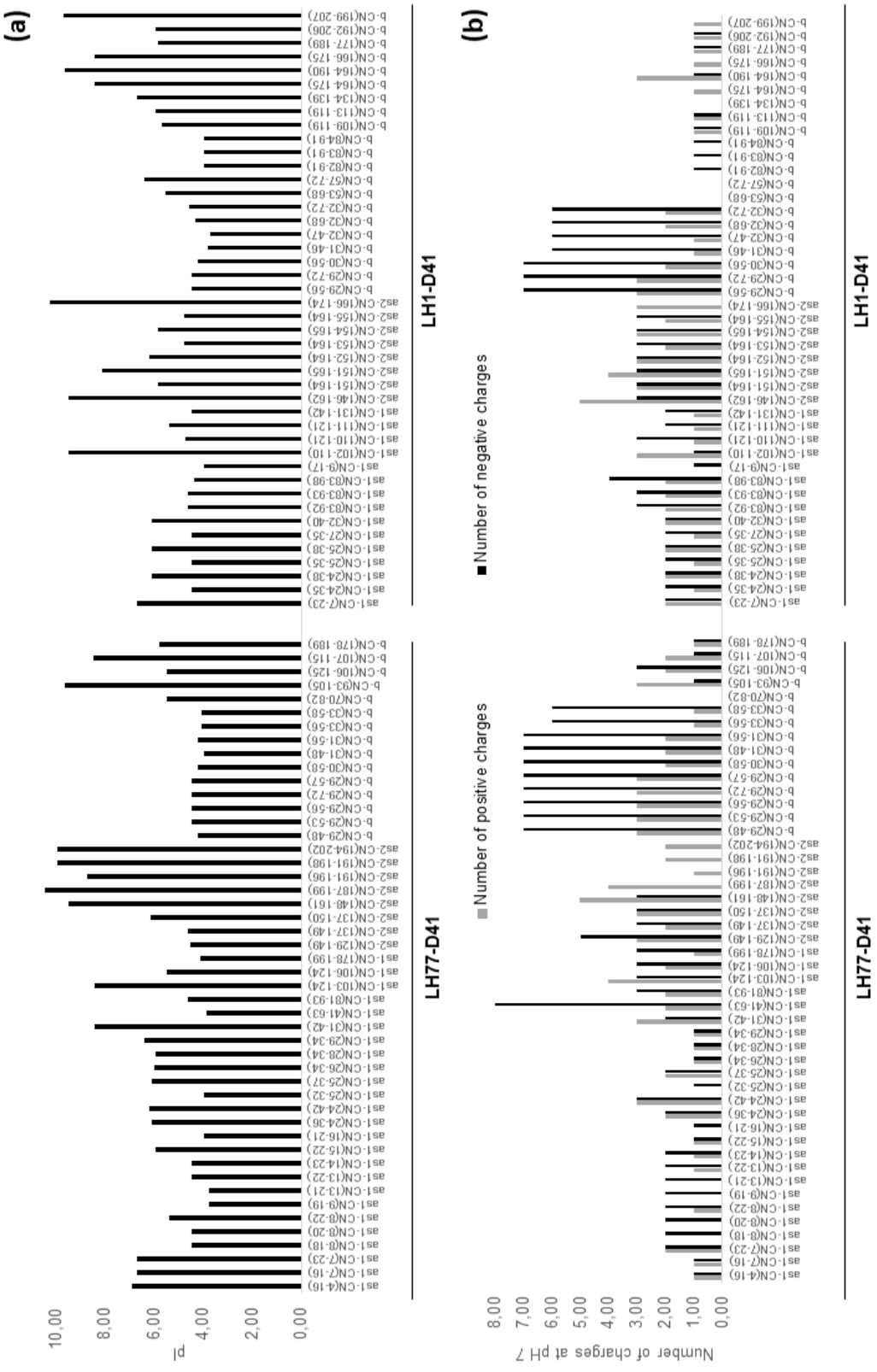
Table A. Peptides belonging to LH1-D41 cheese and LH77-D41 cheese on the CA factor map (Fig. 3). One number is linked to one peptide for the representation on the Fig. A of the supplementary data.

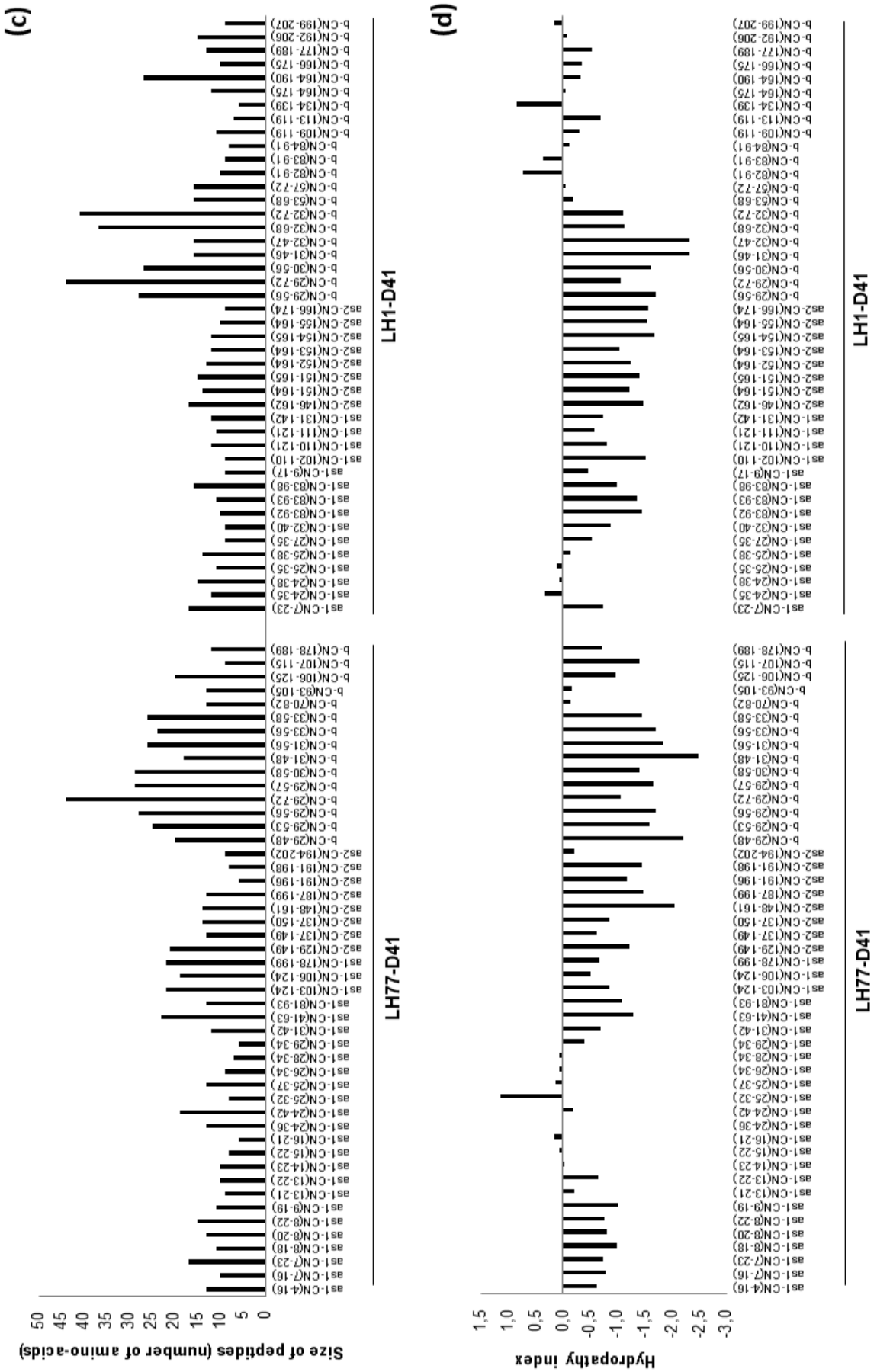
Cheese	Number of peptides on the supplementary data	Name of peptide
LH1-D41	1	α_{s1}-CN(102-110)
LH1-D41	2	α_{s1}-CN(110-121)
LH1-D41	3	α_{s1}-CN(111-121)
LH1-D41	4	α_{s1}-CN(131-142)
LH1-D41	5	α_{s1}-CN(24-35)
LH1-D41	6	α_{s1}-CN(24-38)
LH1-D41	7	α_{s1}-CN(25-35)
LH1-D41	8	α_{s1}-CN(25-38)
LH1-D41	9	α_{s1}-CN(27-35)
LH1-D41	10	α_{s1}-CN(32-40)
LH1-D41	11	α_{s1}-CN(83-92)
LH1-D41	12	α_{s1}-CN(83-93)
LH1-D41	13	α_{s1}-CN(83-98)
LH1-D41	14	α_{s1}-CN(9-17)
LH1-D41	15	α_{s2}-CN(146-162)
LH1-D41	16	α_{s2}-CN(151-164)
LH1-D41	17	α_{s2}-CN(151-165)
LH1-D41	18	α_{s2}-CN(152-164)
LH1-D41	19	α_{s2}-CN(153-164)
LH1-D41	20	α_{s2}-CN(154-165)
LH1-D41	21	α_{s2}-CN(155-164)
LH1-D41	22	α_{s2}-CN(166-174)
LH1-D41	23	β-CN(109-119)
LH1-D41	24	β-CN(113-119)
LH1-D41	25	β-CN(134-139)
LH1-D41	26	β-CN(164-175)
LH1-D41	27	β-CN(164-190)
LH1-D41	28	β-CN(164-191)
LH1-D41	29	β-CN(166-175)
LH1-D41	30	β-CN(166-191)
LH1-D41	31	β-CN(166-192)
LH1-D41	32	β-CN(166-209)
LH1-D41	33	β-CN(177-189)
LH1-D41	34	β-CN(183-209)
LH1-D41	35	β-CN(192-206)
LH1-D41	36	β-CN(193-207)
LH1-D41	37	β-CN(196-209)
LH1-D41	38	β-CN(199-207)
LH1-D41	39	β-CN(30-56)
LH1-D41	40	β-CN(31-46)
LH1-D41	41	β-CN(32-47)
LH1-D41	42	β-CN(32-68)

LH1-D41	43	β-CN(32-72)
LH1-D41	44	β-CN(53-68)
LH1-D41	45	β-CN(57-72)
LH1-D41	46	β-CN(57-77)
LH1-D41	47	β-CN(74-87)
LH1-D41	48	β-CN(82-91)
LH1-D41	49	β-CN(83-91)
LH1-D41	50	β-CN(84-91)
LH77-D41	51	α_{s1}-CN(103-124)
LH77-D41	52	α_{s1}-CN(106-124)
LH77-D41	53	α_{s1}-CN(13-21)
LH77-D41	54	α_{s1}-CN(13-22)
LH77-D41	55	α_{s1}-CN(14-23)
LH77-D41	56	α_{s1}-CN(15-22)
LH77-D41	57	α_{s1}-CN(16-21)
LH77-D41	58	α_{s1}-CN(178-199)
LH77-D41	59	α_{s1}-CN(24-36)
LH77-D41	60	α_{s1}-CN(24-42)
LH77-D41	61	α_{s1}-CN(25-32)
LH77-D41	62	α_{s1}-CN(25-37)
LH77-D41	63	α_{s1}-CN(26-34)
LH77-D41	64	α_{s1}-CN(28-34)
LH77-D41	65	α_{s1}-CN(29-34)
LH77-D41	66	α_{s1}-CN(31-42)
LH77-D41	67	α_{s1}-CN(4-16)
LH77-D41	68	α_{s1}-CN(41-63)
LH77-D41	69	α_{s1}-CN(6-23)
LH77-D41	70	α_{s1}-CN(7-16)
LH77-D41	71	α_{s1}-CN(7-23)
LH77-D41	72	α_{s1}-CN(8-18)
LH77-D41	73	α_{s1}-CN(81-93)
LH77-D41	74	α_{s1}-CN(8-20)
LH77-D41	75	α_{s1}-CN(8-22)
LH77-D41	76	α_{s1}-CN(9-19)
LH77-D41	77	α_{s2}-CN(129-149)
LH77-D41	78	α_{s2}-CN(137-149)
LH77-D41	79	α_{s2}-CN(137-150)
LH77-D41	80	α_{s2}-CN(148-161)
LH77-D41	81	α_{s2}-CN(187-199)
LH77-D41	82	α_{s2}-CN(191-196)
LH77-D41	83	α_{s2}-CN(191-198)
LH77-D41	84	α_{s2}-CN(194-202)
LH77-D41	85	β-CN(106-125)
LH77-D41	86	β-CN(107-115)
LH77-D41	87	β-CN(178-189)
LH77-D41	88	β-CN(28-48)
LH77-D41	89	β-CN(29-53)

LH77-D41	90	β-CN(29-56)
LH77-D41	91	β-CN(29-57)
LH77-D41	92	β-CN(29-72)
LH77-D41	93	β-CN(31-48)
LH77-D41	94	β-CN(31-56)
LH77-D41	95	β-CN(33-58)
LH77-D41	96	β-CN(70-82)
LH77-D41	97	β-CN(70-93)
LH77-D41	98	β-CN(78-101)
LH77-D41	99	β-CN(78-107)
LH77-D41	100	β-CN(83-105)
LH77-D41	101	β-CN(93-105)

Appendix B. Supplementary data




(c)
Size of peptides (number of amino-acids)
0
5
10
15
20
25
30
35
40
45
50
LH77-D41
LH1-D41
(d)
Hydropathy index
-3,0
-2,5
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
LH77-D41
LH1-D41

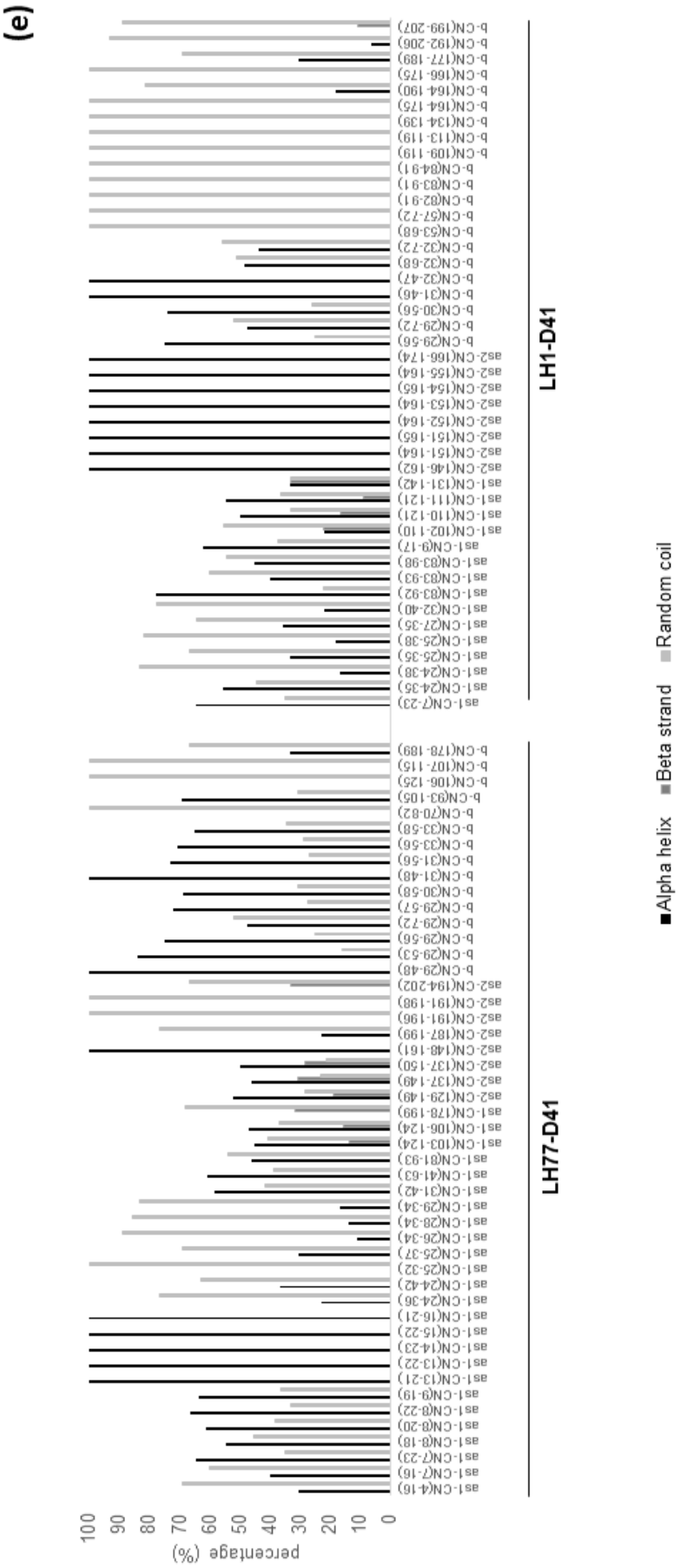

(e)
LH1-D41
LH77-D41
Alpha helix
Beta strand
Random coil
percentage (%)
0
10
20
30
40
50
60
70
80
90
100

Fig. B. Physicochemical and structural characteristics of peptides found in both LH1-D41 cheese and LH77-D41 cheese on the CA factor map (Fig. 5) and in the chromatographic fraction B. (a) the isoelectric point (pI) of peptides; (b) the number of both positive (in grey) and negative (in black) charges calculated at pH7 of peptides; (c) the size of peptides; (d) the hydropathy index of peptides; (e) structural characteristics of peptides. On the left, peptides from LH77 cheese at day 41; on the right, peptides form LH1 cheese at day 41.

Appendix C. Supplementary data

Table C. List of phosphorylated peptides identified in fractions A, B and C during cheese ripening. P: one phosphorylation; 2P: two phosphorylations; 3P: three phosphorylations.

Bacterial strain	Day of ripening	Fraction	Peptide sequence	Phosphorylations
LH1	13	A	β-CN(32-46)	P
LH1	41	A	β -CN(32-46)	P
LH77	13	A	β -CN(33-48)	P
LH77	13	A	α_{s2}-CN(14-24)	P
LH77	13	A	α_{s2}-CN(123-142)	P
LH77	41	A	β -CN(33-48)	P
LH1	41	B	α_{s1}-CN(111-121)	P
LH1	41	B	α_{s1}-CN(110-121)	P
LH1	13	B	α_{s2}-CN(141-150)	P
LH1	41	B	α_{s2}-CN(141-150)	P
LH1	41	B	β -CN(31-46)	P
LH1	13	B	β -CN(33-52)	P
LH1	13	B	β -CN(30-46)	P
LH1	13	B	β -CN(30-52)	P
LH1	13	B	β -CN(32-52)	P
LH1	13	B	β -CN(32-56)	P
LH1	13	B	β-CN(32-58)	P
LH1	13	B	β-CN(29-46)	P
LH1	13	B	β-CN(29-52)	P
LH1	13	B	β-CN(28-46)	P
LH1	13	B	β-CN(35-52)	P
LH1	13	B	β-CN(35-56)	P
LH1	41	B	β-CN(30-46)	P
LH1	41	B	β-CN(30-52)	P
LH1	41	B	β-CN(30-56)	P
LH1	41	B	β-CN(30-58)	P
LH1	41	B	β-CN(30-72)	P
LH1	41	B	β-CN(32-46)	P
LH1	41	B	β-CN(32-47)	P
LH1	41	B	β-CN(32-52)	P
LH1	41	B	β-CN(32-56)	P
LH1	41	B	β-CN(32-72)	P
LH1	41	B	β-CN(32-68)	P
LH1	41	B	β-CN(29-46)	P
LH1	41	B	β-CN(29-48)	P
LH1	41	B	β-CN(29-52)	P

LH1	41	B	β-CN(29-56)	P
LH1	41	B	β-CN(29-72)	P
LH1	41	B	β-CN(28-46)	P
LH1	41	B	β-CN(35-52)	P
LH77	13	B	α_{s1}-CN(114-123)	P
LH77	13	B	α_{s1}-CN(112-123)	P
LH77	13	B	α_{s1}-CN(112-124)	P
LH77	41	B	α_{s1}-CN(41-63)	2P
LH77	41	B	α_{s1}-CN(103-124)	P
LH77	41	B	α_{s1}-CN(112-124)	P
LH77	41	B	α_{s1}-CN(106-124)	P
LH77	13	B	α_{s2}-CN(6-24)	P
LH77	13	B	α_{s2}-CN(123-149)	P
LH77	13	B	α_{s2}-CN(123-150)	P
LH77	41	B	α_{s2}-CN(137-149)	P
LH77	41	B	α_{s2}-CN(137-150)	P
LH77	41	B	α_{s2}-CN(123-142)	P
LH77	41	B	α_{s2}-CN(123-142)	2P
LH77	41	B	α_{s2}-CN(123-149)	3P
LH77	41	B	α_{s2}-CN(123-150)	3P
LH77	41	B	α_{s2}-CN(129-149)	2P
LH77	41	B	α_{s2}-CN(129-150)	2P
LH77	13	B	β-CN(31-52)	P
LH77	41	B	β-CN(31-48)	P
LH77	41	B	β-CN(31-56)	P
LH77	41	B	β-CN(33-48)	P
LH77	13	B	β-CN(33-52)	P
LH77	13	B	β-CN(30-48)	P
LH77	13	B	β-CN(30-52)	P
LH77	13	B	β-CN(30-55)	P
LH77	13	B	β-CN(30-58)	P
LH77	13	B	β-CN(30-46)	P
LH77	13	B	β-CN(32-48)	P
LH77	13	B	β-CN(32-52)	P
LH77	13	B	β-CN(32-58)	P
LH77	13	B	β-CN(29-46)	P
LH77	13	B	β-CN(29-48)	P
LH77	13	B	β-CN(29-52)	P
LH77	13	B	β-CN(29-55)	P
LH77	13	B	β-CN(29-56)	P
LH77	13	B	β-CN(29-58)	P
LH77	13	B	β-CN(29-66)	P
LH77	13	B	β-CN(29-68)	P
LH77	13	B	β-CN(29-68)	P
LH77	13	B	β-CN(29-72)	P
LH77	13	B	β-CN(28-48)	P

LH77	13	B	β-CN(28-52)	P
LH77	13	B	β-CN(28-72)	P
LH77	13	B	β-CN(7-28)	P
LH77	13	B	β-CN(34-52)	P
LH77	13	B	β-CN(34-56)	P
LH77	13	B	β-CN(35-52)	P
LH77	41	B	β-CN(33-52)	P
LH77	41	B	β-CN(33-56)	P
LH77	41	B	β-CN(33-58)	P
LH77	41	B	β-CN(30-48)	P
LH77	41	B	β-CN(30-52)	P
LH77	41	B	β-CN(30-58)	P
LH77	41	B	β-CN(30-72)	P
LH77	41	B	β-CN(32-46)	P
LH77	41	B	β-CN(32-48)	P
LH77	41	B	β-CN(32-52)	P
LH77	41	B	β-CN(29-48)	P
LH77	41	B	β-CN(29-52)	P
LH77	41	B	β-CN(29-53)	P
LH77	41	B	β-CN(29-55)	P
LH77	41	B	β-CN(29-56)	P
LH77	41	B	β-CN(29-57)	P
LH77	41	B	β-CN(29-58)	P
LH77	41	B	β-CN(29-68)	P
LH77	41	B	β-CN(29-72)	P
LH77	41	B	β-CN(28-48)	P
LH77	41	B	β-CN(34-52)	P
LH77	41	B	β-CN(35-52)	P
LH1	41	C	β-CN(29-72)	P
LH77	13	C	β-CN(29-56)	P
LH77	41	C	β-CN(29-72)	P

Epilogue

This study showed that there was a correlation between the nature of peptides produced and the techno-functional property of the cheese. It has permitted to select a profile of peptides: mix of large peptides (more than fifteen amino acids) hydrophilic and hydrophobic.

In order to be able to produce hydrolysates with similar profiles, we chose first to use *in silico* hydrolysis of the caseins by proteolytic enzymes with known specificities. This allows having direct information on the sequence of peptides that could be theoretically produced with intermediate and/or end-products depending on the parameters used in the software. Thus, knowing the sequence of the four types of caseins, α_{s1}, α_{s2}, β and κ-caseins, we were able to hydrolyze them by 9 enzymes with known cutting sites (Table 2) according to PeptideMass software on ExPASy website (http://www.expasy.org). We compared with the same statistical tools the peptides produced in order to select the enzymes the most appropriate to produce peptide with the corresponding profile.

Table 2. Cleavage rules for PeptideMass program.

Enzyme or Reagent	Cleaves where?	Exceptions
Trypsin	C-terminal side of K or R	if P is C-term to K or R
Trypsin (C-term to K/R, even before P)	C-terminal side of K or R	
Trypsin (higher specificity)	C-terminal side of K or R	if P is C-term to K or R; after K in CKY, DKD, CKH, CKD, KKR; after R in RRH, RRR, CRK, DRD, RRF, KRR
Lys C	C-terminal side of K	
Lys N	N-terminal side of K	
Arg C	C-terminal side of R	if P is C-term to R
Asp N	N-terminal side of D	
Asp N / Lys C	N-terminal side of D, C-terminal side of K	
Asp N + N-terminal Glu	N-terminal side of D or E	
Asp N / Glu C (bicarbonate)	N-terminal side of D, C-terminal side of E	
Glu C (bicarbonate)	C-terminal side of E	if P is C-term to E, or if E is C-term to E
Glu C (phosphate)	C-terminal side of D or E	if P is C-term to D or E, or if E is C-term to D or E
Glu C (phosphate) + Lys C	C-terminal side of D, E and K	if P is C-term to D or E, or if E is C-term to D or E
Microwave-assisted formic acid hydrolysis (C-term to D)	C-terminal side of D	
Chymotrypsin (C-term to F/Y/W/M/L, not before P, not after Y if P is C-term to Y)	C-terminal side of F, L, M, W, Y	if P is C-term to F, L, M, W, Y, if P is N-term to Y
Chymotrypsin (C-term to F/Y/W/, not before P, not after Y if P is C-term to Y)	C-terminal side of F, Y, W	if P is C-term to F, Y, W, if P is N-term to Y
Trypsin/Chymotrypsin (C-term to K/R/F/Y/W, not before P, not after Y if P is C-term to Y)	C-terminal side of K, R, F, Y, W	if P is C-term to K, R, F, Y, W, if P is N-term to Y
Pepsin (pH 1.3)	C-terminal side of F, L	
Pepsin (pH > 2)	C-terminal side of F, L, W, Y, A, E, Q	
Proteinase K	C-terminal side of A, F, Y, W, L, I, V	
Thermolysin	N-terminal side of A, F, I, L, M, V	if D or E is N-term to A, F, I, L, M, V

From the various *in silico* hydrolysis conditions (different types of enzymes and different buffers to obtain different pH of hydrolysis), a list of various peptides was obtained from each casein and each enzyme (data not shown). Their physico-chemical characteristics, such as molecular weight (MW),

isoelectric point (pI), hydrophobic index (GRAVY), Aliphatic Index (AI), number of positive and negative charges and their size were calculated using ProtParam software on ExPASy website and compared to those present in the cheeses. Two enzymes, commercially available, were able to produce peptides with physico-chemical characteristics sufficiently close to the characteristics of the targeted peptides: (i) Trypsin and (i) Glu-C (Table 3). It appears that the ratio enzyme/substrate has to be low enough to favor the presence of intermediate, *i.e* large, peptides instead of final peptides, smaller ones. The cleavage specificities of both these enzymes are presented in the Table 3. The Figure 9 shows that the cleavage sites of Trypsin and Glu-C are very different. Glu-C cleaves more the C-term of α_{s1}-casein and κ-casein than Glu-C.

Table 3. Main characteristics of the selected enzymes

Enzyme	EC	Reasons to choose this enzyme	Conditions	Remarks
Glu-C	EC 3.4.21.19	- Production of large peptides (with miss cleavages >1) - Hydrophobic peptides: (-1.4< GRAVY <0) - Several amphiphilic peptides - large peptides are produced near the N-terminal of β-casein	Optimal pH = 4.0–9.0	Synonym: V8 Protease Exceptions if P is C-term to D or E or if E is C-term to D or E
Trypsin	EC 3.4.21.4	- Production of large peptides (with miss cleavages >3) - hydrophobic and hydrophilic peptides (-1.6<GRAVY<0.4) - large peptides are produced near the N-terminal of κ- and α_{s1}-casein	Optimal pH = 7-9	Small ratio enzyme/substrate to increase the miss cleavages Exceptions if P is C-term to K or R

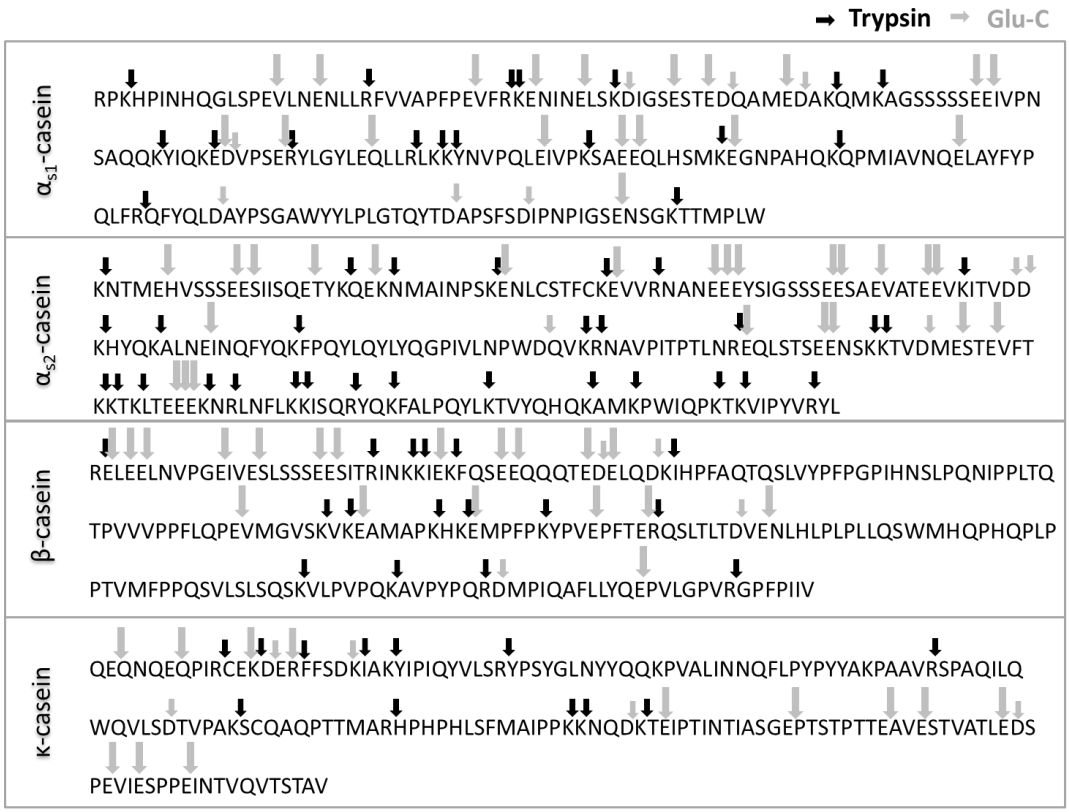


Figure 9. Cleavage sites of Glu-C and Trypsin on α_{s1}, α_{s2}, β and κ-caseins (www.uniprot.org/uniprot/P02662; P02663; P02666 and P02668, respectively). Endoproteinase GluC cleaves selectively peptide bonds C-terminal to glutamic acid residues (large arrow). Endoproteinase GluC also cleaves at aspartic acid residues at a rate 100-300 times slower than at glutamic acid residues (small arrow).

Specific conditions of hydrolysis were available on the PeptideMass program (ExPasy), particularly the pH. For example, Glu-C, in presence of bicarbonate buffer at pH 7.8 or ammonium acetate buffer at pH 4, cleaves caseins at the carboxyl side of glutamic acid whereas, in presence of phosphate buffer pH 7.8, Glu-C cleaves caseins at the glutamic acid and at the aspartic acid sides (Figure 9). Endoproteinase Glu-C is active over the pH range of 3.5 to 9.5 and exhibits maximal activity from pH 4.0 to 7.8. The enzyme exhibits an optimal activity at pH of 7.8 with casein as substrate. On the contrary, Trypsin cleaves proteins at the carboxyl side of lysine and arginine, regardless of the pH used.

To determine the physico-chemical profile of the mix of peptides obtained for each casein and for the two enzymes and to determine the differences between all these mix of peptides, PCA were realized, using peptides as individuals and their characteristics as variables.

The peptides produced from Glu-C, in presence of phosphate buffer, were mostly hydrophobic (mean of GRAVY near -0.847), as their sequence contains many hydrophobic amino-acids such as glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan or proline. Their sizes were very heterogeneous from 2 to 109 amino-acids, depending on the number of missed cleavages, with a mean of 28 amino-acids. With a missed cleavage more than 4, the size of peptides varied from 27 to 65 amino-acids for α_{s1}-casein, 36 to 81 for α_{s2}-casein and from 54 to 109 amino-acids for β -casein. The peptides produced from Glu-C, in presence of phosphate buffer, were mostly hydrophobic (mean of GRAVY near -0.837), as their sequence contains many hydrophobic amino-acids such as glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan or proline. Their sizes were very heterogeneous from 2 to 165 amino-acids, depending on the number of missed cleavages, with a mean of 34 amino-acids. Three peptides were larger with the hydrolysis by Glu-C in bicarbonate buffer conditions compared to in phosphate buffer conditions (α_{s2}-CN(85-207), β-CN(45-209) and β-CN(92-209)). With a missed cleavage more than 4, the size of peptides varied from 34 to 93 amino-acids for α_{s1}-casein, 49 to 123 for α_{s2}-casein and from 60 to 165 amino-acids for β-casein. To summarize, there was very few differences between the physico-chemical characteristics of peptides produced from Glu-C with phosphate or bicarbonate buffer (Figure 10). The common point between these conditions was the pH. Both the hydrolysis were realized at pH 7.8, pH which we used to produce our casein hydrolysates with Glu-C.

For the casein hydrolysis with Trypsin, the sizes of peptide sequence varied from 1 to 121. The size of peptides varied from 4 to 94 amino-acids for α_{s1}-casein, 1 to 72 for α_{s2}-casein and from 1 to 121 amino-acids for β-casein.

Peptides issued form the casein hydrolysis with Trypsin were more hydrophobic (mean of GRAVY of -0.9) than peptides issued from the casein hydrolysis with Glu-C. Moreover, the biggest peptides issued from the hydrolysis with Glu-C were principally issued from α_{s2}- and β-casein whereas with Trypsin, they came from α_{s1}- and α_{s2}-casein (Figure 11).

As for Trypsin as for Glu-C, with a small ratio enzyme/substrate, the miss cleavages increase and peptides could be large.

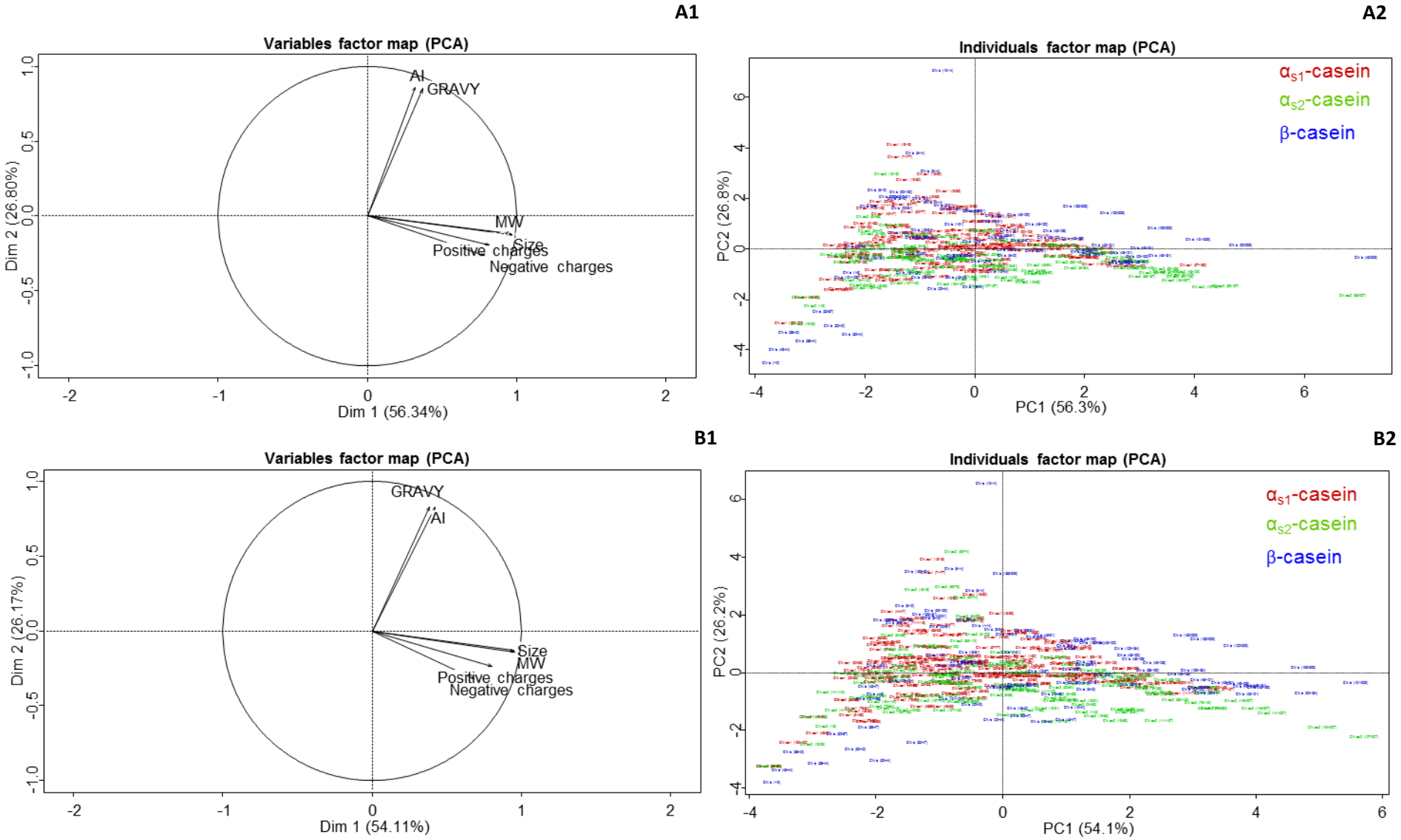


Figure 10. Repartition of peptides, obtained from the hydrolysis of α_{s1}-, α_{s2}-, β- and κ-caseins with Glu-C, on a PCA according to their physico-chemical characteristics, size, molecular weight (MW), isoelectric point (pI), hydropathy index (GRAVY), Aliphatic Index (AI), number of positive and negative charges.

A, in condition of bicarbonate buffer at pH 7.8 or pH 4; B, in condition of phosphate buffer at pH 7.8. A1: variables factor map of the two first dimensions, PC1 represents 56.34% and PC2 represents 26.8% of the total variability. A2: individual factor map. B1, variables factor map of the two first dimensions, PC1 represents 54.11% and PC2 represents 26.2% of the total variability. B2: individual factor map.

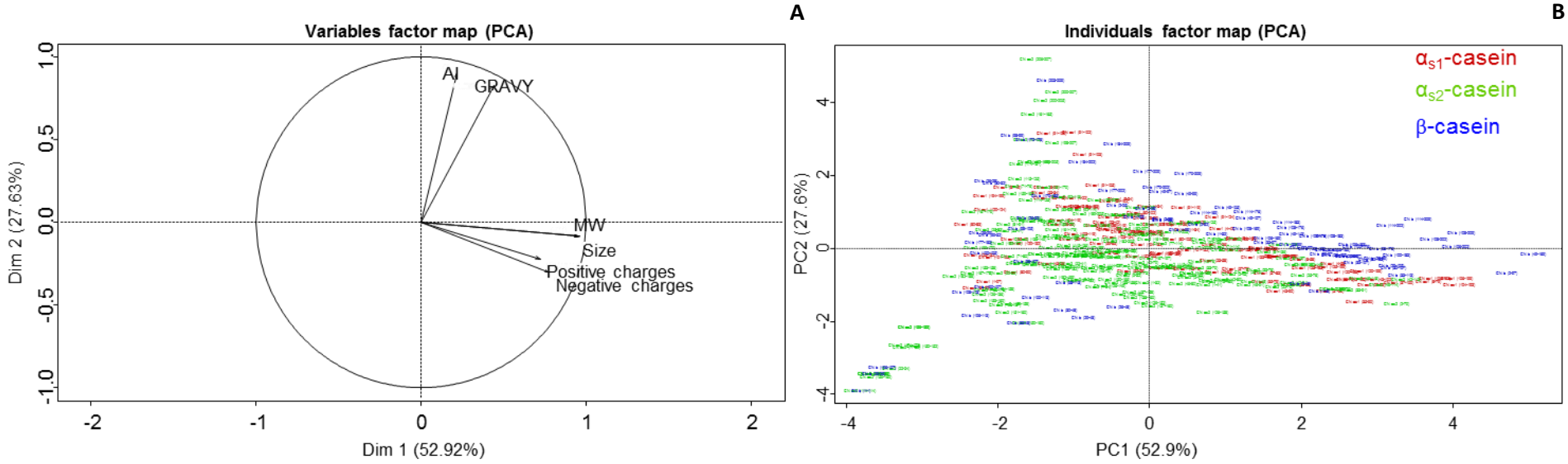


Figure 11. Repartition of peptides, obtained from the hydrolysis of α_{s1}-, α_{s2}-, β- and κ-caseins with Trypsin, on a PCA according to their physico-chemical characteristics, size, molecular weight (MW), isoelectric point (pI), hydropathy index (GRAVY), Aliphatic Index (AI), number of positive and negative charges. A: variables factor map of the two first dimensions, PC1 represents 52.92% and PC2 represents 27.6% of the total variability. B: individuals factor map.

There were more peptides with the hydrolysis by Trypsin (447 peptides) than by Glu-C in presence of phosphate buffer (369 peptides) and in presence of bicarbonate buffer (285 peptides). Glu-C hydrolyzed preferably α_{s1}-casein (165 peptides in presence of phosphate buffer and 123 peptides in presence of bicarbonate buffer), α_{s2}-casein (111 peptides in presence of phosphate buffer and 87 peptides in presence of bicarbonate buffer) and then β-casein (93 peptides in presence of phosphate buffer and 75 peptides in presence of bicarbonate buffer). Contrariwise, Trypsin hydrolyzed more β-casein (185 peptides), α_{s2}-casein (165 peptides) and then α_{s1}-casein (97 peptides). Consequently, the main difference between the peptide profiles of these two enzymes came from the nature of casein hydrolyzed, more than the physico-chemical characteristics of the peptides.

This work had permitted to select two enzymes: Trypsin and Glu-C in order to obtain large peptides rather hydrophobic. Moreover, these enzymes are marketable as immobilized enzymes, so easy to use for this thesis. The conditions of casein hydrolysis were developed in the part II - Materials and Methods of this manuscript.

Following this study, peptides were produced and added in casein micelle suspensions in order to study their biochemical, rheological and textural properties. In order to ensure the well understanding of the impact of protein hydrolysis on the microstructure, the textural and rheological properties of the protein network, we had simplified the matrix in which peptides were incorporated.

Chapter 2: Properties of milk peptides in liquid casein matrices

We showed in the foregoing chapters, in the bibliographic part and the chapter 1, that the peptide nature impacts the rheological and the textural properties of food products. Nevertheless, we do not know which peptide characteristics and how many peptides are needed to influence the rheological properties of dairy systems. Moreover, it will be interesting to determine if the chapter 1 conclusions can be applied to other milk systems than a real cheese. We do not known also whether some general rules can be put forward or not to explain the building-up of the interactions between several peptides and between peptides and caseins and the subsequent reorganization of the dairy system that has to be done to induce new or modified functional properties in various dairy matrices.

The aim of this chapter was to explore the rheological behavior modifications of model systems containing casein micelle with the hydrolysate incorporation at different ratios hydrolysates:casein micelle suspensions (HS:CMS). Particular focus was paid on the hydrolysate nature (6 hydrolysates with various degrees of hydrolysis T1, T2, T3, G1, G2, G3 and a control sodium caseinate), the secondary structure of the peptides and the changes observed in terms of casein micelle organization at microscopic scale and in rheological measurements.

The study presented in this chapter was performed on model dairy systems of different concentrations, from 50 to 200 g of total proteins.kg^{-1}, at neutral pH and 20°C.

We chose to study the microstructure, the viscosity, the viscoelasticity and the gelation capacity of these several model systems. The ratios HS:CMS selected included 25%, 50%, 75% and 100%. On the contrary to the literature, where the impact of hydrolysates was studied on the texture or rheology of model or real food systems according to the degree of hydrolysis as a sole criterion of characterization of the hydrolysates, we chose to work with well-known hydrolysates in controlled dairy systems.

In this study, we adapted a method of preparation of samples for the transmission electronic microscopy (TEM), the cryo-fixation coupled to a step of cryo-substitution. This method was recently developed in cellular imaging as it allows preserving the native structure of cell tissue compared to the classical methods of dehydration and substitution. The preparation of samples and the image acquisition by TEM were realized in collaboration with Irinia Kolotuev (Plateform MRic-TEM of Rennes) and Chantal Cauty (STLO).

Moreover, prior to measuring the impact of hydrolysates on the rheological properties of CMS at pH 7, these tests were realized in milliQ water condition with variations of pH values in order to:

(i) Validate the protocol of solubilization of hydrolysates and casein micelle powder at high concentrations;

(ii) Observe the impact of pH variation on the microstructure and rheological properties of the dairy systems containing hydrolysates.

This chapter is presented under the form of an article which is in preparation for *Journal of Agricultural and Food Chemistry*[5].

[5] Lacou, L. et al. Peptide-casein interactions. Part I- Changes in rheological behavior and structural properties of high-concentrated casein matrices. In preparation for *Journal of Agricultural and Food Chemistry*.

Chapitre 2 : Propriétés des peptides laitiers en matrices liquides concentrées en caséines

Nous avons précédemment montré, dans la partie bibliographique et dans le chapitre 1, que la nature des peptides impacte les propriétés rhéologiques et texturales des produits alimentaires. Néanmoins, nous ne savons toujours pas quelles caractéristiques et quelle teneur en peptides sont nécessaires pour influencer les propriétés rhéologiques des systèmes laitiers. De plus, il serait intéressant de déterminer si les conclusions du premier chapitre peuvent être validées et applicables à des systèmes laitiers autres qu'un fromage. Nous ne savons pas non plus si les règles générales peuvent être proposées ou expliquer autrement les interactions existantes entre plusieurs peptides ou entre des peptides et la caséine et la réorganisation ultérieure du système laitier qui doit induire de nouvelles propriétés fonctionnelles dans diverses matrices laitières.

L'objectif de ce chapitre a été d'explorer la modification du comportement rhéologique de systèmes modèles contenant des micelles de caséines par l'ajout d'hydrolysats à différents ratios. Une attention particulière a été donnée sur la nature des hydrolysats (6 hydrolysats avec divers degrés d'hydrolyse T1, T2, T3, G1, G2, G3 et un témoin caséinate de sodium), la structure secondaire des peptides et les changements observés en terme d'organisation des micelles de caséines à l'échelle microscopique et au mesures rhéologiques.

L'étude présentée dans ce chapitre a été menée sur des systèmes laitiers modèles de différentes concentrations, variant de 50 à 200 g de protéines totales.kg^{-1} à pH neutre et à 20°C.

Nous avons choisi d'étudier la microstructure, la turbidité, la viscosité, la viscoélasticité et les capacités gélifiantes de ces systèmes modèles. Les ratios hydrolysats :micelles de caséine sélectionnés comprennent 25%, 50%, 75% et 100%. Contrairement à la littérature, où l'impact des hydrolysats a été étudié sur la texture et la rhéologie des aliments modèles ou réels seulement en utilisant le degré d'hydrolyse comme critère de caractérisation des hydrolysats, nous avons ici choisi de travailler avec des hydrolysats connus dans un système laitier contrôlé.

Dans cette étude, nous avons adapté une méthode de préparation des échantillons pour la microscopie électronique à transmission (MET), la cryo-fixation couplée à une étape de cryo-substitution. Cette méthode a récemment été développée en imagerie cellulaire dans le domaine biomédical car elle permet de mieux préserver la structure native des tissus ou objets par rapport

aux méthodes classiques de déshydratation et de substitution. La préparation des échantillons et l'acquisition des images ont été réalisées en collaboration avec Irinia Kolotuev (Plateform MRic-TEM of Rennes) et Chantal Cauty (STLO).

De plus, avant de mesurer l'impact de l'ajout d'hydrolysats sur les propriétés rhéologiques des suspensions de micelles de caséines à pH 7, ces tests ont été réalisés dans de l'eau avec un pH dérivant afin de:

(i) Valider le protocole de solubilisation des hydrolysats et des poudres de micelles de caséine à hautes concentrations ;

(ii) Observer l'impact de la variation de pH sur la microstructure et les propriétés rhéologiques des systèmes laitiers contenant des hydrolysats.

Ce chapitre est présenté sous forme d'article qui est en cours d'écriture pour soumission dans *Journal of Agricultural and Food Chemistry*[6].

[6] Lacou, L. et al. Peptide-casein interactions. Part I- Changes in rheological behavior and structural properties of high-concentrated casein matrices. En préparation pour *Journal of Agricultural and Food Chemistry*.

Peptide-casein interactions. Part I- Changes in rheological behavior and structural properties of high-concentrated casein matrices

In preparation for Journal of Agricultural and Food Chemistry

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Abstract

Hydrolysis of proteins into peptides during food processes or storage can enhance the functional properties of food products. It is difficult to determine how, and to which extent peptides can modify the functional properties of proteins. Hence, we produced various groups of peptides by hydrolysis of sodium caseinate with Trypsin and endoproteinase Glu-C. Three peptide groups quantitatively and qualitatively different were obtained per enzyme. Controlled amount of these peptides were incorporated into high-concentrated casein matrices at pH 7 and 20°C, on which flow and oscillatory shear measurements were applied and microstructure was determined by TEM, FTIR and circular dichroism. The nature of the peptides produced clearly influenced the rheological properties and the microstructure of the different matrices. However, the changes in rheological behaviors were also dependent on the proportion of the peptides incorporated, showing complex interactions that could lead to adverse rheological behaviors.

Keywords

Peptide, protein, hydrolysis, identification, sequence, secondary structure, functional properties, gel, dilute solution, complex system, rheology, microstructure

Introduction

Incorporation of milk proteins or their protein-derived peptides as hydrolysates in various food products, i.e. dairy, meat and bakery products, are increasingly used in human diets due to their large contribution to the final texture, organoleptic properties and health-promoting activities *(1, 2)*. Actually, protein hydrolysates, containing native proteins and a mix of peptides, were shown to improve functional properties such as interfacial activity, gelation and solubility, which ultimately impact food texture *(1, 3-6)*. Such an improvement can be related to the fact that some peptides can bind specifically to proteins through non-covalent hydrophobic interactions, as shown for whey protein hydrolysates obtained by Alcalase *(7-9)* or glycinin hydrolysates, obtained by hydrolysis with subtilisin Carlsberg, chymotrypsin, bromelain, and papain *(10)*. Nevertheless, the specificity of the enzyme, as in the case of trypsin, can change the ability of the protein to form gel or not, depending on the protein used as substrate *(7, 10)* and consequently the peptide produced and the way they are able to interact with each other and with the residual native proteins. Therefore, some studies used defined peptides to determine how the amino acid sequence can affect functionality and to what extent *(11)*. The authors showed that an equilibrium has to be maintained between hydrophobic and charged amino acids in order to improve not only the peptide self-assembly but the gelation rate, notably at neutral and basic pH.

However, when hydrolysates are used, most of the time the peptide sequence is unknown. Therefore, it is difficult to relate the change in functional properties with the overall physico-chemical characteristics of the peptide, such as size, pI, charges and hydropathy index, their secondary and tertiary structure depending on both their sequence and the environmental conditions used. This is magnified in some food products, as fermented ones, when the production of peptides takes place *in situ* through various enzyme activities which gives the final expected texture to the food product.

Peptides are produced during manufacture and conservation of dairy products. Actually caseins, representing 80% of the total protein content in cow milk, are usually the network backbone of numerous dairy products. Casein-derived peptides, produced during cheese making or cheese ripening, were already shown to induce softening of cheese matrix during aging *(12)* for example or to enhance stretching properties in hard type cheeses. However, the amount and the type of peptides needed to influence the rheological and the textural properties of milk systems are still under question. Moreover, it is difficult to establish whether some general rules can be put forward or not to explain the building-up of the interactions between peptides themselves and between peptides and caseins. Consequently, it is still difficult to understand the subsequent reorganization of

the dairy system that has to be done to induce new or modified functional properties in various dairy matrices as gelation in the present case.

In the present study, we investigated how both the casein networks and the rheological properties of casein micelles can be changed in the presence of various concentrations of peptides, in three systems from liquid to gel, containing (i) 50 $g.kg^{-1}$, (ii) 100 $g.kg^{-1}$ and (iii) 200 $g.kg^{-1}$ under neutral pH conditions and at 20°C. Peptides were produced from the hydrolysis of caseins by two enzymes: (i) Trypsin and (ii) *Staphylococcus aureus* V8-protease also referred as endoproteinase Glu-C. The peptides were identified, physico-chemically and structurally characterized. Particular focus was paid on the hydrolysate nature, the secondary structure of the peptides and the changes observed in terms of turbidity, casein micelles organization at microscopic scale and in rheological measurements.

Materials and methods

Protein samples: sodium caseinate and casein micelles

Sodium caseinate (NaCas) powder (Armor-Protéines, France) contained 87% (w/w) of proteins, determined by both Kjeldhal method and UV spectrophotometry (UVmc2, Monaco SAFA, France) at λ = 280 nm using extinction coefficient of 0.81ml. mg^{-1}. cm^{-1} *(13)*, including only minor amount of whey proteins as shown by SDS PAGE electrophoresis (protocol described in "Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis"). This powder was dispersed in milliQ water using motor agitation in order to obtain a solution at 10 $g.kg^{-1}$ of caseins.

Native phosphocaseinate powder, Promilk 825B (IDI SAS, France) that contained 80% (w/w) of native casein micelles determined by Kjeldhal method was dissolved at 50, 100 and 200 $g.kg^{-1}$ of caseinsin maleate buffer (80 mM of maleate, 25 mM of $CaCl_2$) pH 7.1 at 20°C in presence of sodium azide (NaN_3, 3mM) as bacteriostatic agent under a weak stirring during 24 hours. Then, these suspensions were heated at 50°C for 3 hours and continuous stirring was kept for the further 21 hours for complete rehydration at ambient temperature. The complete solubilization of the powder was checked by analyzing particle size with the Mastersizer (Malvern Instruments, France) according to both the number and the volume of the particles in order to detect the presence of insoluble aggregates. Solutions were conserved at 4°C maximum during two weeks.

Enzyme digestion

NaCas was hydrolyzed by two immobilized enzymes with different specificities: (i) Tosyl-phenylalanine-chloromethyl-ketone (TPCK) - treated trypsin cross-linked on 4% beaded agarose (Thermo Fisher Scientific, France) that cleaves proteins at the carboxyl side of arginine and lysine

residues and (ii) *Staphylococcus aureus* V-8 protease, also referred as endoproteinase Glu-C (Thermo Fisher Scientific, USA), cross-linked by covalent bonds on 6% beaded agarose that cleaves proteins after glutamic acid residues or at glutamic and aspartic acid residues *(14)*. Immobilized enzymes were selected to easier separate the enzyme from the hydrolysate by filtration to be reusable. For that purpose, enzymes were washed after each hydrolysis period by various washing steps i) with 4M urea for both enzymes, and with 1% Triton X100 only for Glu-C, ii) Phosphate Buffer Saline 1X and iii) MilliQ water.

Prior to each hydrolysis, the activity of the Trypsin was measured by spectrophotometry at 247 nm using 0.01 M 4-toluene-sulphonyl-arginine-methyl-ester, TAME (Sigma Aldrich, France), according to the method of Worthington Laboratories, using the UV-mc2 spectrophotometer (Monaco-SAFAS, France). The activity of Glu-C was measured by the pH-stat method using sodium caseinate (NaCas) (Armor-Protéines, France) as substrate with a ratio of 100 µl immobilized enzyme / 0.2 g of NaCas. Activity control was made for both enzymes to check any of the enzymes was released from the beads during treatments and that activity not changed.

Next, the solution a NaCas at 10 $g.kg^{-1}$ was preheated to 37°C. The ratios enzyme:substrate used were: 1049 U/29 g of NaCas for Trypsin and 34.8 mL of commercial Glu-C/14.5 g NaCas. The reaction was performed at 37°C at a constant pH value of 7.1, using the pH-stat (Mettler Toledo, France) and 1M NaOH as a titrating agent. The hydrolysis reaction was stopped at three defined times corresponding to different compositions both in peptides and in native caseins: (i) 70% caseins + 25% peptides, (ii) 50% caseins + 50% peptides and (iii) 5% caseins + 95% peptides. They were referred respectively as to T1, T2 and T3 for hydrolysis with trypsin and G1, G2, and G3 for hydrolysis with Glu-C. The solution was freezed at -20°C, lyophilized (S.G.D. – SERAIL type CS 10-0.8, France) and stored under vacuum at 20°C under darkness until further use.

The degree of hydrolysis of each hydrolysate was determined according to the pH-stat method *(15)*.

$$DH = B \times Nb \times {}^{1}/_{a} \times {}^{1}/_{mp} \times {}^{1}/_{htot} \times 100$$

$${}^{1}/_{a} = 1 + 10^{pk-pH}$$

$$pk = 7.8 + \frac{298 - T}{298 \times T} \times 2400$$

B= volume of NaOH

Nb= NaOH molarity

mp = protein mass

h_{tot}= 8,2 $meq.g^{-1}$ for casein

T = temperature (Kelvin)

Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis

The extent of casein degradation was evaluated by Urea-PAGE of the insoluble pH 4.6 nitrogen fractions. The peptide pattern was analyzed by SDS-PAGE as described by Schägger and von Jagow *(16)* using Protean II system (16 × 16 × 0.1 cm; BioRad, Marnes-la-Coquette, France) with SDS/Tris/Tricine buffer and a concentration gradient from 12 to 18% of acrylamide used according to Sadat-Mekmene et al. *(17)*. The gels were scanned by the Image scanner III fitted with the LabScan software (GE Healthcare, France) and analyzed by ImageQuant software (GE Healthcare, France), band intensities were estimated by the net volume parameter.

Analysis of peptides by nano-RPLC coupled online to tandem mass spectrometry (ESI/MS/MS)

Mass spectrometry (MS) experiments were performed using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, USA) equipped with a nanoelectrospray ion source. A preliminary sample concentration step was performed on a nanotrap PepMap 100 (C18, 3 µm Inner Diameter 5ID) x 20 mm Length (L)) (Dionex, Netherlands). Separation was performed on a reverse-phase column PepMap RSLC C18 3 µm, 100 Å (75 µm ID, 150 mm L) (Dionex, Netherlands) at 35°C, using solvent A (2 % v/v acetonitrile, 0.08 % v/v formic acid and 0.01 % TFA in deionized water) and solvent B (95 % v/v acetonitrile, 0.08 % v/v formic acid and 0.01 % v/v TFA in deionized water). 5 – 35% of solvent B in 67 min and 35 – 8 % in 2 min was applied as separation gradient at a flow rate of 0.3 µL.min^{-1}. 3 µL were injected onto the column corresponding approximately to 60 ng of peptides. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode and a voltage of 2 kV with the help of a Proxeon Nanospray Flex ion source (Thermo Scientific, USA). Spectra were recorded in full MS mode and selected in a mass range 250-2000 m/z for MS spectra with a resolution of 70.000 at m/z 200. For each scan, the ten more intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17.500 at m/z 200 and the parent ion was subsequently excluded of the analysis during 15 sec. The instrument was externally calibrated according to the supplier's procedure. To identify peptides, all data (MS and MS/MS) were submitted to X! Tandem using the X! Tandem pipeline developped by PAPPSO (Plateforme d'Analyse Protéomique de Paris Sud-Ouest, INRA-Jouy-en-Josas, France, htp://pappso.inra.fr). The search was performed against a database composed of a homemade database named STLO containing milk and egg proteins and cRAP database allowing excluding list of proteins such a human keratin for example. Database search parameters were specified as follows: unknown enzyme cleavage was used with one missed cleavage and the peptide mass tolerance was set to 10 ppm for MS and 0.05Da for MS/MS. As caseins are phosphorylated, phosphorylation (corresponding to mass adduct of 79.96633 Da) were chosen as variable modification as well as oxidation of methionine (corresponding to mass adduct of 15.99494 Da). For each peptide identified,

a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation. Only peptides containing more than 5 amino-acid residues and less than 5 500 Da can be identified by this methodology.

Physicochemical characteristics of the identified peptides

Physico-chemical characteristics of each quantified peptide were considered, including: the number of positive and negative charges at pH 7, the size, the isoelectric point (pI) and the hydrophobic index (GRAVY). The isoelectric point was predicted using ExPASy "Compute pI/Mw" tool *(18)*. The number of positive and negative charges was calculated with Python software from EMBOSS software suite (http://emboss.sourceforge.net). The GRAVY was calculated according to the method of Kyte and Doolittle *(19)*.

Size exclusion chromatography

Tryptic and GluC peptides were suspended at 5 $g.L^{-1}$ in 50 mM phosphate buffer and 150 mM sodium chloride, pH 7.2 and 50 µl samples were injected onto a Superdex Peptide 10/300 GL column (GE healthcare, VWR, France). Isocratic elution was performed at 0.5 ml/min in the same buffer at room temperature and detection was performed at 214 nm. For the molecular mass calibration, β-casein (23 960 Da, 5 $mg.mL^{-1}$ phosphate buffer pH7.2) was used to determine the exclusion volume of the column and synthetic peptides were used as standards: lysine (146.2 Da) , bradykinin (1060.2 Da), Ala-Pro-Gly (243.3 Da), Gly-Leu-Leu-Gly (406.48 Da), polypeptides standard (BioRad, France) containing triose phosphate isomerase (26 626 Da), myoglobin (16 950 Da), α-lactalbumin (14 437 Da), aprotinin (6512 Da), insulin B chain oxidized (3496 Da) and bacitracin (1423 Da).

Mixtures NaCas:CMS and hydrolysates:CMS

Casein micelle suspension (CMS) and NaCas and hydrolysates solutions (in maleate buffer pH 7 containing 25 mM Maleic acid (Sigma-Aldrich, France), 2 mM calcium Chloride.$2H_2O$ (AnalaR, France) and 3mM sodium azide as bacteriostatic agent (Sigma-Aldrich, France) in order to reach the 80 mM ionic strength of milk)) were mixed at a mass ratios hydrolysate:CMS of 1:3 (w/w) (25 % of hydrolysate), 1:1 (w/w) (50 % hydrolysate), 3:1 (w/w) (75 % hydrolysate) and 1:0 (w/w) (100% hydrolysate) at 20°C. The same ratios were used for the control NaCas:CMS suspensions. All suspensions were prepared by gently rotating the tube to mix well, without introducing too much air bubbles. The mixtures were conserved at 20°C during maximum 48h.

Secondary and tertiary structures: Circular dichroism & Fourier Transform Infrared Spectroscopy

The overall tertiary and secondary structures of hydrolysates were examined using far- (190 – 250) and near-UV circular dichroism (CD) spectroscopy (250 – 350 nm) with a Jasco J-810 automatic recording circular dichroism spectropolarimeter fitted with a xenon lamp (Jasco, France) using casein solutions prepared to reach a maximum of absorbance of 0,8 at 280 nm. Spectra were recorded in the wavelength interval 185-260 nm (optical pathway of 2 mm) and 250-360 nm (optical pathway of 10 mm), respectively, using a quartz cuvette STARNA (Starna Scientific, England) of path length 2 mm and 10 mm, respectively. The scan parameters were: 100 nm/min scan rate, 1 nm bandwidth and 2 s averaging time. Spectra shown are averages of 3 accumulations for each sample, corrected by subtracting the baseline scan of the appropriate buffer and subjected to noise reduction.

Fourier Transform Infrared spectroscopy (FITR) was used to elucidate the conformational changes in protein and peptide structure upon the different hydrolysate:CMS ratios. Infrared light absorbance spectrum of each protein/peptide sample was measured in the attenuated total reflection (ATR) mode between 4 000 cm^{-1} and 850 cm^{-1}, with a resolution of 4 cm^{-1}, using the Tensor 27 spectrophotometer (Bruker, France), equipped with a Germanium ATR crystal (mono-reflection) and a mercury-cadmium-telluride detector cooled with liquid nitrogen, and controlled by the OPUS software. Background spectra were recorded for the maleate buffer used to prepare protein solutions. Each spectrum was reported form the average result of 256 scans. The protein solutions were at ~30 g of caseins.kg^{-1}. Before implementing the multivariate statistical analysis, spectra were corrected in automatic way contributions of the atmospheric steam and by some liquid water. To focus on conformational changes, the differences in overall infrared absorbance due to differences in concentration between samples needed to be compensated. In addition, a major drawback of using IR spectroscopy on aqueous solution is that H – O – H bending vibration of liquid water absorbs strongly around 1643 cm^{-1}, which interferes with the amide I band *(20)*. Since the background spectra were recorded using buffer, differences in sample protein concentration resulted in differences in contribution of this liquid water absorption, which also needed to be compensated. The Extended Multiplicative Scatter Correction (EMSC) was used for these automatic compensations *(21)*. EMSC consists in calculating a mean spectrum and in expressing each sample spectrum as a linear combination of the mean spectrum and of the liquid water "interference" spectrum. The coefficients estimated b linear regression then allow the water contribution to be subtracted and the overall absorbance to be normalized. The spectral variability among corrected spectra was then

analyzed using principal component analysis (PCA). Spectral corrections and statistical computations were performed using the R software *(22)*.

Rheological measurements

Flow measurements were performed with a Low Shear 400 (Lamy Rheology, France) for dispersions with low viscosity (< 1000 Pa.s). This instrument was operated with Couette geometry using the system MS-LS 2T-2T Titan with inner and outer radii of 5.5 and 6 mm respectively. The measurements were realized at 20°C, by using a shear rate gradient from 1 to 100 s^{-1}. Measurements were repeated twice or three times showing very good reproducibility (error rate of 5%).

Flow measurements were performed with the MCR301 rheometer (Anton-Paar France S.A.S., France) for dispersions with viscosity > 1 000 Pa.s. These measurements were operated with cone plate geometry. The measurements were realized at 20°C with a shear rate gradient from 0.01 s^{-1} to 1 000 s^{-1}. Measurements were repeated two or three times showing very good reproducibility (error rate of 2%).

Oscillatory shear measurements were performed using the MCR301 rheometer (Anton-Paar France S.A.S., France). A cone-plate geometry was used. The mix hydrolysate:CMS was prepared and then quickly transferred onto the plate, and the upper plate was gently lowered until the sample filled the gap of a constant normal force was reached (about 0.7 N). The storage modulus (G') and loss modulus (G'') were first measured as function of time at a frequency of 1Hz. The frequency-dependent response was then measured from 0.001 Hz to 100 Hz at a strain of 0.01, % within the region of linear response. All rheological experiments were performed at a fixed temperature of 20°C. The measuring system was carefully regulated to the measuring temperature before each measurement. The solution was also kept for 10 minutes in the rheometer prior to performing the measurements. To prevent evaporation from the sample, a closed cover setup was used and the surface of the sample was covered with low-viscosity paraffin oil. Measurements were repeated two or three times, showing very good reproducibility (error rate of 3%).

Microstructure

The microstructure dairy systems of the seven (NaCas:CMS + six hydrolysates:CMS) studied was characterized from transmission electron microscopy (TEM) micrographs.

The prepared concentrated casein-hydrolysate suspensions and the hydrolysate suspension were frozen using a Leica EM PACT2 high-pressure freezer (Leica Microsystems, Austria). The sample carriers, cellulose microcapillary tubes or flat gold-plated specimen carriers~~, were~~ pre-coated with 1%

phosphatidylcholine (Sigma Aldrich Ltd., France) diluted in chloroform to avoid sample sticking. No cryo-protecting agent was added to the samples. For the freeze-substitution step, Leica EM AFS2 freeze substitution machine (Leica Microsystems, Austria) was used. The frozen samples were transferred in liquid nitrogen to a processing container equipped with a flat spacer (Leica Microsystems, Austria). The frozen samples were freeze-substituted in 2% osmium tetroxide diluted in anhydrous acetone. After an initial incubation of 8 h at 90 C the temperature was gradually (5 C/h) rose to 30°C and samples were then left for another 8 h at this temperature. The solvent fixative solution was replaced with pre-chilled mix of ethanol 3:1 resin (epon-araldite mix; Sigma Aldrich Ltd). The temperature was then gradually brought up to room temperature and further ethanol: resin substitutions were done following the resin manufacturer instructions. Thin sections (90 nm) of embedded samples were cut with a diamond knife using a Reichert ultramicrotome. The sections were contrasted with 4% aqueous solution of uranyl acetate.

Hydrolysates were also analyzed by negative coloration.

The support is a TEM copper grid recovered with polymer film formvar glow-discharged is flipped over a droplet of 0.005 mM protein suspensions and followed of the contrast agent– aqueous solution of 2% uranyl acetate.

All the samples casein suspensions and gels and the hydrolysates were observed using a JEM-1400 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) operating at 120 kV accelerating voltage. Digital images were acquired using the Gatan SC1000 Orius® CCD camera (4008 x 2672), set up with the imaging software Gatan DigitalMicrograph™ (Gatan, USA) at Microscopy Imaging Center platform (MRic), situated in Rennes.

Several TEM micrographs were taken on different parts of two independent samples of each of the seven dairy systems.

Statistical analysis

To represent the heatmaps of the quantities of peptides according to the nature of enzyme and the time of hydrolysis, the relative quantification of peptides were normalized. For each hydrolysate, the mean of relative quantity of each peptide was calculated. This value was divided by the maximal mean value of each peptide. Then, Log+1 of this normalized value was included in the process of heatmap construction. The agglomerative hierarchical clustering with the "hclust" function was used to cluster treatment depending on the quantification of peptides in each hydrolysate. ANOVA, followed by a t-test were realized taking into account the mean in category and the mean overall of each physico-chemical characteristic (for all the clusters).

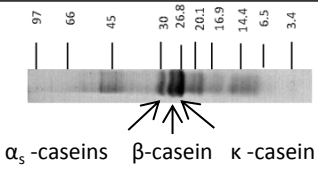
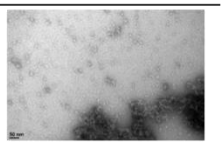
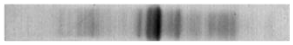
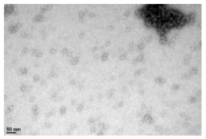
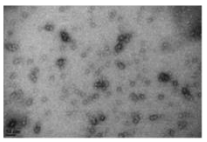
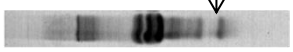
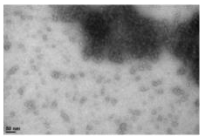
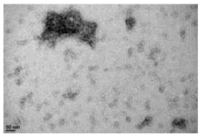
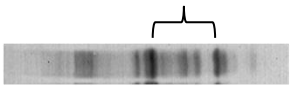
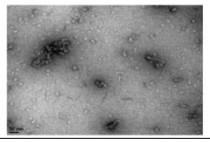
To differentiate the suspensions according to their secondary structure, a methodology based on multivariate exploratory analyses, such as Principal Component Analysis was performed using spectroutils, an R package (internally designed in the STLO by Stéphane Pezennec). The first two PCs were retained as the totality of the inertia explained by both of them, represented a large part of the total inertia. Then, ANOVA was realized on the PC1 scores of secondary structures taking account the nature of hydrolysate, the ratio HS:CMS, the time of hydrolysis and their interactions of order 2.

Results and Discussion

The hydrolysis of sodium caseinate by Trypsin and Glu-C generate peptides with different physico-chemical characteristics

Each hydrolysate was produced from NaCas which contains all types of caseins, i.e. α_{s1}-, α_{s2}-, β- and κ-caseins not linked together by calcium phosphate bonds in contrast to casein micelles present in the CMS suspensions. Trypsin and Glu-C were chosen because their specificities are well-known and in order to obtain peptides with very different sizes and sequences, and consequently different physico-chemical and structural characteristics. The hydrolysis conditions of 37°C and pH 7 were chosen as they were optimal temperature and pH for both enzymes. The six hydrolysates, with different degrees of hydrolysis (Table I), presented different quantities of residual native caseins (Table I) and peptides having different molecular weights (Table I) and charges (Figure 1A). The appropriate enzyme-to-substrate (E/S) ratios were chosen to produce a plateau at 7.5% DH for the hydrolysis with Trypsin and 3% DH for the hydrolysis with Glu-C. The hydrolysis reactions progressed rapidly for the first 2 500 sec with Trypsin and 5 000 sec for Glu-C and then were relatively slowly over time reaching a plateau (data not shown). These exponential reactions were typical of protease hydrolysis *(23)*.

Table I. Degree of hydrolysis (DH) of each hydrolysate, corresponding profile of proteins and peptides by Tris-tricine/SDS-PAGE and the microstructure observed by TEM. a1, a2, b and c represent bands of peptides that are different between the samples.

Hydrolysate	DH (%)	α_{s2}-casein (%)	α_{s1}-casein (%)	β-casein (%)	κ-casein (%)	Tris-Tricine/SDS-PAGE	TEM (X 200 000)
T1	2.5	100	70	95	100		
T2	4.5	70	30	65	100		
T3	7.5	60	5	10	80		
G1	0.5	95	90	95	100		
G2	2	70	55	40	100		
G3	3	5	5	5	85		

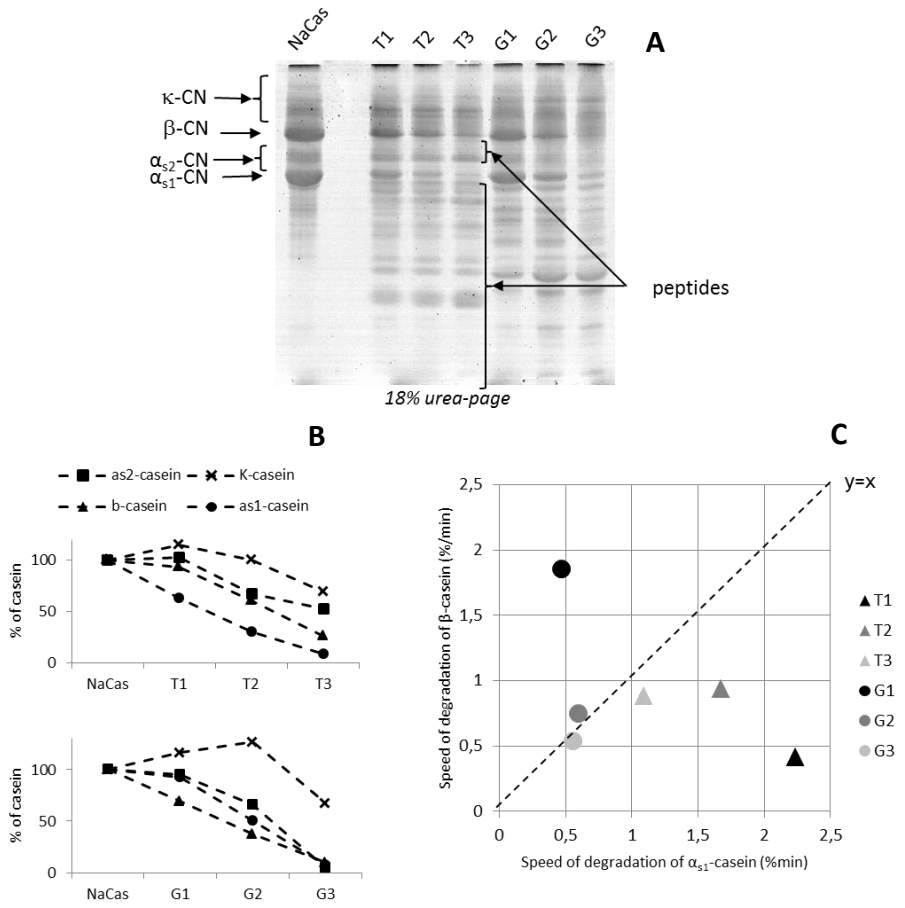


Figure 1. Degradation of caseins and apparition of peptides for each enzyme, according to the time of hydrolysis. (A) Urea-PAGE of NaCas and hydrolysates; (B) Percentage of casein degradation according to the enzyme and the time of hydrolysis, quantified by Urea-PAGE; (C) Rates of degradation of α_{s1}-casein compared to the speed of degradation of β-casein according to both the time of hydrolysis and the type of enzyme. CN is the abbreviation for casein.

As seen in the Table I and Figure 1, α_{s1}-, α_{s2}- and β-caseins were hydrolyzed both by Trypsin and Glu-C. Trypsin was able to degrade more quickly α_{s1}-casein compared to Glu-C (Figure 1B and Figure 1C) whereas Glu-C did not have preferential substrates since the rate of degradation of α_{s1}- and β-caseins were equivalent for the two last times of hydrolysis (Figure 1). On the contrary, at G1 the rate of degradation of β-casein was largely higher than the rate of degradation of α_{s1}-casein (Figure 1). α_{s1}-casein was more hydrolyzed by Trypsin than by Glu-C. The degradation of β-casein was more pronounced with Glu-C at the beginning of the hydrolysis and was similar for the other times.

As expected, the peptide profiles were different between the hydrolysis of NaCas by Trypsin and the hydrolysis of NaCas by Glu-C. There were more peptides under 14.4 kDa in T hydrolysates than in G hydrolysates (Table I). This was confirmed by the size exclusion chromatographic profile of the hydrolysates (Figure 2) that shows a distribution of the peptides ranging from 50 to 10 000 Da for peptides present at the end of hydrolysis time for Trypsin and from 180 to 20 000 Da for those arising

from Glu-C hydrolysis. Moreover, it clearly appears that small peptides were formed early in the hydrolysis by Trypsin whereas small peptides were only visible on the chromatogram from the hydrolysis time G2. The change in the profile was also different according to the type of enzyme. The profile is the same for Glu C hydrolysate throughout hydrolysis time suggesting a continuous hydrolysis of the same bonds.

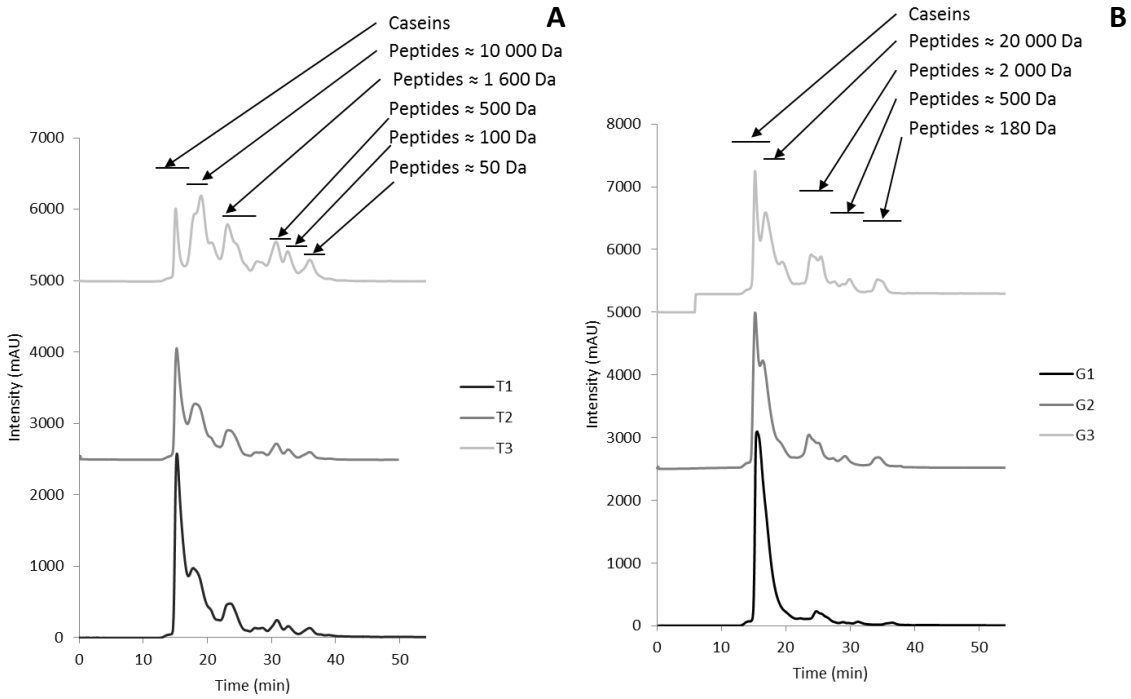


Figure 2. Size exclusion chromatography of (A) Trypsin hydrolysates and (B) Glu-C hydrolysates.

Regarding the type of peptides with high molecular weight that were produced and visible on the SDS tris tricine gels (Table I), it appears that some bands were specifically present in T or G hydrolysates. This was the case of the band a2 (Table I) which was present only in T hydrolysates and decreased according to the time of hydrolysis. G hydrolysates had some bands more intense than T hydrolysates in the region b (Table I) and more numerous in the region c (Table I). Moreover, the molecular weight observed was higher for the peptides produced with Glu-C in agreement with the chromatographic profile (Figure 2).

To deeply characterize the hydrolysates, peptides were identified by tandem mass spectrometry and their intensity determined. This identification/quantification was rarely realized. In several studies, the authors linked a degree of hydrolysis to a functional property of proteins but did not quantify the peptides produced. 352 peptides were identified for Trypsin and 283 peptides were identified for Glu-C. Most of them were intermediate peptides containing at least two or more amino acid residues corresponding to the specificity of Trypsin and GluC, i.e. Lys / Arg and Glu / Asp respectively showing

that the hydrolysis was not extensive with both enzymes. Presumably some sites were less accessible to the enzymes than theoretically predicted as shown in the figure 1 of supplementary material. It is also possible that the time of hydrolysis was not sufficient to produce mainly final peptides or that some of the final peptides that have sequence less than five amino acids were not identified by the mass spectrometer analysis.

For the NaCas hydrolysis by Trypsin, 37% of the peptides were present in T1, 29% in T2 and 34% in T3. T1 (129 peptides) included 40% of peptides derived from α_{s1}-casein, 34% from α_{s2}-casein, 20% from β-casein and 6% from κ-casein. T2 (103 peptides) included 35% of peptides derived from α_{s1}-casein, 36% from α_{s2}-casein, 23% from β-casein and 6% from κ-casein. T3 (120 peptides) included 40% of peptides derived from α_{s1}-casein, 33% from α_{s2}-casein, 21% from β-casein and 7% from κ-casein. G1 (94 peptides) included 53% of peptides derived from α_{s1} casein, 13% from α_{s2}-casein and 20% from β -casein. G2 (92 peptides) included 50% of peptides derived from α_{s1}-casein, 14% from α_{s2}-casein and 20% from β-casein. G3 (97 peptides) included 52% of peptides derived from α_{s1}-casein, 15% from α_{s2}-casein and 22% from β-casein. The identified peptides were, for most of them, produced as early as T1 and G1 as shown in the overview of the peptides (Figure 2). All caseins exhibited series of highly and weakly hydrolyzed regions all along their sequence and these series of alternated regions were relatively well-conserved at all sampling times, including peptides that are phosphorylated.

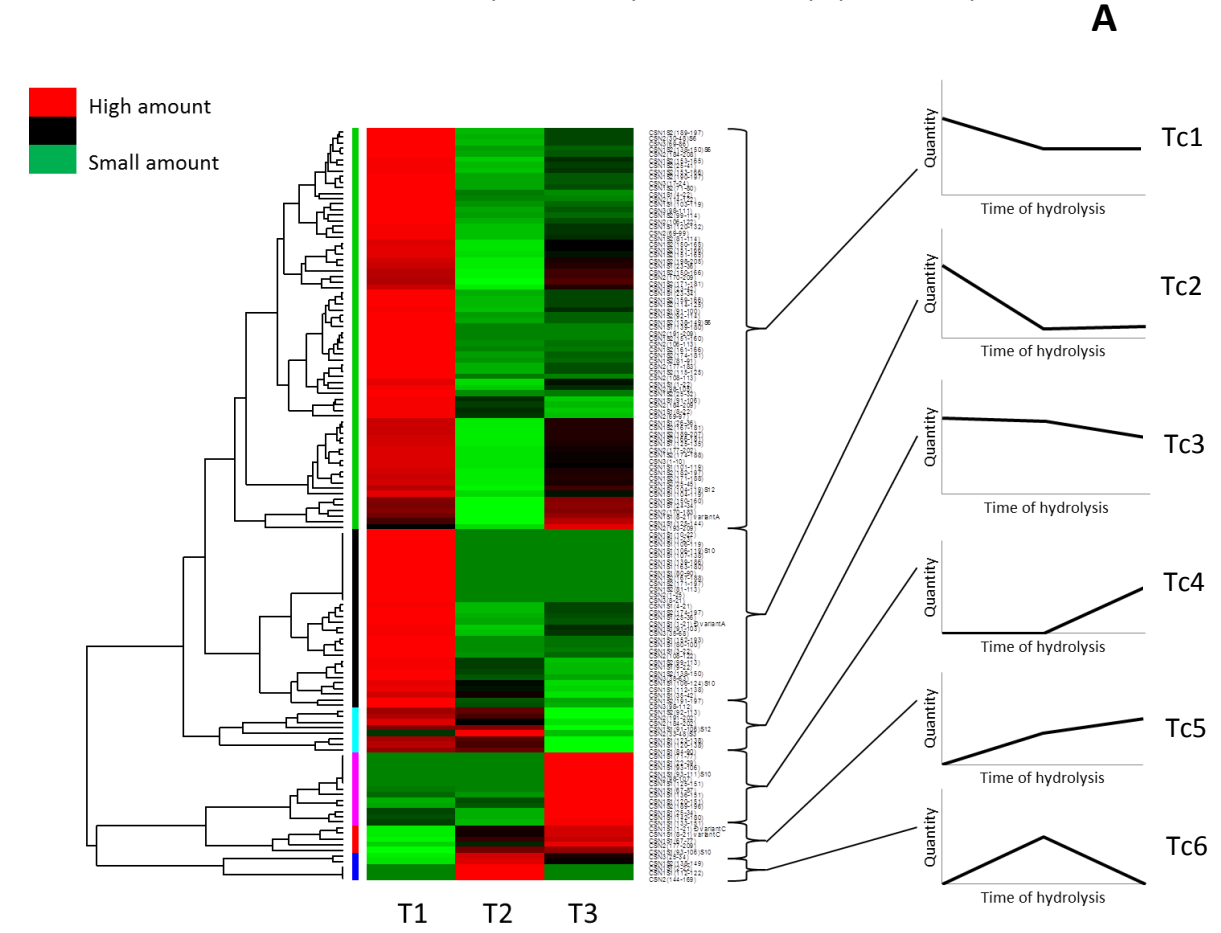

A
High amount
Small amount
T1
T2
T3
Quantity
Time of hydrolysis
Tc1
Tc2
Tc3
Tc4
Tc5
Tc6
B
High amount
Small amount
G1
G2
G3
Gc1
Gc2
Gc3

Figure 3. Heat maps representing relative quantification of peptides contained for each hydrolysates T1, T2, T3 (A) and G1, G2, G3 (B). Green bands represent small amount of peptide; red bands represent high amount of peptide and black bands represent the intermediary amount of peptide. On the heatmap, the y axis represents the peptides and the x axis represents the hydrolysates. A dendrogram was added to the left side to show the clusters of peptides. Each cluster was represented on graphics (on the right of the Figure) where the quantity of peptides was represented on the y axis and the time of hydrolysis on the x axis.

Peptides were also quantified, as shown on the heat maps of the Figure 3. From these results we can have explanation of the chromatographic profiles shown above. Actually, peptides were grouped on the heat maps according to the kinetics of peptide production. Clusters of peptides with the same kinetics were formed. Then, it clearly appears that the numbers of clusters was higher in the case of Trypsin hydrolysis than in the case of Glu-C, with 6 clusters (Tc1, Tc2, Tc3, Tc4, Tc5, Tc6) against 3 (Gc1, Gc2, Gc3) respectively. The same bonds were hydrolyzed by Glu-C and gradually released the same peptides. It was also showed by the majority of the peptides present in the cluster Gc1, compared to the other two clusters that contained intermediate peptides in the cluster Gc3 further hydrolyzed into final ones in cluster Gc1 and Gc2. In the case of Trypsin, the mode of action of the enzyme can be related to the “zipper” mode in which all the molecules of caseins are simultaneously degraded, leading to the concomitant formation of intermediate and final peptides in the reaction mixture *(25)*. The way that the intermediate peptides were gradually degraded was represented in four different clusters Tc1, Tc2, Tc3 and Tc6 while the peptides that continuously increased are represented in clusters Tc4 and Tc5, even if some peptides were also intermediate ones that maybe have a rate of production higher than the rate of degradation.

Thanks to the knowledge of the sequence of all identified peptides, it was possible to calculate their physico-chemical characteristics in terms of size, negative and positive charges at pH 7, pI, molecular weight, number of aromatic amino-acids and average hydropathy scores (GRAVY) which indicated whether a peptide is hydrophilic (negative values) or hydrophobic (positive values) *(19)*. Such an approach is rarely done in the literature, as thin characterization of the peptides present in hydrolysates by tandem mass spectrometry is usually not performed.

From the heat maps we found that hydrolysates were separated into various clusters according to the kinetics of production. A t-test was performed to highlight the main physico-chemical traits related to each cluster and in turn to characterize each type of hydrolysates according to the hydrolysis time.

Table II: Main physico-chemical characteristics of peptides ranging by cluster. GRAVY represents grand average hydropathy score; pI represents isoelectric point; MW represents molecular weight.

Cluster	Hydrolysate	Characteristics	Mean in category	Mean overall	SD in category	SD overall	p value
Tc1	T1	pI	7.50	6.75	2.05	2.06	2.68E-09
		GRAVY	-0.65	-0.78	0.75	0.62	8.09E-04
		Aliphatic index	78.17	73.78	32.41	32.01	2.65E-02
		Number of negative charges at pH 7	2.58	3.22	1.36	1.43	3.37E-13
Tc2	T1	Number of aromatic amino-acids	3.34	2.11	2.63	1.85	1.27E-07
		MW (Da)	2405.34	1927.68	1051.59	853.11	9.36E-06
		Size (number of amino-acids)	20.25	16.42	9.41	7.54	5.65E-05
Tc3	T1-T2	Aliphatic index	89.20	73.78	36.01	32.01	1.95E-02
		Number of positive charges at pH 7	2.11	3.18	0.34	1.17	7.80E-06
		pI	4.73	6.75	0.76	2.06	2.22E-06
Tc4	T3	Number of aromatic amino-acids	3.14	2.11	2.10	1.85	8.19E-03
		MW (Da)	2300.27	1927.68	1104.10	853.11	3.84E-02
		GRAVY	-0.52	-0.78	0.52	0.62	4.42E-02
		pI	5.80	6.75	1.82	2.06	2.95E-02
Tc5	T3	GRAVY	-0.34	-0.78	0.80	0.62	1.76E-02
		Number of positive charges at pH 7	2.43	3.18	0.64	1.17	3.10E-02
Gc1	G3	Number of negative charges at pH 7	3.35	3.22	1.35	1.43	5.49E-03
		Number of aromatic amino-acids	1.79	2.11	1.68	1.85	4.71E-08
		Size (number of amino-acids)	15.09	16.42	6.89	7.54	1.05E-08
		Aliphatic index	67.75	73.78	27.01	32.01	1.01E-09

		MW (Da)	1766.88	1927.68	773.09	853.11	1.00E-09
		GRAVY	-0.91	-0.78	0.51	0.62	2.55E-12
Gc2	G1-G3	Number of negative charges at pH 7	3.95	3.22	1.60	1.43	3.49E-07
		Aliphatic index	86.24	73.78	43.23	32.01	9.94E-05
		MW (Da)	2222.00	1927.68	947.30	853.11	5.60E-04
		Size (number of amino-acids)	18.74	16.42	8.11	7.54	2.11E-03
		Number of aromatic amino-acids	2.67	2.11	2.36	1.85	2.25E-03
		Number of positive charges at pH 7	2.81	3.18	0.85	1.17	1.56E-03
		pI	5.84	6.75	2.23	2.06	1.11E-05
Gc3	G1	Size (number of amino-acids)	21.32	16.42	5.72	7.54	4.74E-08
		MW (Da)	2459.50	1927.68	658.69	853.11	1.62E-07
		Number of positive charges at pH 7	3.85	3.18	1.50	1.17	1.77E-06
		GRAVY	-0.49	-0.78	0.89	0.62	7.75E-05
		Number of aromatic amino-acids	2.68	2.11	1.36	1.85	8.83E-03
		pI	7.23	6.75	1.39	2.06	4.77E-02

Among the parameters tested (pI, GRAVY, number of positive charges, size, Aliphatic index, number of aromatic amino-acids, molecular weight), only some of them were highlighted as predominant for each cluster and are presented in the Table II.

The hydrolysis of NaCas by Trypsin involved the production of hydrophobic peptides with few negative charges at pH 7 and a high pI value as shown for T1 that mainly contains the clusters Tc1, Tc2 and Tc3. This peptide profile was kept throughout the hydrolysis time except that the average size of the peptides decreased and the pI tends to more acid values as shown by the predominance of the clusters Tc5 and Tc6 in the T3 hydrolysate.Tc1 and Tc2 contained intermediate peptides which were hydrolyzed later but in peptides that kept the same overall characteristics.

In contrast, the peptide profile greatly changed during caseinate hydrolysis by Glu-C. G1 contains predominantly cluster Gc3 that generated hydrophobic peptides whereas G3 mainly contained hydrophilic peptides and G2 will have specific behavior since it contains few amount of peptides from the different clusters rendering it atypical *(7-10)*. The size of peptides decreased with the time of hydrolysis, as expected *(26)*. Consequently, specific interactions depending on the hydrolysates used can be expected to occur when the peptides will be reincorporated into the various casein micelle suspensions *(11)*.

Peptide secondary and tertiary structures changes in the presence of casein micelles suspension

In order to highlight which and how hydrolysates can impact the structuration of the casein micelle suspensions, circular dichroism (Figure 4) alone and in combination with Fourier Transform Infrared spectroscopy (Figure 5) were performed as a way to characterize the main differences between secondary and tertiary structures of peptides, caseins with different organizations as caseinate and casein micelles, and mixture of peptides and casein micelles.

Considering the near-UV CD spectra, there were no significant modification of the CMS spectra in presence of hydrolysates (data not shsown). This was not surprising as caseins had few tertiary structures *(27)*. Considering the far-UV CD soectra, some differences between the spectra were observed when hydrolysates were added to the CMS. As expected, the changes in conformation of the caseins is low comparatively to that could be observed for globular proteins that contain well-defined α-helices with negative minima at 208 and 222 nm, β-sheets and random coils with positive band in the 185-190 nm range *(28)*. However, Figure 4A shows distinct shapes of the ellipticity for the samples regarding whether they contained or not casein hydrolysates T and G in the 190-250 nm range. Such spectral changes indicated conversion of random coil conformations into more ordered secondary structural elements *(29, 30)* mainly for the peptides. In order to highlight the differences between samples and to find the main parameters that drive these changes, PCA was performed on the different spectra, colored according to the time of hydrolysis (Figure 4B and Figure 5D), tha quantity of native caseins (Figure 4B and Figure 5D) or the ratio hydrolysate:CMS (Figure 4E and Figure 5B). Figure 4F and Figure 5F represent the coordinates of PC1 and PC2 according to the wavelength. Thus, when the loadings on the two main principal components PC1 and PC2 were reported as a function of the wavelengths (Figure 4F), we showed the PC1 had a shape corresponding to a protein with two negative minimums at 206 and 217, corresponding to α-helix conformation and the maximum at 195 nm, while PC2 had a negative minimum at 195, and an overall shape corresponding to random coil *(28)*. Consequently, high coordinates on PC1 were correlated with the formation of α-helices. Regarding the distribution of the different samples, we

confirmed a distinct separation on PC1 (84.47% of the variation) into two groups. Nevertheless, it was not so clear what are the main parameters that led to such a separation: the time of hydrolysis (Figure 4B), the type of hydrolysate (Figure 4C), the percentage of peptides into the casein micelle suspension (Figure 5E), the residual percentage of the native caseins in the different suspensions (Figure 5F) and likely interactions between all. It appears that only the presence of the peptides from T and G hydrolysates induced changes of the conformational states of the casein micelles suspensions since the addition of caseinate did not change the spectrum of the casein micelle suspensions (Figure 4B). However, the impact of the peptides was not a linear effect, as confirmed by another statistical treatment of the PC1 score by ANOVA (Figure 6). It shows that for each enzyme, the impact of the hydrolysate proportion is strongly dependent on the time of hydrolysis. In a general way, when caseins were hydrolyzed, α-helices were formed except when time of hydrolysis is excessive as T3. Consequently, hydrolysis of caseins produced large peptides which were able to structure each other and to change also the structure of the caseins when they were reincorporated into the casein micelles suspension. However, the quantity of peptides sufficient to induce conformational changes in mixed matrices was not the same regarding the type of hydrolysates, 25 % of T1 was sufficient to form α-helices whereas 75 % of G1 was needed to induce such conformational changes. In contrast incorporation of 75 % T3 and 75 % G3 led to adverse behaviors, with formation of α-helices with G3 and no more with T3. Moreover, for the T hydrolysates, there was a continuous decrease in the ability of forming α-helices that can be related to the evolution of the peptide composition throughout hydrolysis time, in that sense, the overall physico-chemical characteristics were kept except that the size of the peptide diminished rendering more difficult conformational changes to occur. In contrast, G1 had a complete different behavior than the other also shown on the ANOVA, highlighting the deep changes in the peptide composition that occurred during hydrolysis. This was shown by significant minima observed for intermediate ratios, which was changing with the time of hydrolysis and for Glu-C these minima were moving closer to the values observed for the weak ratio HS:CMS.

Considering the near-UV CD spectra there were only minor changes observed that were not considered to be reliable indicator of alterations in their tertiary packing (data not shown).

Hydrolysates reincorporated or not in the casein micelles suspension were also analyzed for their secondary structural parameters by FTIR spectra. The analysis by Fourier transformed infrared spectroscopy was coupled to the analysis by circular dichroism (Figure 6). The spectra were so close that loadings on the PC had to be multiplied by a 10 times standard deviation factor to highlight differences in the FTIR spectra (Figure 6A). Some bands were visible with specific wavelengths as 1400 that was close to values corresponding to free carboxylates, and related to the increase in peptides in the casein micelles suspensions. The bands 206 and 217 were specific wavelength of α-

helix close to values determined in the literature *(31)*. However, the complexity of the sample with huge number of peptides actually led to extensive overlap of bands arising from the different structure rendering difficult the interpretation of FTIR spectra *(32)* even as a sole analysis (data not shown). It was also apparent that the type of enzyme used to hydrolyze NaCas, the time of hydrolysis and the interactions of CMS with casein hydrolysates cause some supplement but subtle conformational changes. Only few hydrolysates were clearly separated from the other samples 100 % G1 25 % T2, 50% T1, 100 % G3 and 50% G1.

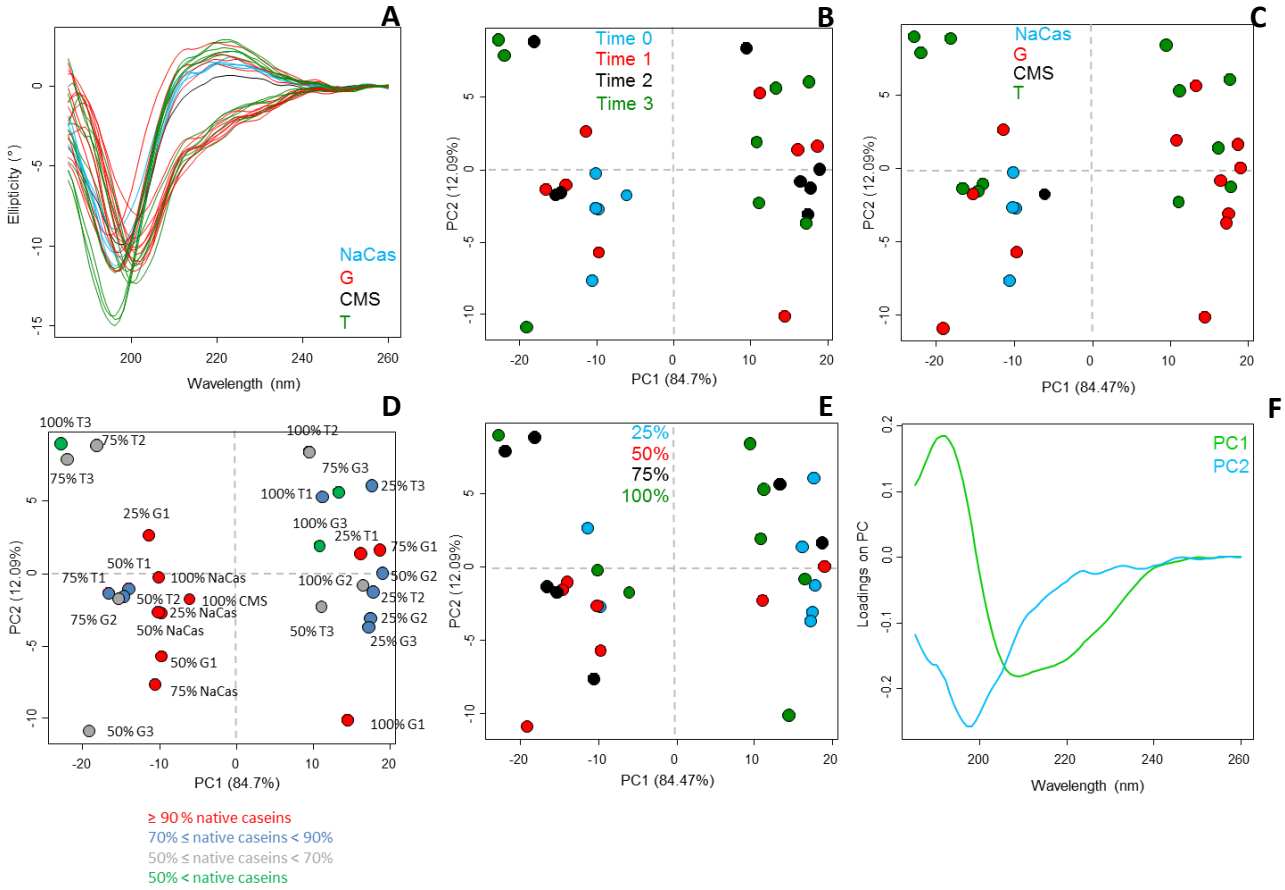


Figure 4. Far-UV CD spectra of hydrolysates T and G in presence of different ratios hydrolysate:CMS. The hydrolysates were scanned from 190 to 250 nm. (A) represents the Far-UV CD spectra according to the type of hydrolysate; (B) represents the individuals according to the time of hydrolysis; (C) represents the individuals according to the type of hydrolysate and casein suspension; (D) represents the individuals according to the quantity of native caseins in the different samples; (E) represents the individuals according to the quantity of hydrolysate; (F) represents the coordinates of PC1 (green) and PC2 (blue) of the spectra according to the wavelength.

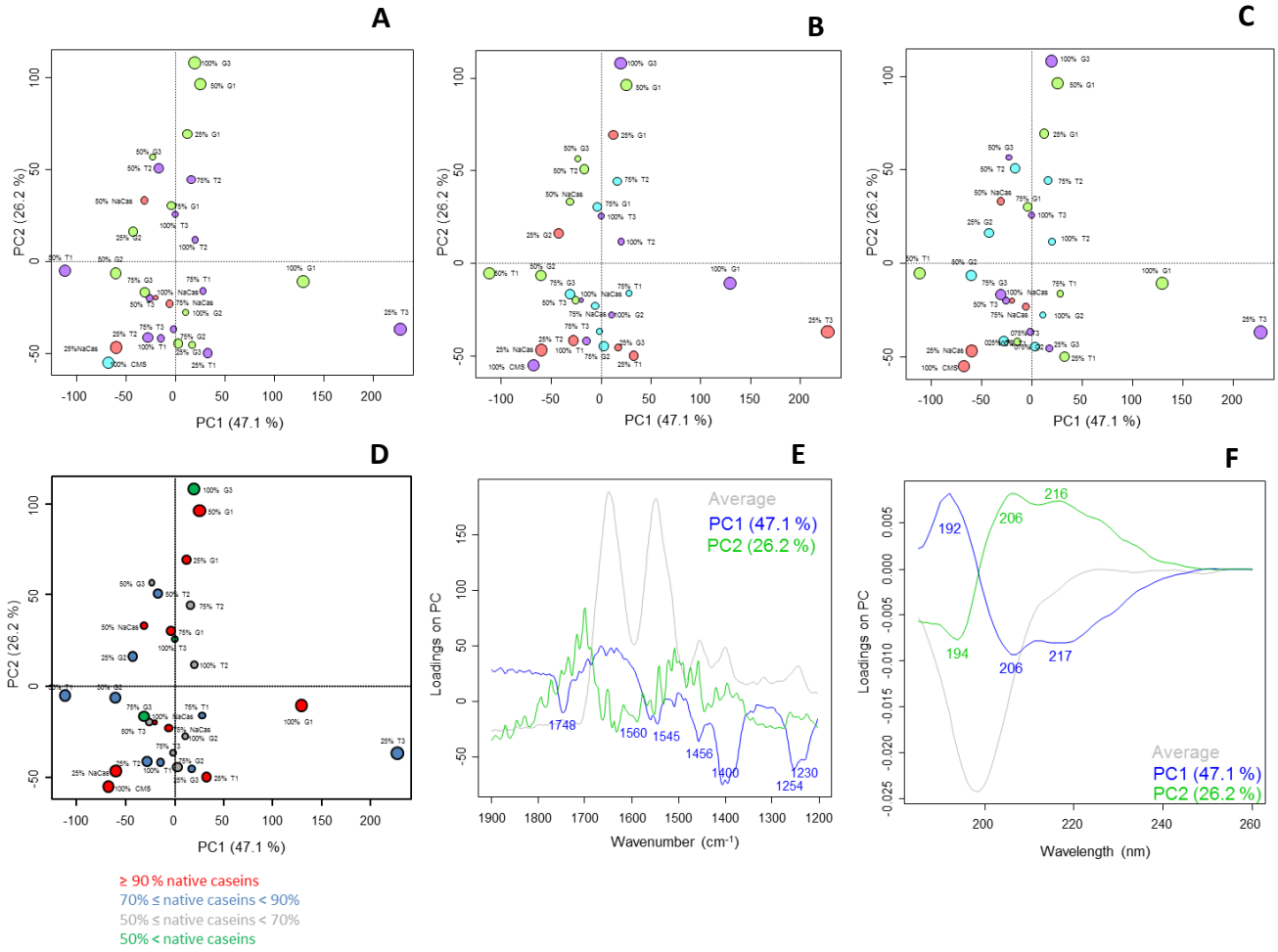


Figure 5. Far-UV CD spectra of hydrolysates T and G in presence of different ratios hydrolysate:CMS coupled with Fourier transformed infrared spectroscopy. (A) represents the individuals according to the type of hydrolysate; (B) represents the individuals of the spectra according to the quantity of hydrolysate; (C) represents the PCA of the spectra according to the time of hydrolysis; (D) represents the individuals of the spectra according to the quantity of native caseins; (E) represents the FTIR part of the spectra according to PC1 and PC2 multiplied by 10 standard deviations; (F) represents the CD part of the spectra according to PC1 and PC2 multiplied by 10 standard deviations. The biggest the circles are, the most the individuals were represented.

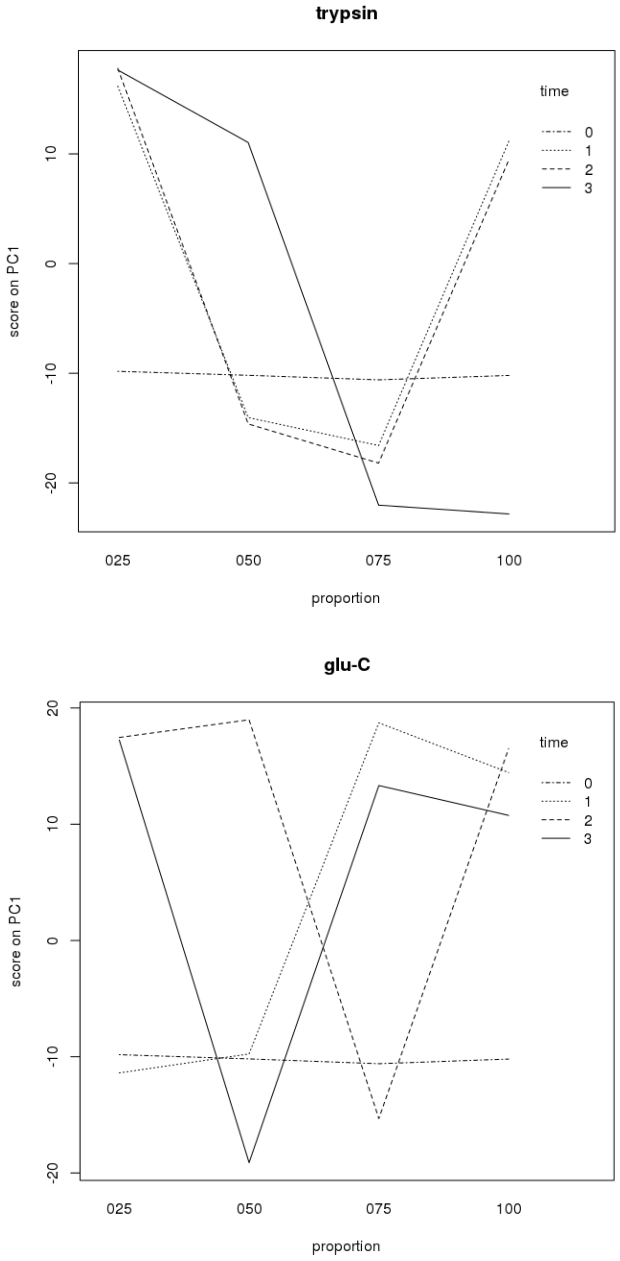


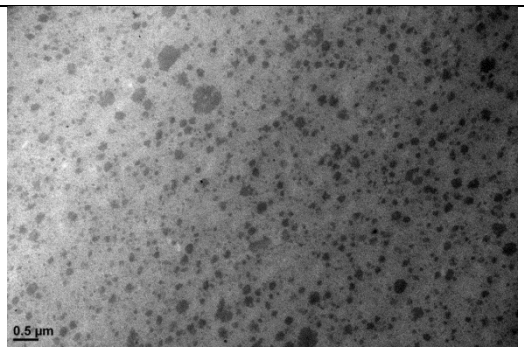
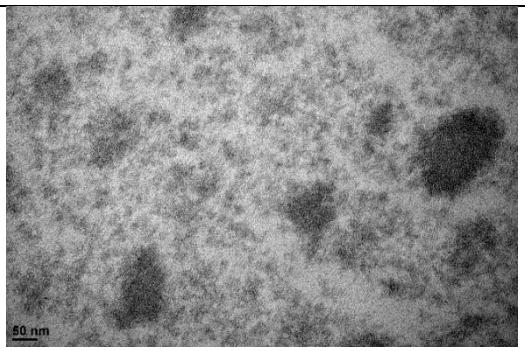
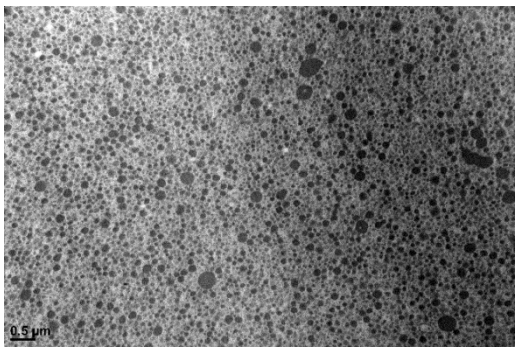
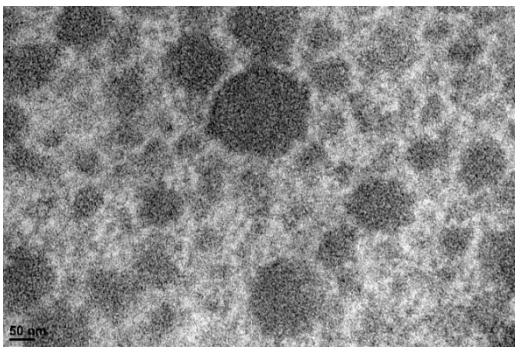
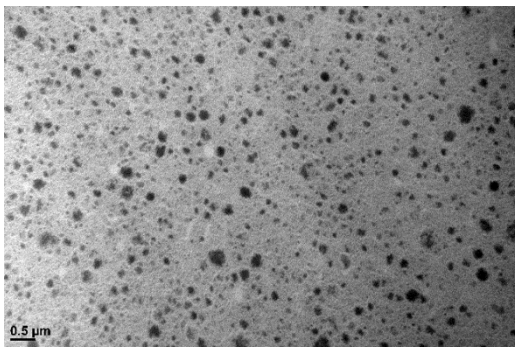
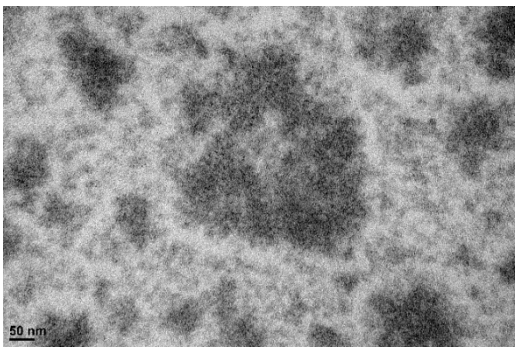
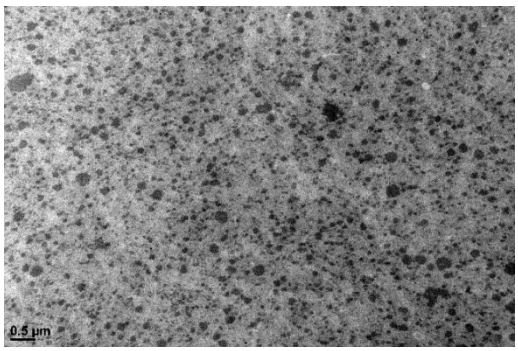
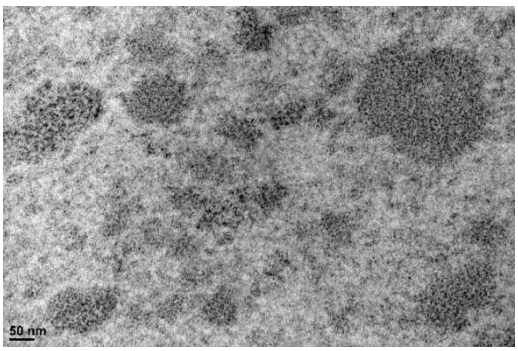
Figure 6. ANOVA on the PC1 scores of secondary structures measured by circular dichroism taking account the nature of hydrolysate, the ratio HS:CMS, the time of hydrolysis and their interactions of second order.

The microstructure of casein micelle network is modified in presence of hydrolysates

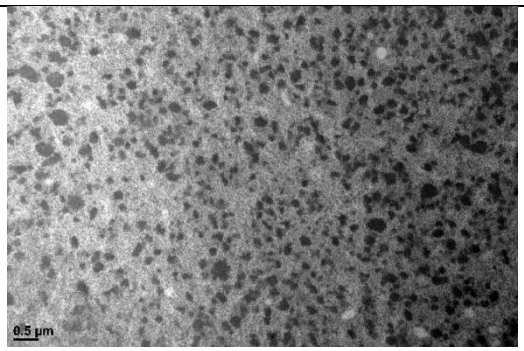
Micrographs of nanostructures formed at pH 7 by CMS with 50% of hydrolysates were captured by transmission electron microscopy (TEM) (Table III). At pH 7, individual caseins were associated in well-defined casein micelles. The calcium phosphate nanoclusters were intact as shown on the micrographs of CMS sample in Table II in agreement with micrographs of casein micelles *(33, 34)*. The addition of 50% NaCas to the 50% CMS induced the formation of a denser and more compact

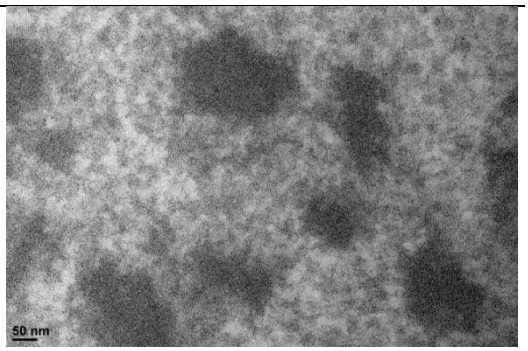
network. Nevertheless, the size of the micelles seemed unchanged. The addition of 50% hydrolysates T1 and G1 did not lead to a change in the casein micelle structure but these mixes were different from the mix 50% CMS:50% NaCas. These hydrolysates contained few quantities of peptides and were mainly composed by caseins. However, the hydrolysis of caseins in these hydrolysates was nonetheless sufficient to form a less dense network than that observed for the mix CMS:NaCas. With the addition of T3 and G3, micelles were bigger than the control CMS: NaCas. This phenomenon was amplified when the casein micelles and the hydrolysates were resuspended in milliQ water, without a monitored pH(data not shown). This could be due to the entrance of small peptides in the casein micelles that is a well-known porous structure, in an easier way than the caseins. As peptides were charged and can be considered as polyelectrolytes, they could act as counter-ions and increase repulsive interactions inside the casein micelles favoring the expansion of the casein micelle volume up to a limit. Actually, the presence of phosphate nanocluster warrants the structuration of caseins as micelles. This was clearly shown with the hydrolysates T2, T3 and G3, for which the casein micelles seemed to be denser than the control sample. The backgrounds of the micrographs, corresponding to these samples, were darker because of the presence of several particles. The sample containing G2:CMS was particularly different from both the control and the other samples. In this sample, the casein micelles were aggregated and were more condensed than the other samples probably because peptides stabilize the structure of casein micelles via ionic or hydrophobic interactions. Peptides could also avoid the interactions between casein micelles as it was known for the interactions between the β-lactoglobulin and the surface of casein micelles in milk *(35)*.

Table III. Electronic microscopy scanning micrographs of mix 50% CMS - 50 % hydrolysates at pH 7.

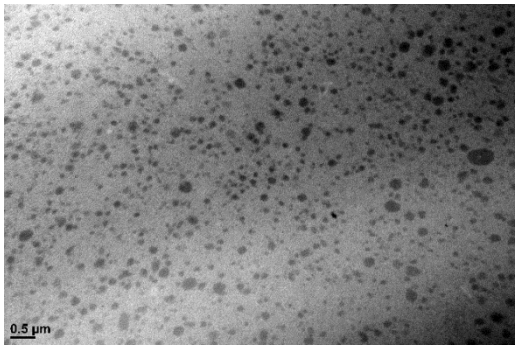
Sample	X 20 000	X 200 000
CMS		
CMS:NaCas		
CMS:T1		
CMS:T2		

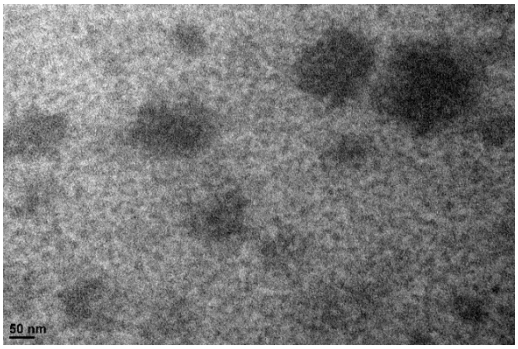
CMS:T3



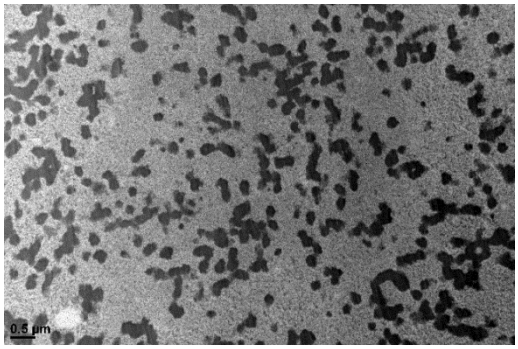


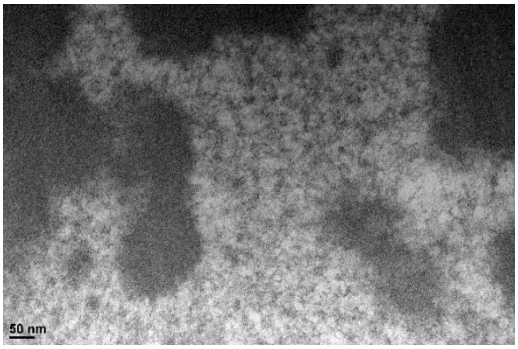
CMS:G1



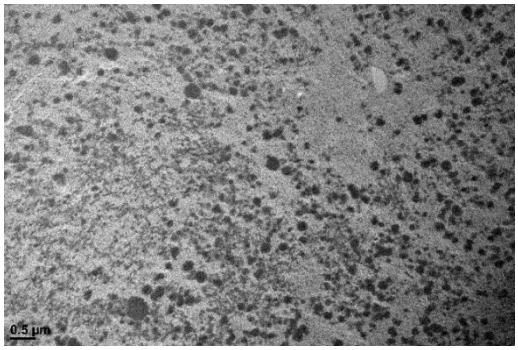


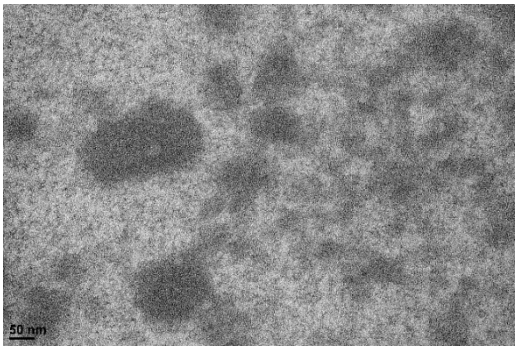
CMS:G2





CMS:G3





These TEM results first demonstrate that changing the type of hydrolysates added to the CMS leads to the formation of quite different nanostructures. Indeed, nearly identical peptides, differing by only their quantity, had significantly different impacts on the casein micelle morphologies. This observation reinforces the idea that peptide physico-chemical characteristics and their interactions were driving force to change the microstructure of the casein micelles. Nevertheless, no good relationship between microstructure and rheological properties of a peptide solution, such as gel capacity, was well established. Guy and Voyer *(11)* showed that peptides with different structures self-assembled into different nanostructures to form different network which led to gel formation of not. However, in this study, it was shown that different nanostructures can induce a gel formation.

Up to a critical concentration, peculiar hydrolysates and mixes were able to form gel

In the previous parts of the paper we showed that hydrolysates T and G were able to differentially induce secondary structural changes of casein micelles suspension. The question is now to evaluate how these hydrolysates were able to modify the rheological behavior of the casein micelle suspension. For that purpose, three concentrations of casein micelle suspensions were tested to modify the rheological behavior of the casein micelle suspsensions: 50, 100 and 200 $g.kg^{-1,}$ in which peptides from T and G hydrolysates, and caseinate were reincorporated at various HS:CMS ratios.

At 50 and 100 $g.kg^{-1}$, 100% NaCas, pure CMS, pure hydrolysates and all mixes CMS-hydrolysates or CMS-NaCas were under liquid form (data not shown). Under a concentration of 100 $g.kg^{-1}$, no gel appeared. At 200 $g.kg^{-1}$, the behaviors of the NaCas, the CMS, the hydrolysates and the mixes were drastically different from the samples at 100 $g.kg^{-1}$ and led to gel formation for most peptides- CMS mixtures (Figure 7).

At 200 $g.kg^{-1}$, the CMS was a very viscous liquid, and looks like a gel, which was instable during the time (Figure 7). Bouchoux et al., *(38)* showed that at a concentration of 178 $g.L^{-1}$ of caseins the dispersion behaved as a viscoelastic gels in which the micelles were forced to deform and deswell as under confinement conditions. At this concentration, casein micelles were connected themselves with bonds. The hydrolysates alone were present as a viscous liquid except T2 that already formed a gel. The behavior of the casein micelles suspensions drastically changed when the hydrolysates or the caseinate were reincorporated into the CMS. As early as 25 % of peptides gels were observed and the gelation was kept at 50% but decreased for 75 % peptides showing that there was a limit of the amount of peptides useful to induce visible changes and to build up new interactions between peptides and caseins, beyond which the adverse effects were observed leading to a destabilization of the gels. This structuration of the gel was also due to the presence of native casein in the hydrolysates and evidenced by the fact that the caseinate control was also able to form gel when

added to the CMS. However, the gels formed at 50 and 75 % were apparently less stable than those formed with hydrolysates.

Trypsin hydrolysates had a behavior different than that observed with the G hydrolysates. It was particularly notable for T2 and T3. Under its original form, T2 was a sticky viscous liquid. The T2 sample contained peptides with a molecular weight under 14.4 kDa which could interact to give a very viscous liquid. This viscosity was reinforced by the presence of CMS in the mixes up to 75 % of peptides. Although T3 was initially liquid, it was also able to form a viscous liquid up to 75 % reincorporation. It could be due to the presence of hydrophobic interactions between hydrophobic peptides of T3 and casein micelles. Moreover, T3 contains many peptides (120) which could link casein micelles between them. Consequently, the addition of hydrolysates increased the viscosity of CMS at a concentration of 200 $g.kg^{-1}$. Nevertheless, more hydrolysates added were, less the viscosity of the liquid was.

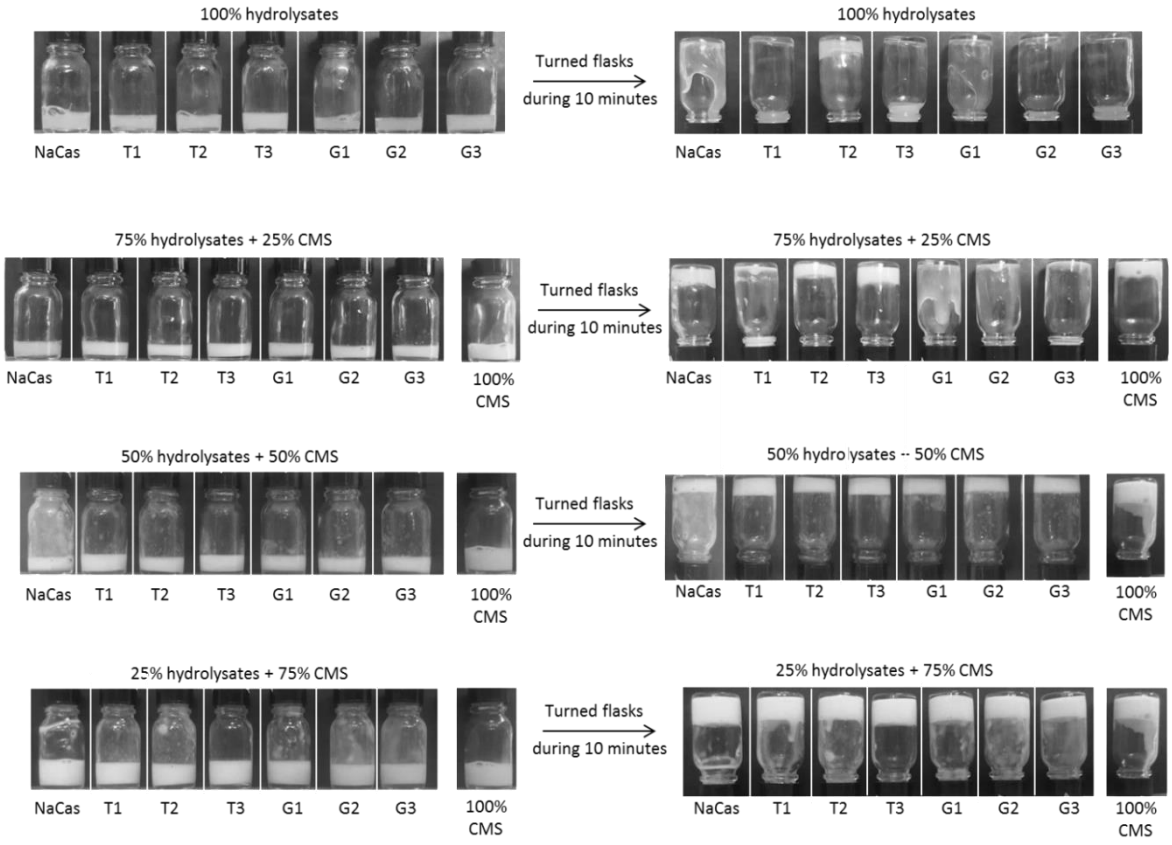


Figure 7. Influence of the ratio hydrolysate:CMS on the appearance and the functional properties of the suspensions at 200 $g.kg^{-1}$, at 20°C and pH 7. NaCas: sodium caseinate; T1, T2 and T3: casein-derived hydrolysates from Trypsin; G1, G2 and G3: casein-derived hydrolysates from Glu-C and CMS: casein micelle suspensions.

The rheological properties of casein micelles were differentially affected by the presence of hydrolysates

Rheology represents the mechanical properties of both solid and liquid foods and is an important monitor of quality control in dairy processing and in scientific research and was used to estimate the strength of the gel formed and their viscoelastic properties. Milk caseins are mainly responsible for the rheological and textural properties of dairy products such as gelation, stretchability and fracture *(39)*. Rheology of fluid is largely influenced by its viscosity. The dynamic viscosity η is a physical property of a fluid to resist to its flow or to pour *(40)*. In normal fluid milk, the viscosity is affected by the state and concentrations of fat, protein, temperature, pH and age of the milk. Under most conditions, milk behaves as a Newtonian liquid, meaning the shear stress is proportional to the shear rate *(41)*. Average milk viscosity has been determined at 1.7 mPa.s for cow milk at 20°C (Park, 2001). At 50 $g.kg^{-1}$, CMS, hydrolysates and mix hydrolysates-CMS had actually a Newtonian behavior (data not shown) with a viscosity ranged from 1.5 to 4 mPa.s. The viscosity did not vary according to the ratio hydrolysate:CMS (Figure 8). The viscosity of NaCas at 50 $g.kg^{-1}$ was divided by 25 compared to the viscosity of NaCas at 100 $g.kg^{-1}$. Consequently, the protein content was mainly responsible for the viscosity value of the solution.

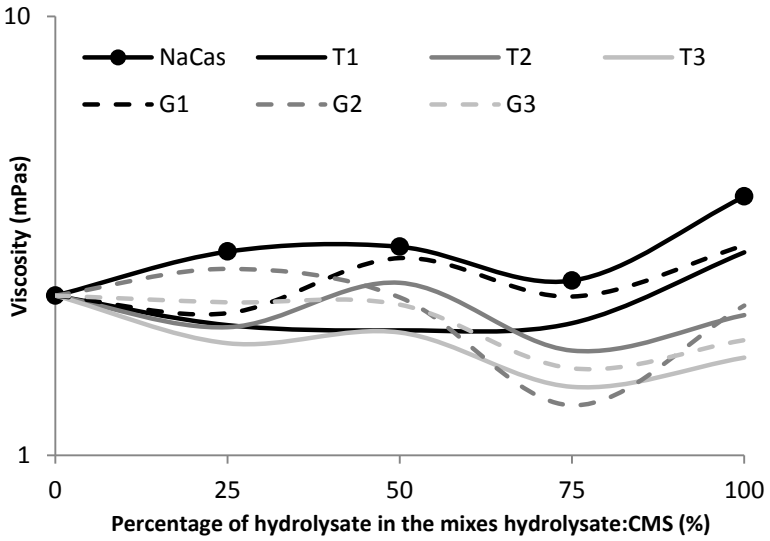


Figure 8. Shear rate dependence of the viscosity (η) of casein micelles with/without peptides at 50 $g.kg^{-1}$ at 20°C and pH 7. The viscosity was measured at a shear rate of 1 s^{-1}.

At 100$g.kg^{-1}$, a distinction can be drawn for the samples containing 100% hydrolysates: (i) the non-hydrolyzed NaCas had a viscosity higher than hydrolysates and (ii) the viscosity of 100% hydrolysates decreased when the time of hydrolysis increased. The viscosity of the hydrolysates, the mixes, the CMS and the NaCas ranged from 2 to 100 mPas (Figure 9). The samples at 100% and 75% of NaCas

had a rheofluidifiant behavior, since a shear rate of 20 s^{-1} and 30 s^{-1} respectively, whereas the hydrolysates had Newtonian behavior. From these shear rates limits, the samples at 100% and 75% of NaCas became rheofluidifiant. When the ratio hydrolysate:CMS decreased, the viscosity value of the mix tended to be near the viscosity value of 100% CMS. Except (i) at 25% of hydrolysate, the mix NaCas:CMS had a viscosity much higher than the mix with hydrolysates and (ii) at 100% and 75% of hydrolysate. Only for 100% and 75% of G1, the mix G1:CMS had a viscosity much higher than the other mixes with hydrolysates. The shear thinning behavior which implies a decrease in viscosity over a range of shear rates was exhibited by most polymeric solutions and melts *(42)* as by a large number of colloidal systems *(43)*. The sample showed Newtonian behavior at low shear rates and at a critical shear rate ($\dot{\gamma}c$) the viscosity of the sample decreased. Thus, at low shear rates the viscosity attained a constant value, the zero-shear viscosity near 100 mPa.s for 100% CMS, 35 mPa.s for 75% CMS and ranged from 4 mPa.s to 20 mPa.s for the other samples.

For the hydrolysates alone and for both Trypsin and Glu-C hydrolysates, the viscosity decreased with the time of hydrolysis. These results were in agreement with the results of Tsumura et al. *(44)* who reported that the apparent viscosity of soy protein-derived papain hydrolysate was lower than the viscosity of unhydrolyzed soy protein. In general, lower apparent viscosity is observed when proteins are hydrolyzed as their molecular mass is reduced by proteolysis. Peptide profiles were dramatically altered after hydrolysis, as discussed earlier, and can explain the changes in viscosities. As previously reported *(45, 46)*, the observed decrease in viscosity after hydrolysis can be attributed in part to increased protein solubility. Increasing the degree of hydrolysis steadily decreased both interfacial shear elasticity and viscosity values as compared to the unhydrolyzed control *(47)*.

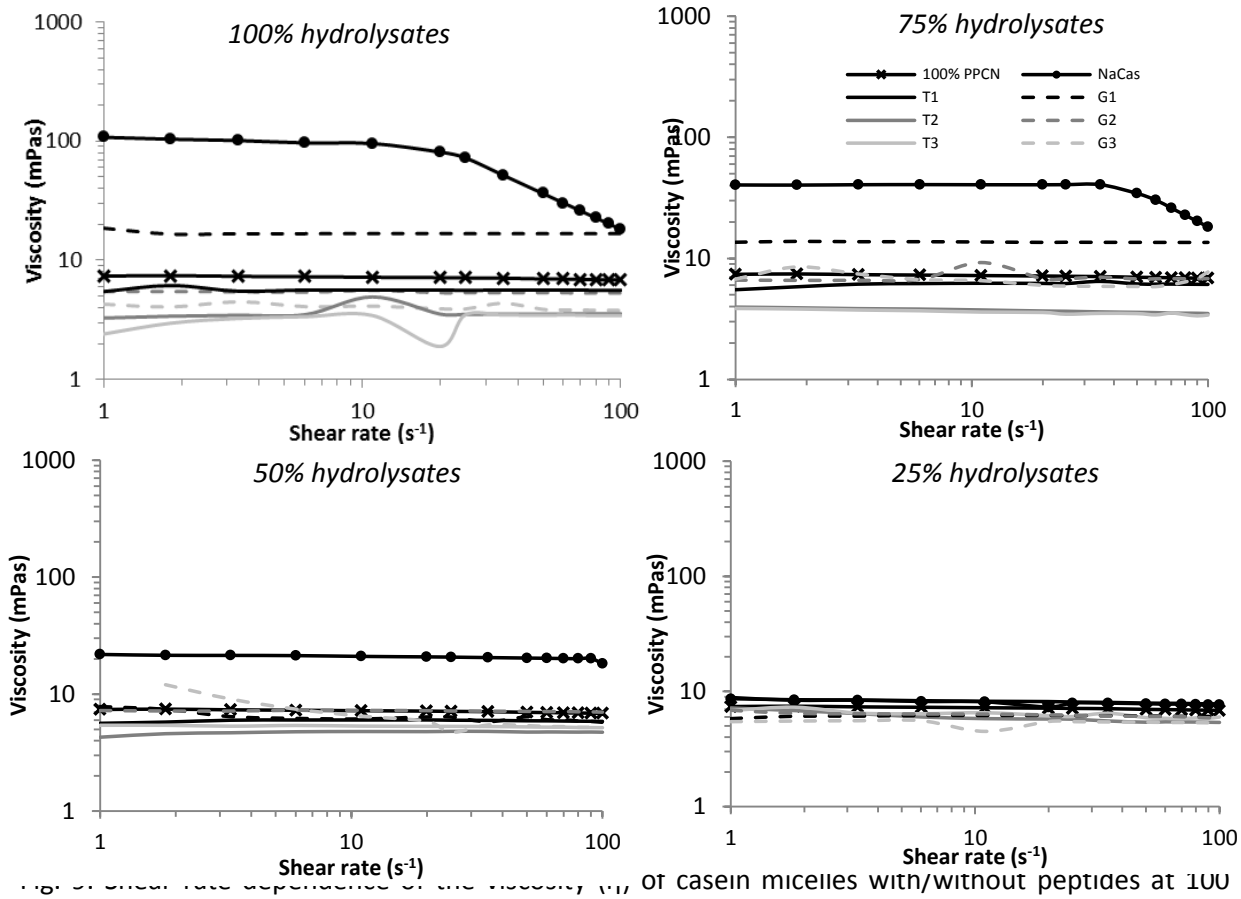


Fig. 9. Shear rate dependence of the viscosity (η) of casein micelles with/without peptides at 100 g.kg^{-1} at 20°C and pH 7.

At 200 g.kg^{-1}, the 100% NaCas had a Newtonian behavior and became rheofluidifiant at a shear rate around 20 s^{-1}, whereas T1. G2 and G3 had a Newtonian behavior along the entire shear rate tested (Figure 10). G1 followed the same behavior of NaCas, firstly Newtonian and finally rheofluidifiant. T2, T3 and CMS had a Newtonian behavior since a shear rate of 0.01 s^{-1}. The more CMS was added to the NaCas or hydrolysate samples, the more the behavior of each sample followed the Newtonian behavior of CMS. Only 25% of CMS (w/w) was sufficient enough to change the behavior of the hydrolysates and to the NaCas. Effectively, the NaCas became rheofluidifiant at around 0.3 s^{-1}. G1 at around 1 s^{-1} whereas G2 and G3 became rheofluidifiant from 0.01 to 1 s^{-1} and T1 became rheofluidifiant on the entire shear rate. The rheological properties of native casein micelles and hydrolysate dispersions have been examined over a wide range of concentrations from dilute liquid-like dispersion (50 g.kg^{-1}) to dense gel-like dispersions (200 g.kg^{-1}). CMS, NaCas and hydrolysates had a rheofluidifiant behavior at high concentration whereas they had a Newtonian behavior at low concentration over a wide range of shear rate. Peptides were charged and could have the same role of than the NaCl in several studies where the viscosity of NaCas suspensions showed a tendency to increase when NaCl was added, in high concentrated systems *(48-51)*. As shown on the TEM images

(Table III), the peptides migrated into the native casein micelles and these micelles were expanded. Bouchoux et al. *(38)* had shown that at a regime below around 170 $g.kg^{-1}$ and at low-shear rates, the casein micelle dispersions behave qualitatively and quantitatively as a polydisperse hard-sphere fluid. At a high shear rates, a slight deviation from the hard-sphere fluid behavior was observed, presumably caused by deformations of the micelles in the flow. The micelles are well separated from each other and interact through excluded volume effects only *(38)*. Interactions were presumably presented between the CMS and NaCas because a strong gel appeared when CMS was mixed with NaCas, a stronger gel than NaCas alone. The viscosity of NaCas solutions increases strongly with increasing protein concentration above about 100 g.L-1, because the particles become close packed *(52)*. The presence of charged peptides can strongly influence the properties of casein micelle suspensions. The caseins can specifically bind peptides reducing the net charge of the proteins and thus the repulsive electrostatic interactions between the particles *(53-55)*.

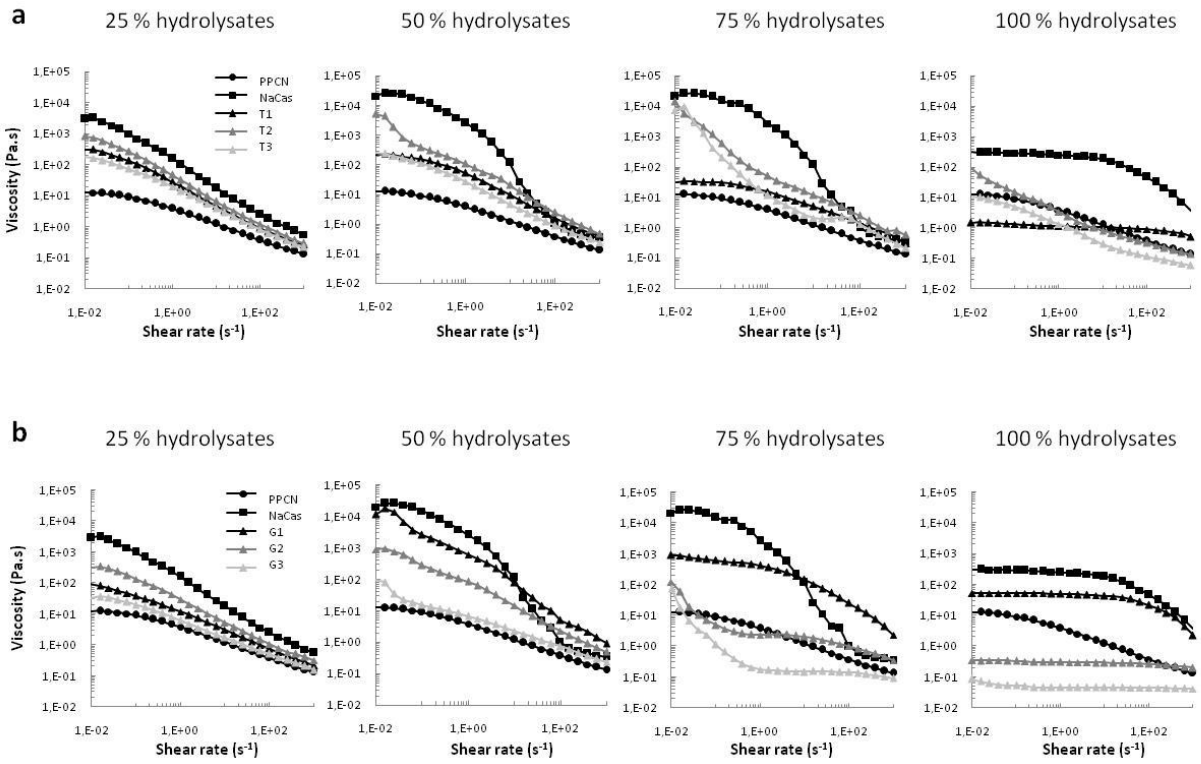


Figure 10. Shear rate dependence of apparent viscosity (η) for 100% CMS and NaCas and hydrolysates at different ratios, at 200g.kg^{-1} - strain=0.1% and 20°C. a: trypsin hydrolysates; b: Glu-C hydrolysates.

Dynamic magnitudes had to be used, such as modulus and phase angle, to describe the consistency and character of each sample. Viscoelastic measurements were also useful for characterizing changes like melting, crystallization and gel formation. The moduli of all samples varied dramatically with frequency. When the loss modulus which describes the viscous properties was much higher than the storage modulus, the sample behaved like a viscous liquid. When the storage modulus became much higher than the loss modulus, the sample had an elastic behavior. Storage modulus (G') and loss modulus (G") were the two viscoelastic parameters indicating gel strength *(46)*. According to the time, at a frequency of 1 Hz, the more hydrolysates were added, the more different were the behaviors of hydrolysates. The results showed on the Figure 11 confirmed the results of the visual tests (Figure 7). NaCas had a gel behavior at 25%, 50% and 75%. T1 had a gel behavior at 25% was liquid and became a gel from 5000 sec at 50% and was as liquid at 75%. T2 became a viscous fluid like a gel when a minimal of 50% of CMS was added. T3 was also like a gel in presence of CMS. The behavior of G1 seems to be identical to what was observed for NaCas, excepted at 25 and 75% where a stransition solution-gelation was observed. This could be related to the fact that a high content in native caseinate is still present in this hydrolysate and consequently the amount of peptides is too low to change the rheological properties. G2 and G3 had close values and behaviors too. Nevertheless, G3 had a weaker G' and G'' values than G2 for each ratio hydrolysate:CMS, as for the

iscosity values. Actually, when the concentration of peptides was increased in the CMS, the behavior was able to change as shown by G1 that was a gel at 75% In some other cases, time is needed to induce gelation as in the case of G2 that initially was a liquid and became a gel from 2000 sec at 50% and at 6000 sec for 75%. In contrast G3 that looks like a gel in visual test but kept a liquid behavior according to the time and the quantity of CMS added throughout rheological measurements. Once again, these examples showed the non-linearity of the phenomena.

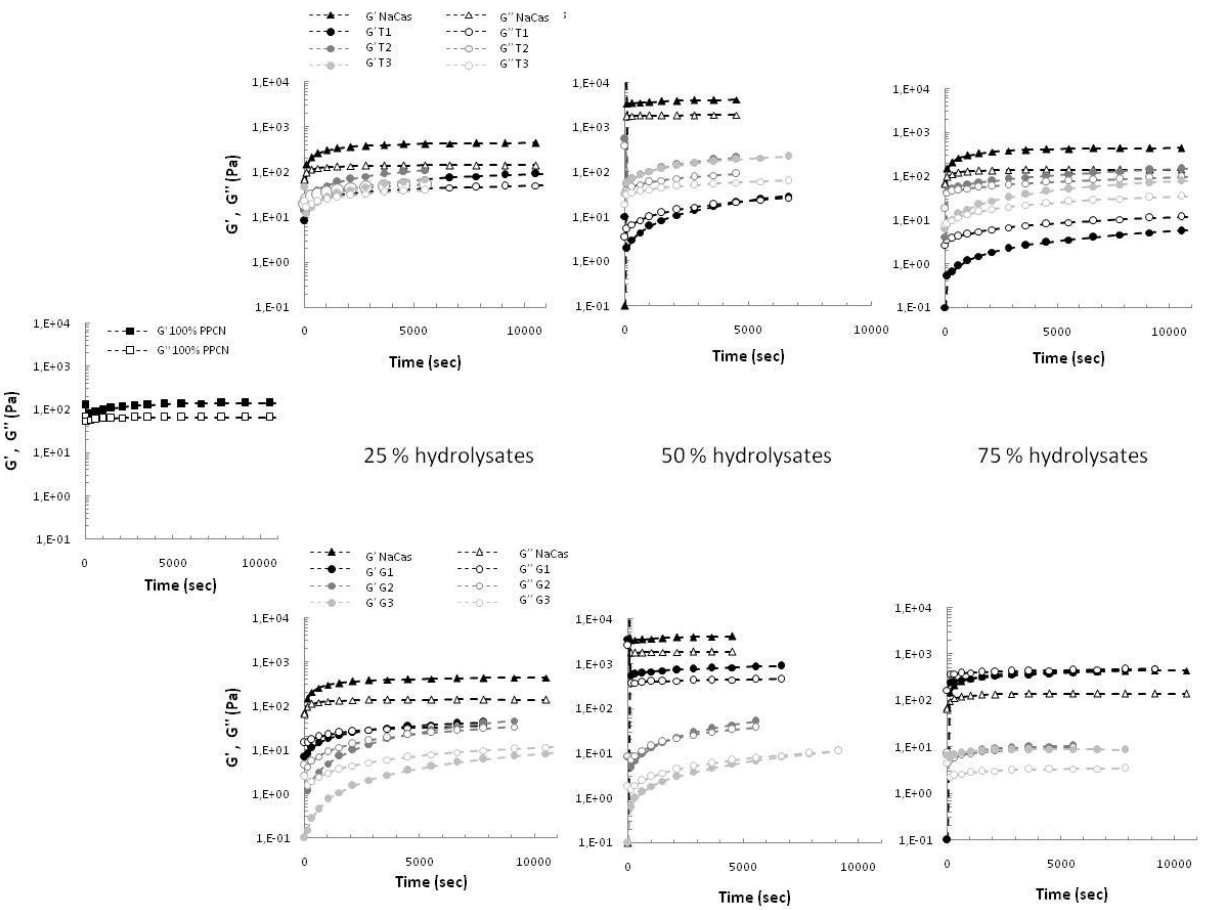


Figure 11. Time dependence of the elastic G' and loss G'' moduli for CMS. NaCas and hydrolysates at different ratios, at 200g.kg^{-1} - frequency = 1Hz. strain=0.1% and 20°C.

At concentrations 200 g.kg^{-1}, CMS, NaCas and hydrolysate suspensions began to show some elastic properties. This viscoelastic behavior was characterized through the response to harmonic stresses. Figure 12 shows the frequency dependence of the elastic modulus G and loss modulus G’ of CMS dispersions with or without addition of NaCas and hydrolysates at a concentration of 200 g.kg^{-1}. At the lowest concentration of hydrolysates (25%), the dispersions (excepted 25% NaCas) behaved as viscoelastic fluids, like 100% CMS, with G and G’ scaling from 10 to 100 Pa. The strong frequency dependence persisted at the other concentrations 50%, 75% and 100% of NaCas or hydrolysates. Then, viscoelastic modulus of the hydrolysates and the mixes hydrolysate-CMS were strongly dependent of the frequency used in the experiments. At 50% of NaCas, T1, T2, T3, G1 and G2, there

was a cross-over of G and G' at frequencies just below 0.01 Hz, indicating a transition to another regime. At this frequency, all these samples became liquid fluids whereas G3 became a liquid fluid only at frequency of 10 Hz. At the concentration of 75% hydrolysates, G' varied only slightly on frequency. T2, T3 and G3 had the behavior of liquid fluids whereas T1 was a viscoelastic fluid. NaCas, T1 and G2 were subjected to a transition to another regime. NaCas and T1 became a liquid fluid at around 0.01 Hz and 1Hz, respectively whereas at around 1 Hz G clearly dominated G' of 75% G2, meaning that the dispersion behaved primarily as liquid and became a viscoelastic fluid. At 100%, CMS, NaCas and G1 became liquids at around 0.01 Hz, 7Hz and 10 Hz respectively whereas T1 was viscoelastic regardless of the frequency and T2, T3, G2 and G3 became viscoelastic at around 0.1 Hz. The changes of behaviors of molecules with the hydrolysis were shown on the study of Doublier and Wood *(56)* showing that at high frequency, the viscoelastic behavior of hydrolyzed oat gum was similar to that of the unhydrolyzed sample, but at low frequency, the system was behaving as a gel as a result of intermolecular interactions.

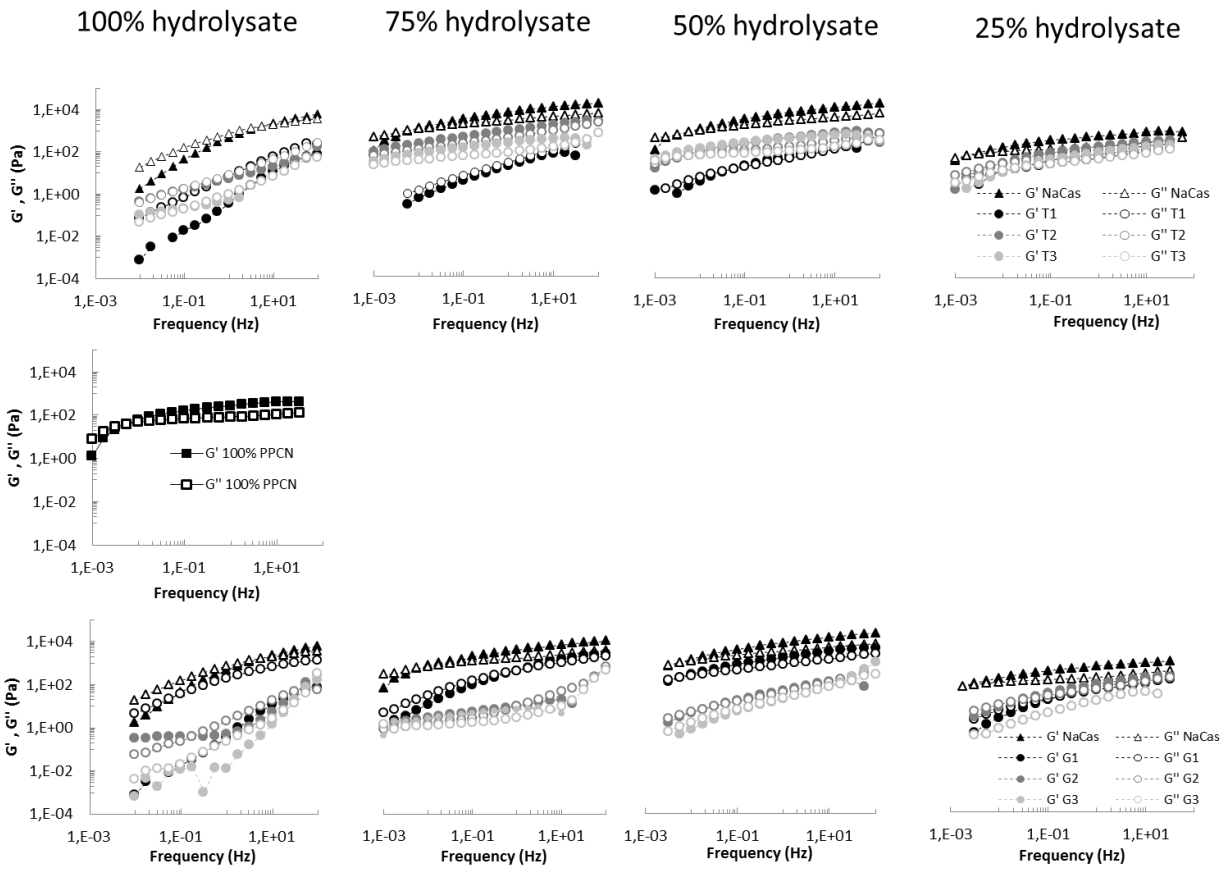


Figure 12. Frequency dependence of the elastic G' and loss G'' moduli for CMS. NaCas and hydrolysates at 200g.kg^{-1} - strain=0.1% and 20°C **.**

Overall, the investigation of gel formation first demonstrates that slightly modifying the time of hydrolysis of peptides or the nature of peptides produced leads to an important diversification of the rheological properties. Indeed, some Trypsin and Glu-C hydrolysates can form gel under neutral conditions, while many analogs underwent gelation either in presence of CMS depending on the nature of peptides and the ratio hydrolysate:CMS used. Additionally, it was highlighted that simple modification on the quantity of peptides in the hydrolysate also substantially impacts the microstructure and the gel formation rates. Indeed, some hydrolysates produced with the same enzyme can form gels in presence of casein micelles instantly, whereas others will form hydrogels later at a lower ratio hydrolysate:CMS.

This resulted in a high implication of the peptides in the reinforcement of the structuration of the matrices up to a limit beyond which the rheological properties were worsened depending i) on the types of peptides produced, involving mainly hydrophobic peptides, and peptides that were able to form α helices; ii) their proportion in the hydrolysate and hence in the matrices; and iii) the overall concentration of the dairy systems in which hydrolysates were added. Such an unexpected non-linearity of the phenomena could explain some adversely evolutions of the rheological properties of the casein systems observed.

This innovative approach gives new ways to study the relationship between physico-chemical and structural characteristics of peptides and their functional properties in matrices. To control the properties of mixed protein-peptide systems, it is important to further identify which peculiar peptides among the hydrolysates are strongly implied in the conformational and textural changes and on which parts of the caseins the interactions occur. The next step would be to select the peptides able to bind to proteins under such concentration and pH conditions.

Supplementary data

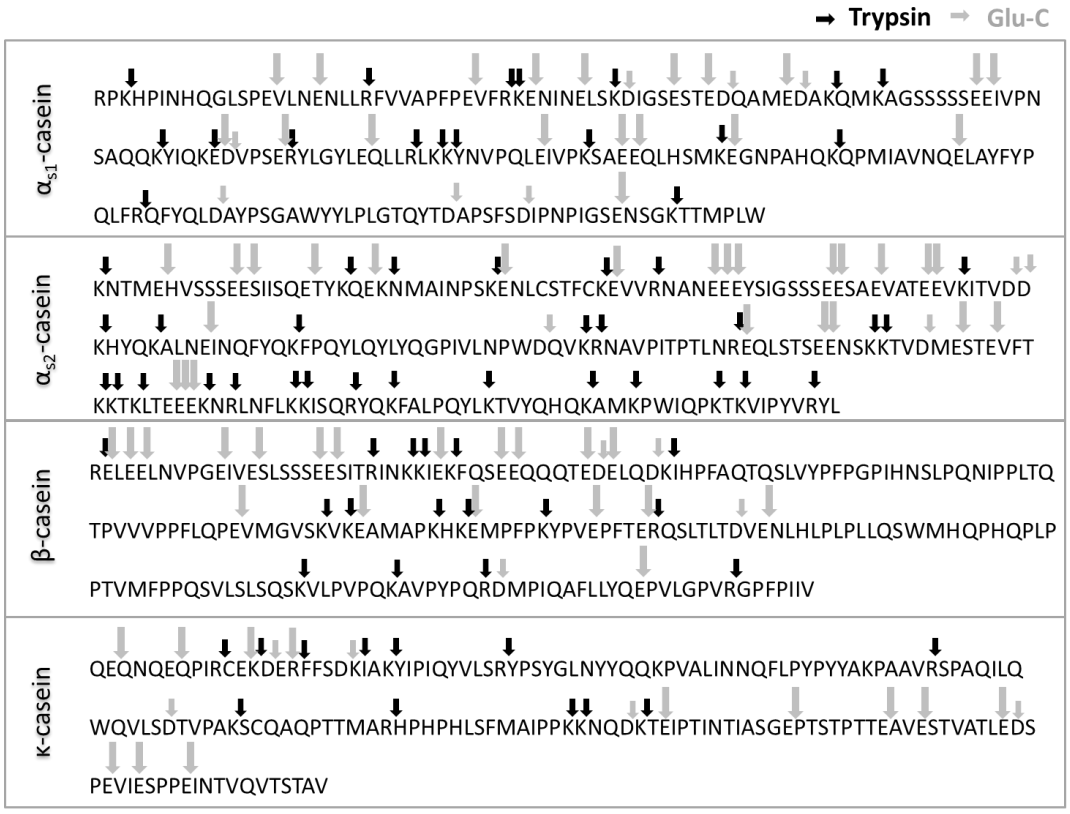
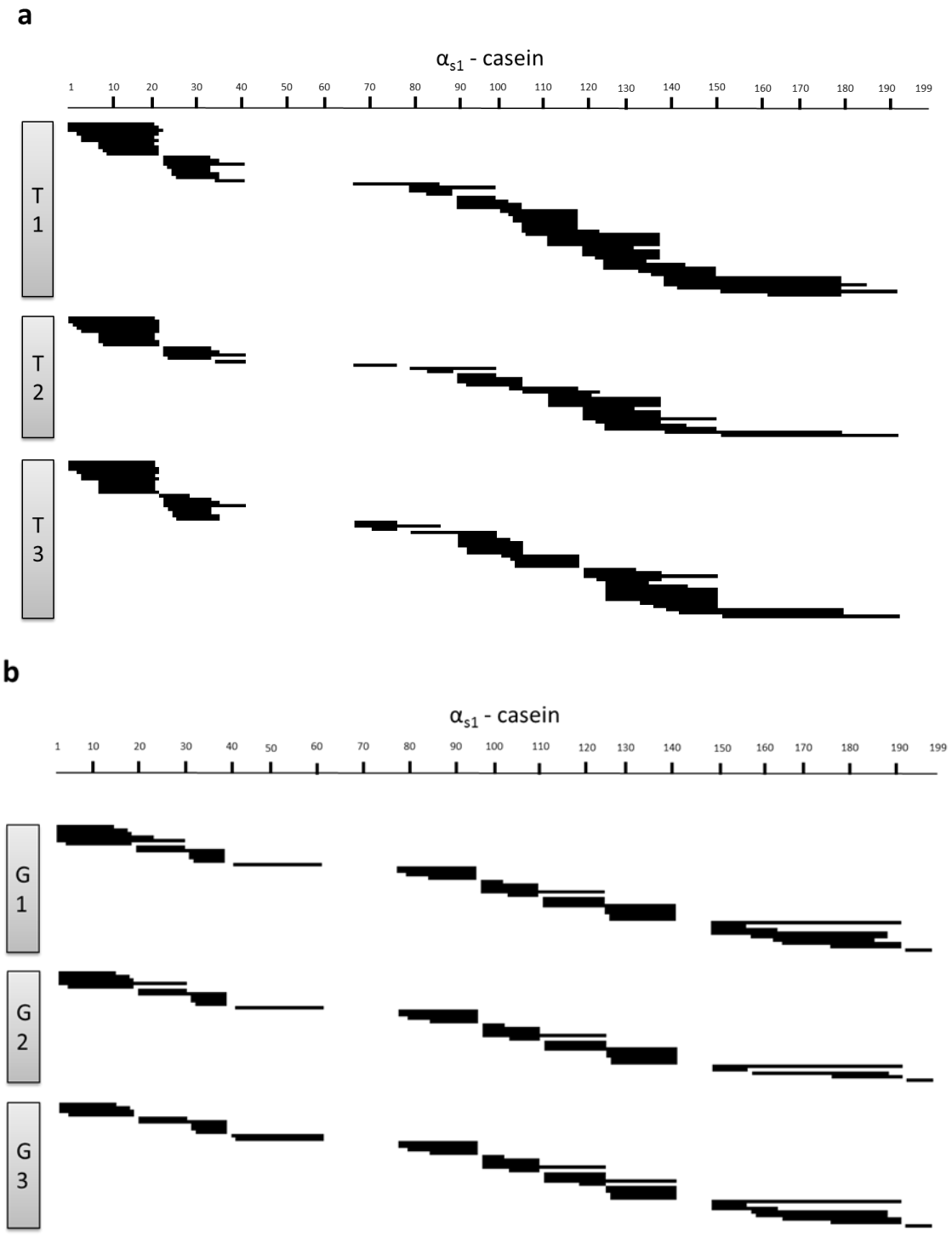
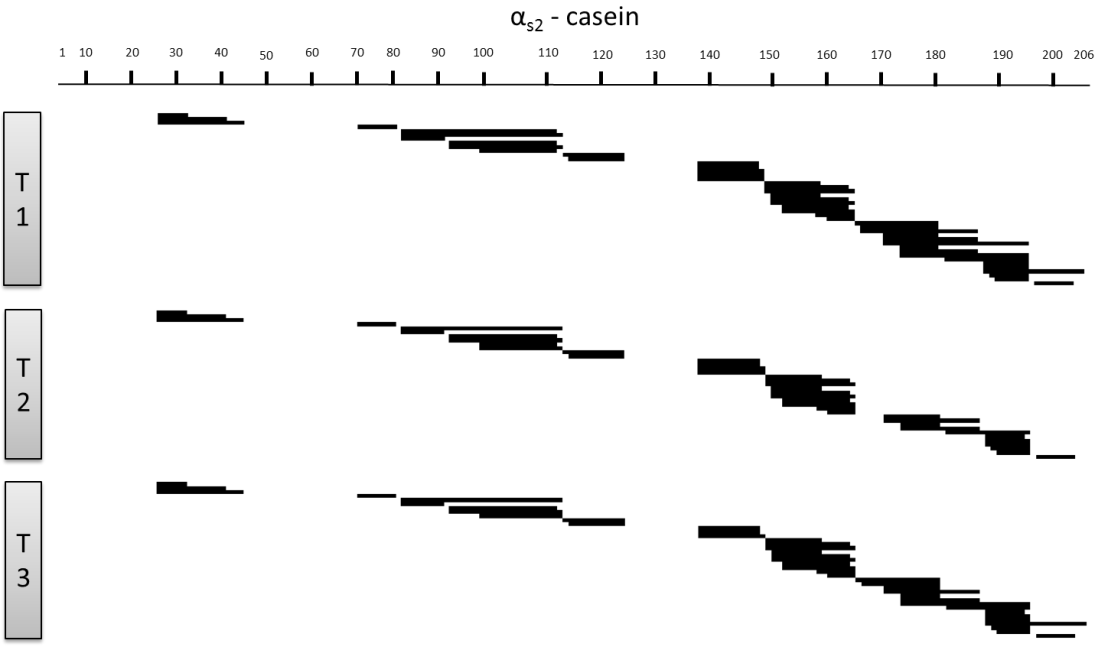


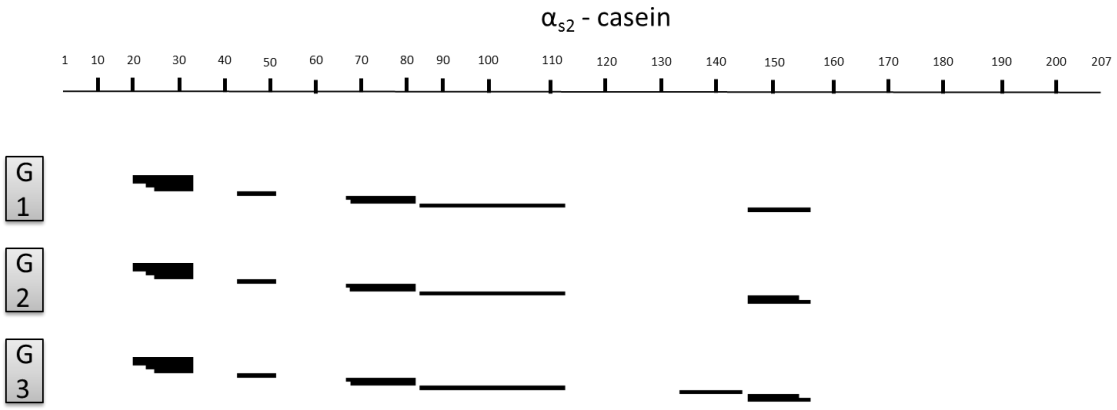
Figure 1. Cleavage sites of Glu-C and Trypsin for α_{s1}. α_{s2}. β and κ-caseins.


a
α_{s1} - casein
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 199
T 1
T 2
T 3
b
α_{s1} - casein
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 199
G 1
G 2
G 3

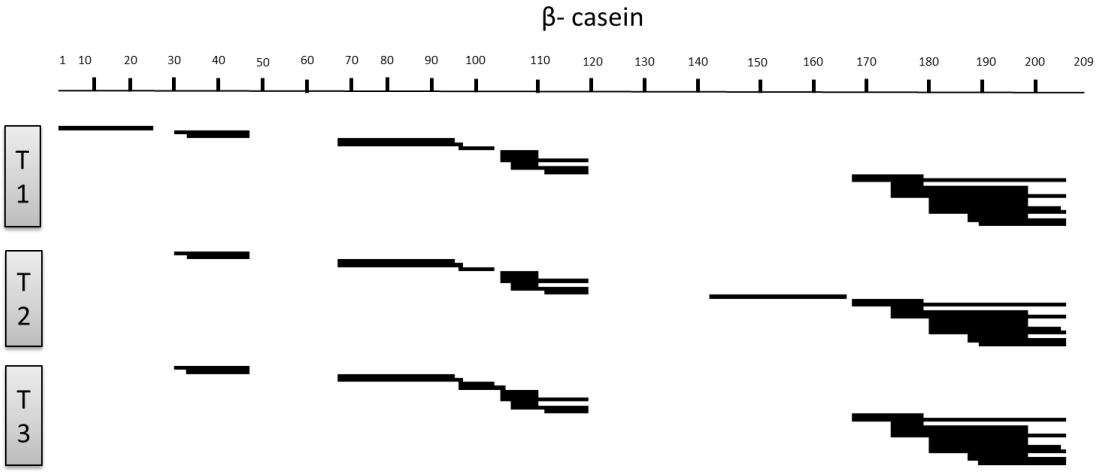
c


αs2 - casein
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 206
T 1
T 2
T 3

d


αs2 - casein
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 207
G 1
G 2
G 3

e


β- casein
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 209
T 1
T 2
T 3

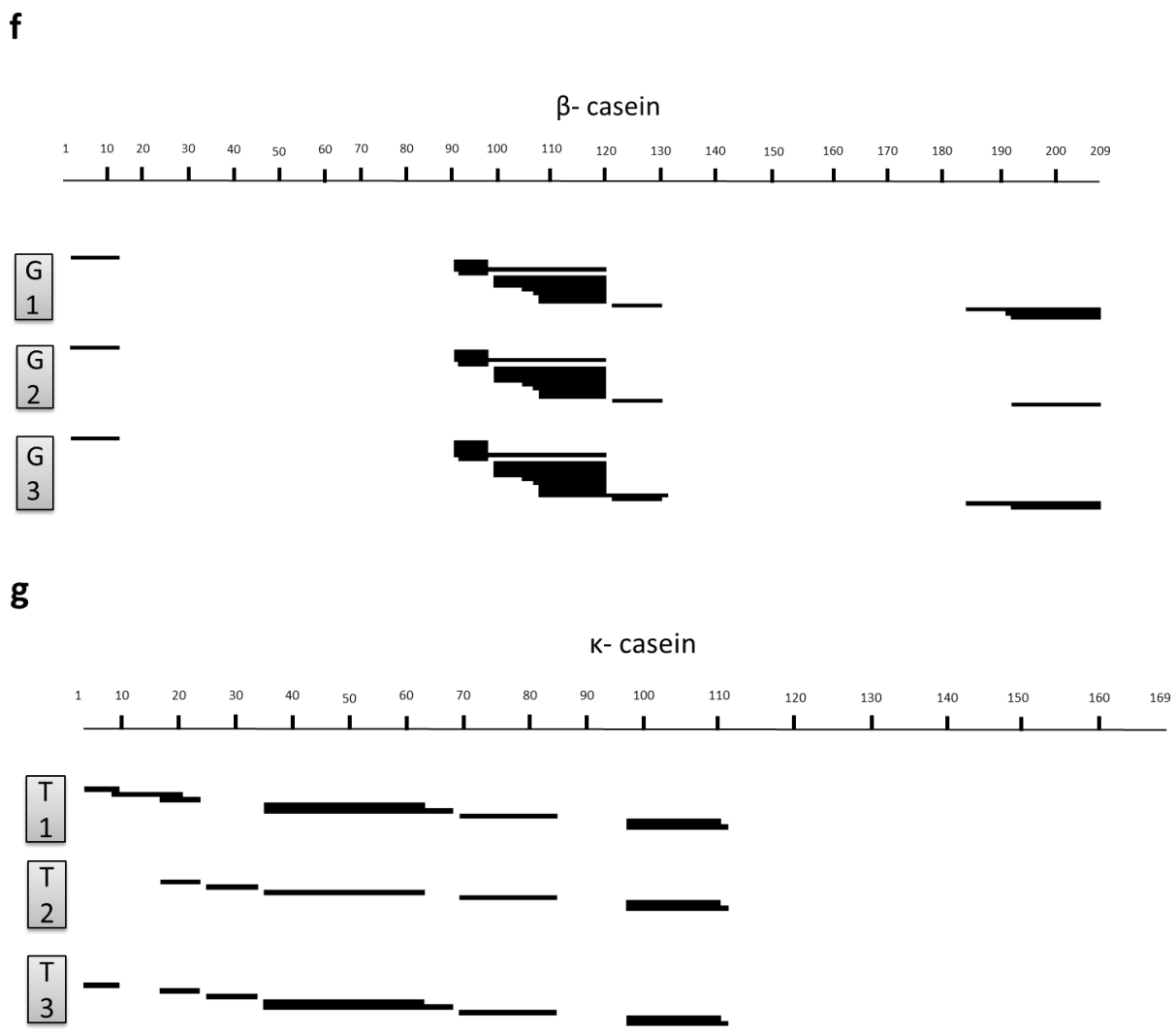


Figure 2. Peptide sequences. Number and location of cleavage sites of Trypsin and Glu-C through time of hydrolysis of α_{s1}- casein, α_{s2}- casein, β-casein and κ-casein.

Table I. Main physico-chemical characteristics of peptides ranging by cluster.

Cluster	Characteristics	Mean in category	Mean overall	SD in category	SD overall	p value
Gc1	Number of K	1.74	1.58	1.01	1.09	4.93E-06
	Number of D	0.48	0.40	0.85	0.76	3.54E-04
	Number of S	0.79	0.67	1.27	1.15	1.42E-03
	Number of H	0.55	0.48	0.70	0.70	1.65E-03
	Number of M	0.54	0.49	0.65	0.65	9.18E-03
	Number of T	0.56	0.50	0.84	0.80	1.61E-02
	Number of C	0.00	0.01	0.00	0.15	1.17E-02
	Number of N	0.66	0.73	0.73	0.83	4.52E-03
	Number of A	0.56	0.64	0.77	0.79	1.52E-03
	Number of W	0.08	0.11	0.27	0.31	9.37E-04
	Number of G	0.59	0.68	0.77	0.81	8.50E-05
	Number of Y	0.64	0.79	1.03	1.19	5.36E-05
	Number of V	1.04	1.18	0.79	1.01	1.33E-05
	Number of P	1.54	1.82	1.69	1.74	7.35E-08
	Number of I	0.65	0.79	0.70	0.79	1.40E-08
	Number of F	0.53	0.73	0.84	0.97	2.57E-11
	Number of R	0.38	0.51	0.52	0.57	6.41E-13
	Number of L	1.00	1.29	1.16	1.28	5.49E-13
	Number of Q	0.90	1.19	0.99	1.28	1.57E-13
Gc2	Number of Q	2.07	1.19	1.72	1.28	8.54E-12
	Number of G	1.18	0.68	0.78	0.81	1.40E-09
	Number of I	1.26	0.79	0.97	0.79	1.76E-09
	Number of E	2.48	1.83	1.76	1.19	3.61E-08
	Number of Y	1.21	0.79	1.46	1.19	4.00E-04
	Number of R	0.69	0.51	0.46	0.57	1.14E-03
	Number of W	0.21	0.11	0.41	0.31	1.35E-03
	Number of T	0.16	0.50	0.45	0.80	3.52E-05
	Number of K	1.00	1.58	0.63	1.09	8.00E-08
Gc3	Number of F	1.55	0.73	1.34	0.97	1.14E-12
	Number of V	2.00	1.18	1.51	1.01	9.62E-12
	Number of P	3.18	1.82	1.64	1.74	6.06E-11

	Number of N	1.18	0.73	0.98	0.83	5.32E-06
	Number of M	0.82	0.49	0.98	0.65	2.13E-05
	Number of K	2.09	1.58	1.62	1.09	8.64E-05
	Number of I	1.14	0.79	0.92	0.79	2.08E-04
	Number of G	1.00	0.68	0.74	0.81	1.09E-03
	Number of A	0.91	0.64	0.60	0.79	4.17E-03
	Number of E	2.23	1.83	1.00	1.19	4.72E-03
	Number of R	0.68	0.51	0.70	0.57	9.91E-03
	Number of D	0.05	0.40	0.21	0.76	8.21E-05
Tc1	Number of C	0.06	0.01	0.33	0.15	1.70E-06
	Number of L	1.52	1.29	1.32	1.28	3.48E-03
	Number of F	0.89	0.73	0.98	0.97	6.35E-03
	Number of R	0.59	0.51	0.60	0.57	2.27E-02
	Number of T	0.60	0.50	0.85	0.80	3.59E-02
	Number of D	0.26	0.40	0.58	0.76	2.55E-03
	Number of S	0.41	0.67	0.82	1.15	1.71E-04
	Number of G	0.49	0.68	0.73	0.81	7.25E-05
	Number of M	0.32	0.49	0.47	0.65	2.33E-05
	Number of H	0.27	0.48	0.61	0.70	1.26E-06
	Number of E	1.32	1.83	1.22	1.19	8.46E-12
Tc2	Number of Y	1.53	0.79	2.20	1.19	8.98E-07
	Number of L	2.07	1.29	1.48	1.28	1.42E-06
	Number of Q	1.81	1.19	1.40	1.28	1.22E-04
	Number of P	2.61	1.82	1.56	1.74	3.61E-04
	Number of W	0.24	0.11	0.46	0.31	1.13E-03
	Number of G	1.00	0.68	1.01	0.81	2.09E-03
	Number of H	0.69	0.48	0.88	0.70	1.63E-02
	Number of N	0.95	0.73	1.05	0.83	3.96E-02
	Number of M	0.29	0.49	0.49	0.65	1.30E-02
Tc3	Number of Q	1.96	1.19	1.60	1.28	3.78E-03
	Number of L	1.96	1.29	1.00	1.28	1.13E-02
	Number of Y	1.30	0.79	0.95	1.19	3.51E-02
	Number of R	0.74	0.51	0.44	0.57	4.84E-02
	Number of T	0.13	0.50	0.34	0.80	2.68E-02

	Number of H	0.00	0.48	0.00	0.70	7.99E-04
	Number of K	0.39	1.58	0.49	1.09	1.04E-07
Tc4	Number of A	1.18	0.64	0.94	0.79	1.10E-03
	Number of Y	1.55	0.79	1.20	1.19	2.51E-03
	Number of I	1.27	0.79	0.69	0.79	3.66E-03
	Number of R	0.82	0.51	0.57	0.57	9.78E-03
	Number of K	0.86	1.58	0.81	1.09	1.70E-03
Tc5	Number of V	2.18	1.18	1.19	1.01	9.68E-04
	Number of P	3.18	1.82	2.41	1.74	9.45E-03
	Number of R	0.91	0.51	0.79	0.57	1.90E-02
	Number of T	0.00	0.50	0.00	0.80	3.91E-02
	Number of K	0.45	1.58	0.50	1.09	5.36E-04
Tc6	Number of T	1.67	0.50	1.37	0.80	3.47E-04

Abbreviations used

CMS: Casein micelle suspension

NaCas: Sodium caseinate

T1: sodium caseinate hydrolyzed by Trypsin at time 1

T2: sodium caseinate hydrolyzed by Trypsin at time 2

T3: sodium caseinate hydrolyzed by Trypsin at time 3

G1: sodium caseinate hydrolyzed by Trypsin at time 1

G2: sodium caseinate hydrolyzed by Trypsin at time 2

G3: sodium caseinate hydrolyzed by Trypsin at time 3

FTIR: Fourier Transform Infrared (spectroscopy)

CD: Circular Dichroïsm (spectroscopy)

SDS-PAGE: Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis

Urea-PAGE: Urea Polyacrylmide gel electrophoresis

PC1: Principal Component 1

PC2: Principal Component 2

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Notes

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Chapter 3: Properties of milk peptides in gel casein matrices

The results presented in the chapter 2 showed that the presence of peptides modified the microstructure and the rheological properties of matrices differentially, depending on several parameters interacting to each other: the hydrolysate nature, the time of hydrolysis and the ratio HS:CMS used. This resulted in a high implication of the peptides in the reinforcement of the matrix structuration up to a limit beyond which the rheological properties were worsened depending on (i) the types of peptides produced, (ii) the peptide proportion in the hydrolysate and hence in the matrices and (iii) the overall concentration of the dairy systems in which hydrolysates were added. Such an unexpected nonlinearity of the phenomena could explain some adversely evolutions of the rheological properties of the casein systems observed.

The question remains as to whether such modification of rheological properties observed under neutral pH and liquid up to viscous suspensions were observed in the case of dairy gels under acid conditions.

In this context, the aim of this chapter was to explore how incorporation of peptides can modify the texture and the microstructure of gels formed under acid conditions by varying hydrolysate nature, the time of hydrolysis, the protein system concentrations and the ratio HS:CMS.

In this study, we chose to get a pH of 4.6 by addition of Glucono-delta-Lactone (GDL) in the various HS:CMS suspensions to reach the proper acidification conditions. This pH mimics the pH of many dairy products as yoghurt and cottage cheeses for example.

This chapter is presented under the form of an article in preparation for *Journal of Agricultural and Food Chemistry*[7] and is presented as a second part of the paper of the chapter 2.

[7] Lacou, L. et al. Peptide-casein interactions. Part II- Changes in texture and structural properties of high-concentrated casein matrices under acid gelation conditions. In preparation for *Journal of Agricultural and Food Chemistry*.

Chapitre 3 : Propriétés des peptides laitiers en gels hautement concentrés en caséines

Les résultats présentés dans le chapitre 2 ont montré que la présence de peptides modifiait la microstructure et les propriétés rhéologiques des matrices différemment, selon plusieurs paramètres en interaction : la nature de l'hydrolysat, le temps d'hydrolyse et le ratio hydrolysat:micelles de caséines utilisé. Cela résulte en une forte implication des peptides au renforcement de la structuration de la matrice jusqu'à une limite au-dessus de laquelle les propriétés rhéologiques ont empiré selon (i) le type de peptides produit, (ii) la proportion de peptides dans l'hydrolysat et donc dans la matrice concentrée en caséines, (iii) la concentration globale du système laitier dans lequel sont incorporés les peptides. Une non-linéarité inattendue des phénomènes pourrait expliquer certaines évolutions défavorables des propriétés rhéologiques de systèmes caséiniques mis en œuvre.

Si une telle modification des propriétés rhéologiques a été observée, à pH neutre et en conditions liquides jusqu'à obtention de suspensions visqueuses, sera-t-elle également observée dans le cas de gels laitiers en conditions acides?

Le but de ce chapitre a été d'explorer comment l'incorporation de peptides modifie la texture et la microstructure de gels formés en conditions acides en variant la nature de l'hydrolysat, le temps d'hydrolyse, les concentrations des systèmes protéiques et le ratio HS:CMS.

Dans cette étude, nous avons choisi d'atteindre un pH 4.6 an acidifiant les systèmes caséiniques *via* la Glucono-delta-Lactone (GDL). Ce pH mime le pH de plusieurs produits laitiers tels le yaourt ou le fromage blanc par exemple.

Ce chapitre est présenté sous forme d'article qui représente la seconde partie de l'article du chapitre 2, tous deux en préparation pour soumission à *Journal of Agricultural and Food Chemistry*[8].

[8] Lacou, L. et al. Peptide-casein interactions. Part II- Changes in texture and structural properties of high-concentrated casein matrices under acid gelation conditions. En préparation pour *Journal of Agricultural and Food Chemistry*.

Peptide-casein interactions. Part II- Changes in texture and structural properties of high-concentrated casein matrices under acid gelation conditions

In preparation for Journal of Agricultural and Food Chemistry

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Abstract

Gelation of dairy products can be modified by the hydrolysis of the caseins. However, to determine how, and to which extent, the functional properties of proteins are modified due to protein hydrolysis is still a matter of questions notably when caseins forms gels under acid pH conditions. Sodium caseinate was hydrolyzed by Trypsin and Glu-C, to obtain peptides with defined and different physico-chemical characteristics. Controlled amount of the three hydrolysates obtained per enzyme were incorporated into high-concentrated casein matrices from 50 to 200 $g.kg^{-1}$. Texture profile analysis (TPA) and microstructure of these matrices by TEM were studied at 20°C, under acid pH conditions. A high discrepancy between matrices was observed at the microstructural scale that was not observed in TPA. Although incorporation of hydrolysates drastically change the casein micelle texture by decreasing gel hardness regardless of the concentration used, there were few differences between the type of hydrolysates T and G in changing the texture properties at pH 4.6.

Keywords

Peptide, protein, hydrolysis, texture, identification, sequence, secondary structure, functional properties, gel

Introduction

Gelation is one of the most important functional properties of proteins as it provides texture and structure to the foods and namely to dairy products, in which the main responsible proteins for gelation are the caseins. Caseins represent 80% of the protein content in cow milk. They are composed of 4 caseins: α_{s1}-, α_{s2}, β and κ-caseins. These molecules are naturally unfolded *(1)* but in native milk, they are associated in the form of casein micelles linked themselves by phosphate and calcium ions. The exact organization of the caseins within these micelles is still unknown but it is admitted that they are "spherical" with a diameter ranging from 50 to 500 nm *(2)*. Casein micelles are dynamic structures and their supramolecular organization is the product of a continuous exchange with the medium. For example, acidification is a possibility to change the structure of the casein micelles and their interactions by extracting colloidal calcium phosphate (CCP) and water from the casein micelles *(3)*. This demineralization involved a partial dissociation of casein micelles *(4)* and the release of almost all the nanoclusters of calcium phosphate *(5)* and lead to formation of casein gels from pH 5.2.

The gel texture can be modified by the hydrolysis of the caseins. In a previous study, Lacou et al. *(6)* showed that incorporation of peptides into casein micelle suspensions were able i) to modify the microstructure of the casein micelle suspension by increasing the voluminosity of the casein micelles, ii) to induce conformational changes implying formation of more ordered secondary structural elements as α-helices and iii) to modify the rheological properties of the casein matrices leading to gel formation under neutral pH conditions, differentially, depending on several parameters interacting to each other as interactions between the hydrolysate nature, the time of hydrolysis and the mass ratio hydrolysate:casein micelles used. This resulted in a high implication of the peptides in the structuration reinforcement of the matrices up to a limit beyond which the rheological properties were worsened. This depends on the types of peptides produced (hydrophobic or not), their proportion in the hydrolysate and hence on the overall concentration of the dairy systems in which hydrolysates were added.

The question remains as to whether such modification of texture observed under neutral pH and liquid up to viscous suspensions were observed in the case of dairy gels under acid conditions. In the present study, we investigated how both the casein network organization and the textural properties of micelle caseins can be changed in the presence of various concentrations of peptides in three systems containing (i) 50 $g.kg^{-1}$ of proteins, (ii) 100 g.kg-1 and (iii) 200 g.kg-1 under acid pH conditions and at 20°C. Peptides were produced from the hydrolysis of caseins by two enzymes: (i) Trypsin and (ii) Staphylococcus aureus V8-protease also referred as endoproteinase Glu-C. The peptides were identified, physico-chemically and structurally characterized. Particular focus is paid on the

hydrolysate nature, the secondary structure of the peptides and the changes observed in terms of casein micelles organization at microscopic scale and in texture profile analyses.

Materials and methods

Hydrolysates

The hydrolysates used for this study were the same than the hydrolysates used in the study of Lacou et al. (article 2a). Briefly, sodium caseinate at 10 $g.kg^{-1}$, preheated to 37°C was hydrolyzed either by Trypsin immobilized on agarose beads (Thermo Fisher Scientific, France) at a ratio enzyme:substrate of 1049 U/29 g of NaCas or by endopeptidase GluC immobilized on agarose beads (Thermo Fisher Scientific, USA) at a E/S ratio of 34.8 mL of commercial Glu-C/14.5 g NaCas. The reaction was performed at 37°C and at a constant pH value of 7.1, using the pH-stat (Mettler Toledo, France) and 1M NaOH as a titrating agent. The hydrolysis reaction was stopped by filtration to separate immobilized enzymes from the hydrolysates and reused them, at three defined times corresponding to different compositions both in peptides and in native caseins: (i) 70% caseins + 25% peptides, (ii) 50% caseins + 50% peptides and (iii) 5% caseins + 95% peptides. They were referred respectively as to T1, T2 and T3 for hydrolysis with trypsin and G1, G2, and G3 for hydrolysis with Glu-C. The solution was freezed at -20°C, lyophilized (S.G.D. – SERAIL type CS 10-0.8, France) and stored under vacuum at 20°C under darkness until further use.

The physico-chemical characteristics of the peptides were estimated from the sequence of the peptides previously identified by tandem mass spectrometry *(6)*. Only the calculation of the charges with Python software from EMBOSS software suite (http://emboss.sourceforge.net) were modified to determine positive and negative charges at pH 4.6 corresponding to the environmental condition used in this study.

Gelation

The lyophilized hydrolysates were resuspended in maleate buffer pH 7 containing 25 mM Maleic acid (Sigma-Aldrich, France), 2 mM calcium Chloride.$2H_2O$ (AnalaR, France) and 3mM sodium azide as bacteriostatic agent (Sigma-Aldrich, France) in order to reach the 80 mM ionic strength of milk. The solution was stirred during 12h at 20°C. Then, this peptide solution was heated at 50°C for 3 hours in a water bath. The casein micelle suspension (CMS) was added at several concentrations (w/w) to the hydrolysate solution (HS) and the mix was stirred during 12h. To reach a pH of 4.6, GDL (Merck, France) was then added to the mix. On addition of GDL to native phosphocaseinate solutions, the mixture was stirred vigorously for approximatively 2 minutes before to put in a water bath at 20°C

during around 24h to reach pH 4.6. After acidification, gels were conserved at 4°C until rheological measurements.

Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis

Gels at 200 $g.kg^{-1}$ including 50% or 100% of hydrolysates were ultracentrifuged at 100 000 g for 1h using a Sorvall Discovery 90 Ultracentrifuge (Hitachi Koki, France). Under these conditions, all undissociated caseins were pelleted. The peptide pattern of the supernatants was analyzed by SDS-PAGE as described by Schägger and von Jagow *(7)* using Protean II system (16 × 16 × 0.1 cm; BioRad, Marnes-la-Coquette, France) with SDS/Tris/Tricine buffer and a concentration gradient from 12 to 18% of acrylamide used according to Sadat-Mekmene et al. *(8)*. The gels were scanned by the Image scanner III fitted with the LabScan (GE Healthcare, France) and analyzed by ImageQuant software (GE Healthcare, France), band intensities were estimated by the net volume parameter.

Microstructure

The microstructure of the gels was analyzed following the same protocol as described in the article of Lacou et al. *(6)*.

Instrumental texture profile analysis

The samples were equilibrated at 20°C for at least 1 hour prior to testing. A two-bite compression test was performed using the Texture Profil Analysis (TPA), described by Henry et al. (1971) and Bourne (1978), at 20°C with a texturometer Lloyd instrument (Ametek, France) with a 5 N load cell. A 50% compression was used and crosshead speed was 10 $mm.min^{-1}$. Nexigen Plus computer program was used for data collection and calculations. The samples were axially compressed in two consecutive cycles at 50% deformation, 30 seconds apart, with a 5 mm diameter cylinder. The crosshead moved at a constant speed of 10 $mm.min^{-1}$. From the TPA scores, the following textural parameters were obtained: (i) hardness (force necessary to attain a given deformation) at 50% deformation, (ii) elasticity (extent to which a deformed material returns to its original condition after deformation force is removed), (iii) cohesiveness (extent to which scallops could be deformed without rupturing), (iv) stickiness, (v) gumminess and (vi) rupture strength *(9)*. TPA test was conducted in triplicate for each of the three replications.

Statistical analysis

The physico-chemical characteristics of the peptides contained in each cluster formed in the study of Lacou et al., (part 1) were analyzed by ANOVA, following by a t-test that was realized taking account the mean in category and the mean overall of all the clusters.

To link cheese texture to a specific hydrolysate, a methodology based on multivariate exploratory analyses, such as Principal Component Analysis was performed using FactoMineR, an R package *(10)*. Two PCA were performed, one with all the gels and the other one without the gels made with NaCas in order to see the differences between the gels only made with hydrolysates. The first two PCs were retained as the totality of the inertia explained by both of them, represented a large part of the total inertia.

Results

Physico-chemical characteristics of hydrolysates at pH 4.6

Gelation of dairy products can be modified by the hydrolysis of the caseins. However, to determine how, and to which extent, the functional properties of proteins are modified due to protein hydrolysis is still a matter of questions notably when caseins forms gels under acid pH conditions. Sodium caseinate was hydrolyzed by Trypsin and Glu-C, to obtain peptides with defined and different physico-chemical characteristics. Controlled amount of the three hydrolysates obtained per enzyme were incorporated into high-concentrated casein matrices from 50 to 200 $g.kg^{-1}$. Clusters were formed (citer article part I) according to the kinetics of peptide apparition in each hydrolysate. A t-test was performed to highlight the main physico-chemical traits related to each cluster and, in turn, to characterize each type of hydrolysates according to the time of NaCas hydrolysis (Table I).

Table I. Number of charges of peptides at pH 4.6 ranging by cluster.

Cluster	Hydrolysate	Number of charges	Mean in category	Mean overall	SD in category	SD overall	p value
Tc1	T1	Negative	2,13	2,63	1,06	1,12	4,59E-13
Tc2	T1	Positive	4,01	3,57	1,64	1,47	1,88E-02
Tc3	T1-T2	Positive	2,13	3,57	0,34	1,47	2,19E-06
Gc1	G3	Negative	2,73	2,63	1,07	1,12	3,90E-03
Gc2	G1-G3	Negative	3,19	2,63	1,22	1,12	5,81E-07
Gc3	G1	Positive	4,18	3,57	1,91	1,47	5,46E-04

Among the parameters tested (pI, GRAVY, number of positive charges, size, Aliphatic index, number of aromatic amino-acids, molecular weight), only some of them were highlighted as predominant for each cluster (citer article part I). Moreover, some differences between the numbers of charges were highlight according to the pH of the dairy systems. The differences of number of charges at pH 4.6 between the clusters and are presented in the Table I. At pH 7, T1 contained hydrophobic peptides

with less negative and positive charges than the clusters contained in the other hydrolysates on the contrary to pH 4.6. The G hydrolysates contained peptides with similar characteristics at pH 4.6 than at pH 7. To conclude, the pH had a net influence only on the number of charges of peptides produced at the beginning of the hydrolysis with Trypsin.

Gel formation at pH 4.6

At pH 4.6, each mix of hydrolysates T and G with CMS formed gels regardless of the concentration in CMS used. The gels had different shapes. Some of them were grainy, especially for gels manufactured with 100% hydrolysates. The gel made with 100% CMS was softer than the gels made with hydrolysates as shown for gels at 200 $g.kg^{-1}$ (Figure 1).

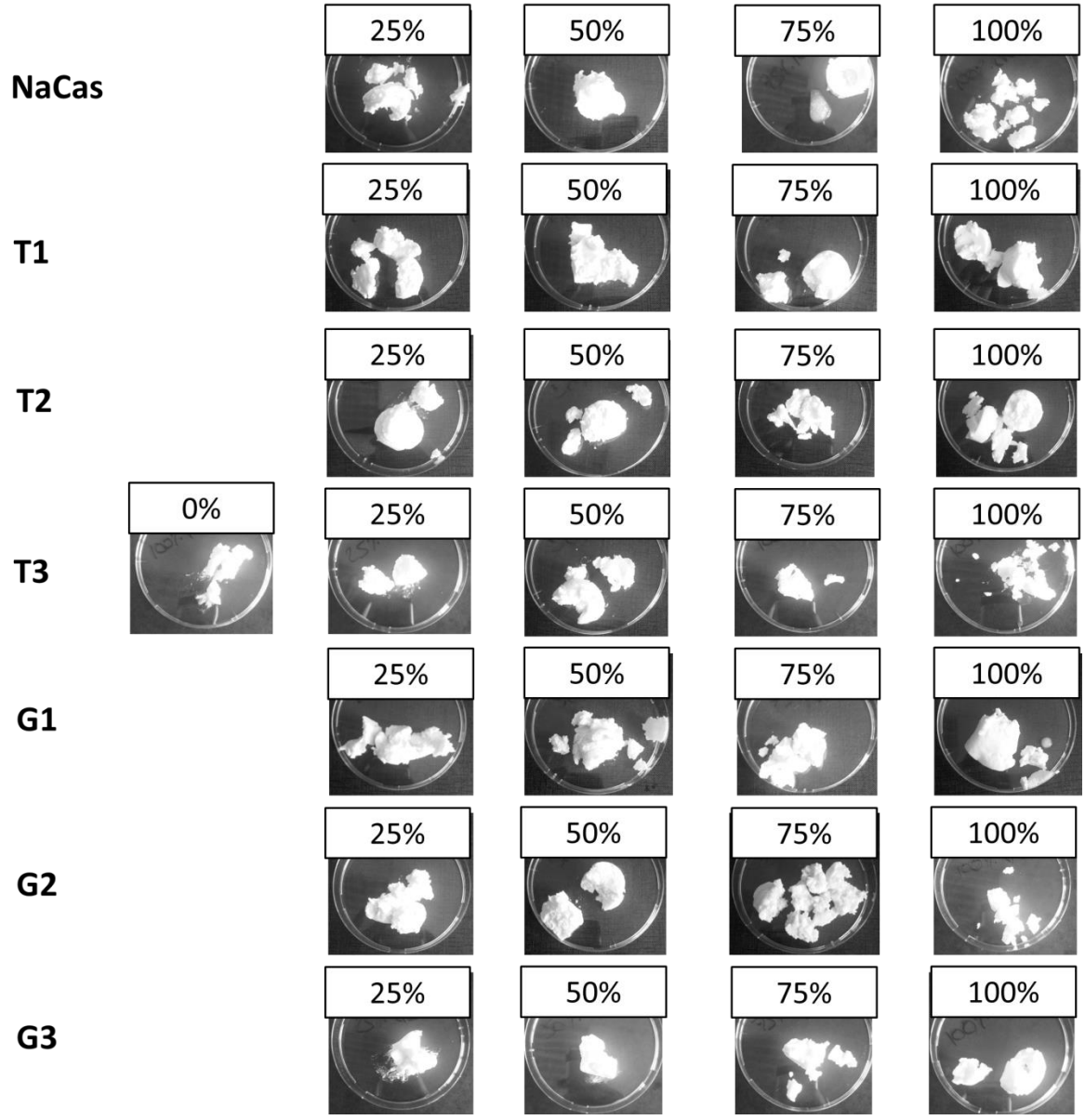


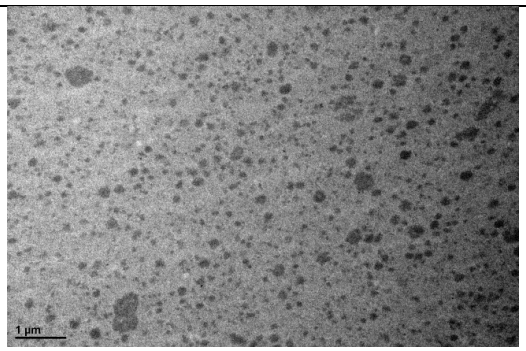
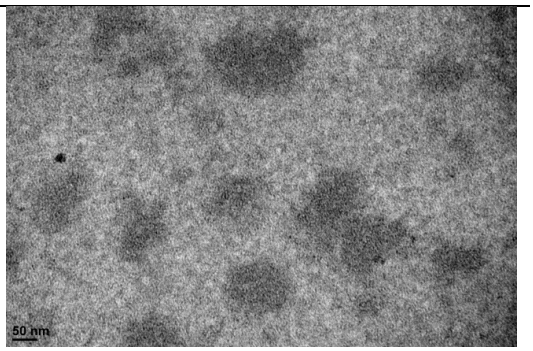
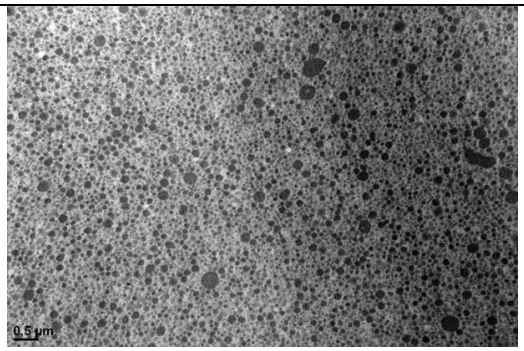
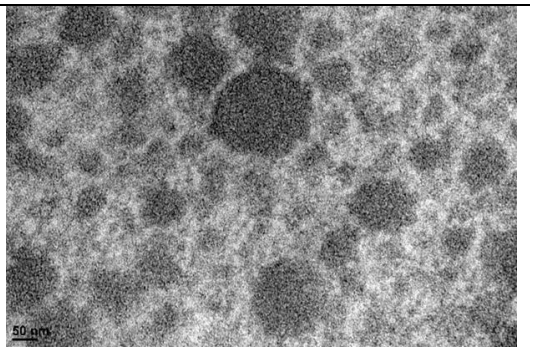
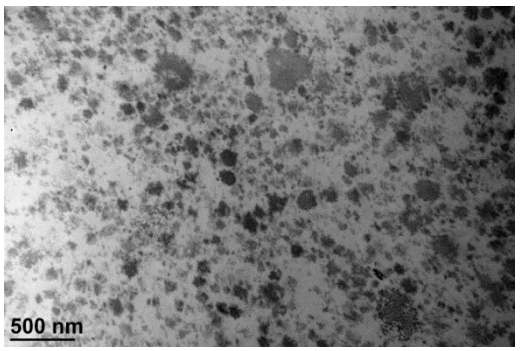
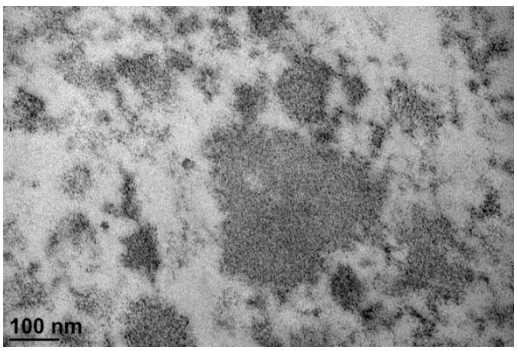
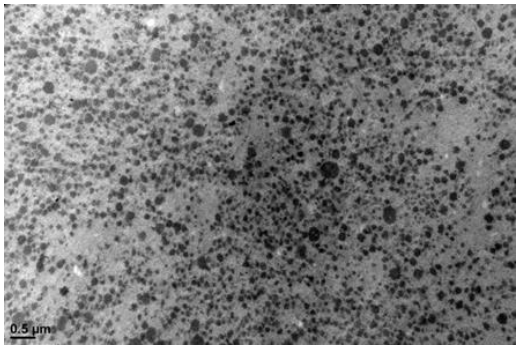
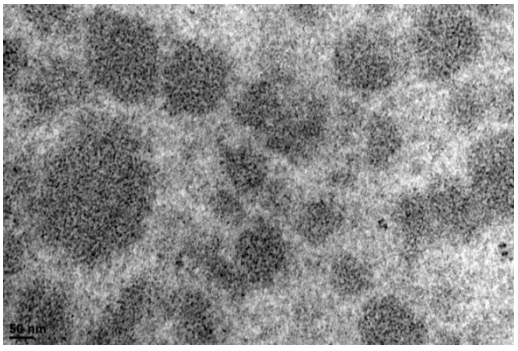
Figure 1. Photographs of gels at 200 g.kg^{-1} made with NaCas, casein-derived hydrolysates from Trypsin (T1, T2 and T3) and from Glu-C (G1,G2 and G3)and casein micelles suspensions (CMS) and various reincorporation levels (from 25 to 100%) of NaCas and hydrolysates T and G in the CMS.

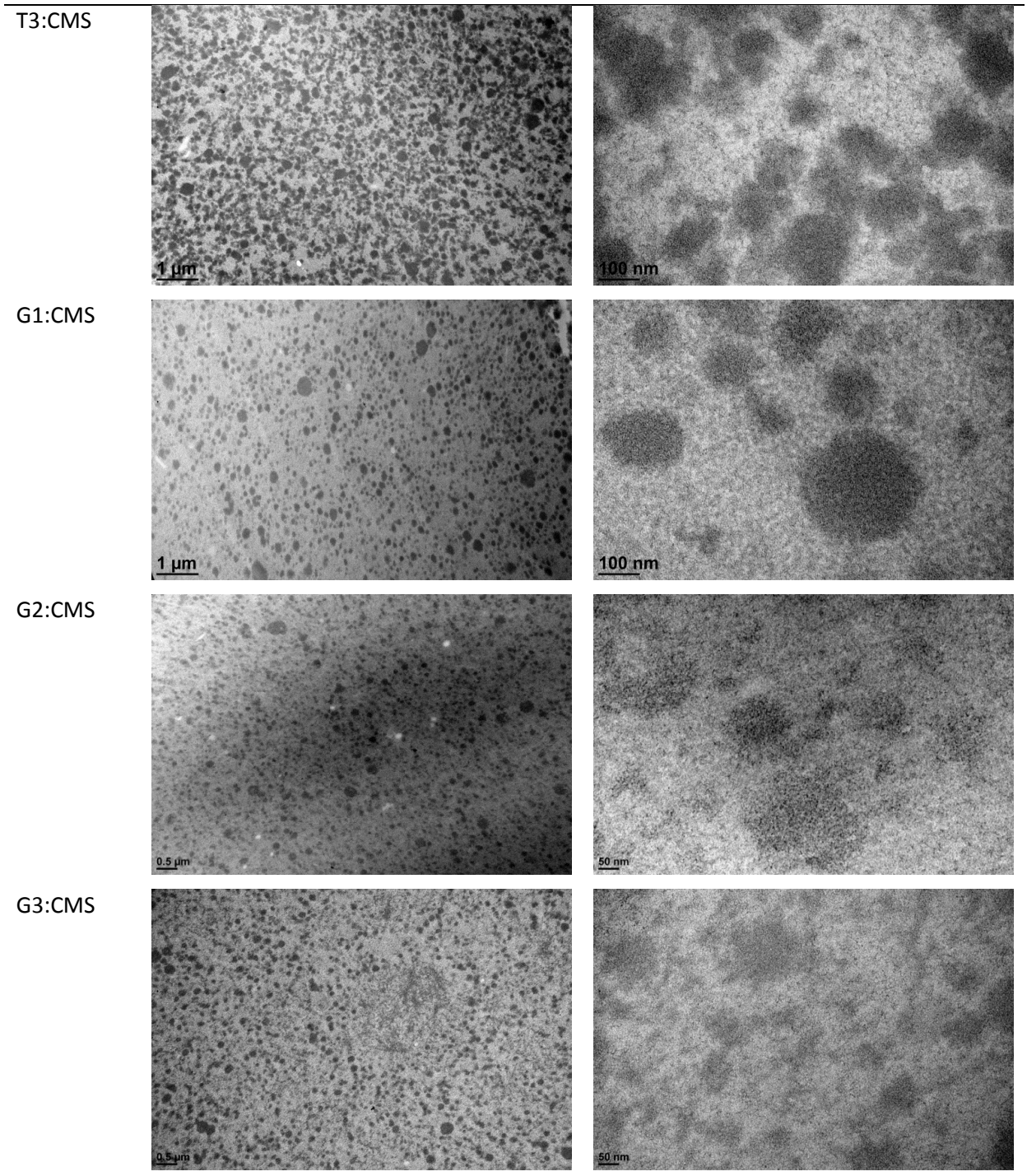
The microstructure of casein micelle network is modified in presence of hydrolysates

The microstructure of gels made with 50% HS and 50% CMS at 200 g.kg^{-1} was analyzed by TEM (Table II). At pH 4.6, the casein micelles were more compact than at pH 7 *(6)*. Under high magnification conditions (× 200 000) some particles of colloidal calcium phosphate also referred as calcium phosphate nanoclusters were visible in our sample in contrast to the study of Marchin et al. *(5)* in which they disappeared at pH 4.6. This is due to the pH which could be at higher value than expected, the very small size of the sample making any direct pH control impossible. When NaCas was incorporated in the CMS, the network was denser as NaCas contains caseins that can auto

associate to form particles of 20 nm and they also can penetrate within the casein micelles. The structure of casein micelle was expanded in presence of T1 hydrolysate, likely because peptides can enter into the micelles. In presence of T2, the structure of native casein micelle stayed intact in size but appeared denser than the CMS, phenomenon that was reinforced with T3. G1 has a microstructure close to that observed with T hydrolysates. However, the gel containing G2 had a very different microstructure, showing interactions between casein micelles. With the presence of G3, which contains hydrophilic peptides with many negative charges, the casein micelles seemed to be exploded.

Table II. Electronic microscopy scanning micrographs of gels made by mix 50% HS and 50% CMS at 200 $g.kg^{-1}$ at pH 4.6.

Sample	× 20 000	× 200 000
CMS		
NaCas:CMS		
T1:CMS		
T2:CMS		



The texture profile analysis of casein micelle gel is affected by the presence of hydrolysates

Texture profile analysis (TPA) and microstructure of these matrices by TEM were studied at 20°C, under acid pH conditions. A high discrepancy between matrices was observed at the microstructural scale that was not observed in TPA. Although incorporation of hydrolysates drastically change the casein micelle texture by decreasing gel hardness regardless of the concentration used, there were

few differences between the type of hydrolysates T and G in changing the texture properties at pH 4.6.

Principal component analysis allowed us to summarize the information related to the changes in texture profile analysis in two dimensions, accounting 8.16%, 87.6% and 89.31% of the total variation for all the gels at 50, 100 and 200 $g.kg^{-1}$, respectively.

Figure 2A and 2D represent correlation circle of the variables and Figures 2B, 2C, 2E and 2F represent the scores of the individuals for the gels prepared at $50g.kg^{-1}$ under various hydrolysate:CMS ratios. Principal component 1 explained most of the variation (71.28% for the Figures A to C and 67.07% for the Figures D to F) and was related to the gumminess, hardness, stickiness and elasticity of the acid gels (Figure 2A and 2D). The different gels were also separated according to the principal component 2, which represents 16.88% of the total variation for the Figures A to C and 17.84% of the total variation for the Figures D to F and was related to the cohesiveness. The gels containing NaCas at a concentration more than 75% (Figure 2B) and the gels made with hydrolysates with a low DH (T1, G1, G2) (Figure 2C) scored positively on PC1 and were correlated with the hardness, the stickiness and the gumminess. When the gels contained few quantities of hydrolysates, they scored negatively on PC1 and were correlated with the elasticity and with a lower hardness value (Figure 2C). The gels containing 75% of hydrolysates were very dispersed on PC1 (Figure 2C). However, the gels made with a low DH were correlated with the hardness, stickiness and gumminess (Figure 2C). The Figures D, E and F represent the PCA without the presence of control gels containing NaCas to deeply explore the differences between the textures of gels containing only hydrolysates. The gels made with 100% CMS had a high hardness and a weak elasticity. The gels manufactured with 100% hydrolysates had specific textures different from the CMS texture. The gels made with G2 and T1 had a good cohesiveness and the gel made with G1 was firmer (Figure 2D). The presence of T1, G1 and G2 at a minimum of 25% in the gels increased the cohesiveness of the gel whereas the presence of T2, T3 and G3 at a minimum of 25% increased the elasticity of the CMS (Figure 2E). Regardless of the ratio hydrolysate:CMS, the gels containing Trypsin hydrolysates were more elastic than the other gels.

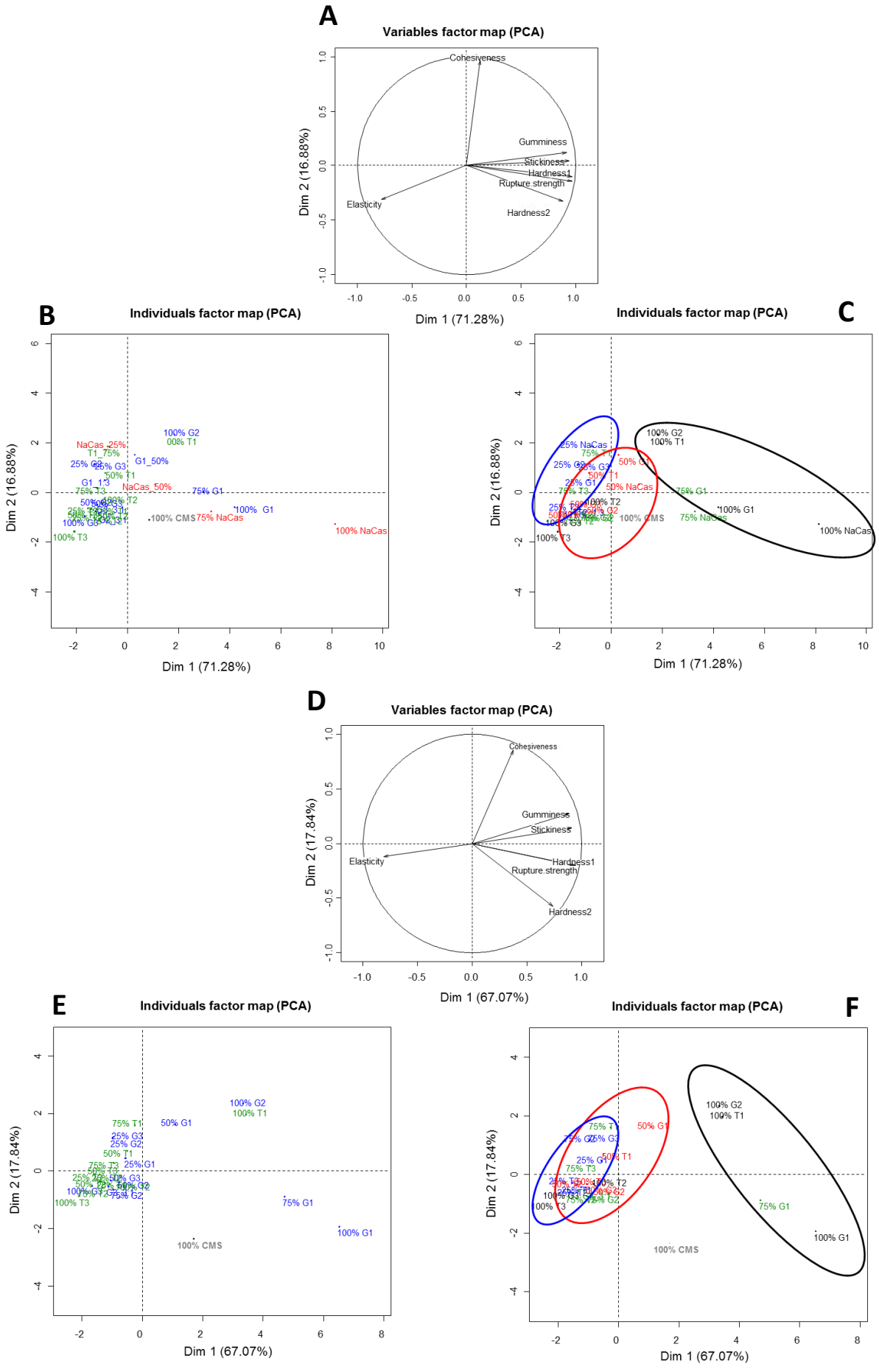

A
Variables factor map (PCA)
Cohesiveness
Gumminess
Stickiness
Hardness1
Rupture strength
Elasticity
Hardness2
Dim 2 (16.88%)
Dim 1 (71.28%)
B
Individuals factor map (PCA)
100% G2
100% T1
NaCas_25%
T1_75%
25% G2
25% G3
G1_50%
50% T1
G1_1.3
NaCas_50%
75% G1
100% G1
100% CMS
75% NaCas
100% NaCas
100% T3
Dim 2 (16.88%)
Dim 1 (71.28%)
C
Individuals factor map (PCA)
100% G2
100% T1
25% NaCas
75% T1
50% G1
50% T1
25% G1
50% NaCas
75% G1
100% T2
100% G1
100% CMS
75% NaCas
100% NaCas
100% T3
Dim 2 (16.88%)
Dim 1 (71.28%)
D
Variables factor map (PCA)
Cohesiveness
Gumminess
Stickiness
Elasticity
Hardness1
Rupture.strength
Hardness2
Dim 2 (17.84%)
Dim 1 (67.07%)
E
Individuals factor map (PCA)
100% G2
100% T1
75% T1
50% G1
25% G3
25% G2
50% T1
25% G1
75% G1
100% G1
100% CMS
100% T3
Dim 2 (17.84%)
Dim 1 (67.07%)
F
Individuals factor map (PCA)
100% G2
100% T1
75% T1
50% G1
25% G1
75% T3
100% T2
75% G1
100% G1
100% CMS
Dim 2 (17.84%)
Dim 1 (67.07%)

Figure 2. Principal Component Analysis of gels manufactured at 50 $g.kg^{-1}$with sodium caseinate (NaCas), Trypsin hydrolysates (T1, T2 and T3), Glu-C hydrolysates (G1, G2, G3) at different ratios hydrolysate:CMS (4:0, 3:1, 2:2, 1:3, 0:4). (A) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 71.28% of the total variability and dim 2 = 16.88% of the total variability). (B) Gels are represented according to their texture parameters and are colored according to the type of hydrolysates used (red: NaCas, green: Trypsin, blue: Glu-C, grey: CMS). (C) Gels are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate, grey: 100% CMS). (D) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 67.07% of the total variability and dim 2 = 17.84% of the total variability) without the presence of NaCas. (E) Gels, except gels made with NaCas, are represented according to their texture parameters and are colored according to the type of hydrolysates (green: Trypsin, blue: Glu-C, grey: CMS). (F) Gels, except gels made with NaCas, are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate, grey: 100% CMS).

Figure 3A and 3D represent a correlation circle of the variables and Figures 3B, 3C, 3E and 3F represent the scores of the individuals for the gels prepared at 100$g.kg^{-1}$ under various hydrolysate:CMS ratios. Principal component 1 explained most of the variability (73.55% for the Figures A to C and 64.61% for the Figures D to F) and was related to the gumminess, hardness and stickiness of the gels (Figure 3A). The different gels were also separated according to the principal component 2, which represents 14.05% of the total variation % for the Figures A to C and 20.05% of the total variation for the Figures D to F and was related to the elasticity (Figure 3A) or to the cohesiveness (Figure 3D). The gels were better separated according to their hardness, stickiness and gumminess than according to their elasticity (Figures 3B and 3C). Contrary to the gels at 50 $g.kg^{-1}$, the elasticity was uncorrelated with the hardness of gels (Figure 3A). As at 50 $g.kg^{-1}$, the gels made with 100% hydrolysates had very specific textures. The gels made with 100% G1, G2, T2 and T3 were firmer than the others whereas the gels manufactured with 100% T1 had a high cohesiveness and the gel at 100% of G3 was slightly sticky (Figure 3F). As at 50 $g.kg^{-1}$, the presence of hydrolysates in the gels rendered the gels firmer than the control one (Figure 3E). Moreover, the more the hydrolysates were present in a high quantity, the more the gels were firm (Figure 3F).

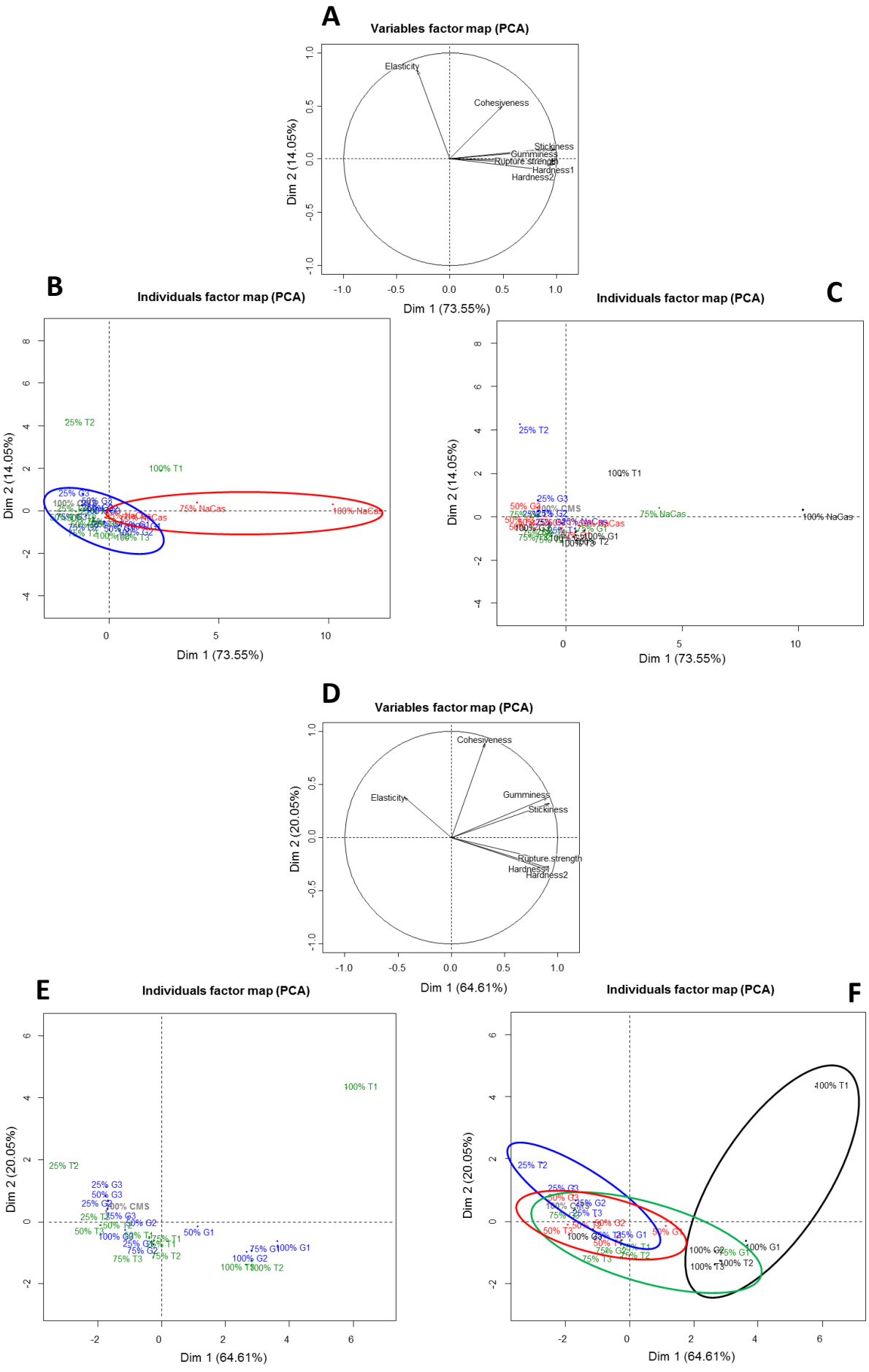

A
Variables factor map (PCA)
Elasticity
Cohesiveness
Stickiness
Gumminess
Rupture strength
Hardness1
Hardness2
Dim 2 (14.05%)
Dim 1 (73.55%)
B
Individuals factor map (PCA)
25% T2
100% T1
75% NaCas
100% NaCas
Dim 2 (14.05%)
Dim 1 (73.55%)
C
Individuals factor map (PCA)
25% T2
100% T1
25% G3
50% G3
100% CMS
75% NaCas
100% NaCas
100% G1
100% T2
Dim 2 (14.05%)
Dim 1 (73.55%)
D
Variables factor map (PCA)
Cohesiveness
Elasticity
Gumminess
Stickiness
Rupture strength
Hardness1
Hardness2
Dim 2 (20.05%)
Dim 1 (64.61%)
E
Individuals factor map (PCA)
100% T1
25% T2
25% G3
50% G3
25% G2
100% CMS
25% T3
50% T3
50% T2
50% G2
100% G3
50% T1
75% T1
50% G1
25% G1
75% G2
75% T3
75% T2
75% G1
100% G1
100% G2
100% T3
100% T2
Dim 2 (20.05%)
Dim 1 (64.61%)
F
Individuals factor map (PCA)
100% T1
25% T2
25% G3
50% G3
25% G2
100% CMS
25% T3
50% T3
50% T2
50% G2
100% G3
50% T1
75% T1
50% G1
25% G1
75% G2
75% T3
75% T2
75% G1
100% G1
100% G2
100% T3
100% T2
Dim 2 (20.05%)
Dim 1 (64.61%)

Figure 3. Principal Component Analysis of gels manufactured at 100 $g.kg^{-1}$with sodium caseinate (NaCas), hydrolysates of Trypsin (T1, T2 and T3), hydrolysates with Glu-C (G1, G2, G3) at different ratios hydrolysate:CMS (4:0, 3:1, 2:2, 1:3, 0:4). (A) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 73.55% of the total variability and dim 2 = 14.05% of the total variability). (B) Gels are represented according to their texture parameters and are colored according to the type of hydrolysate (red: NaCas, green: Trypsin, blue: Glu-C, black: CMS). (C) Gels are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate). (D) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 64.61% of the total variability and dim 2 = 20.05% of the total variability) without the presence of NaCas. (E) Gels, except gels made with NaCas, are represented according to their texture parameters and are colored according to the type of hydrolysate (green: Trypsin, blue: Glu-C, grey: CMS). (F) Gels, except gels made with NaCas, are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate, grey: 100% CMS).

Figure 4A and 4D represent a correlation circle of the variables and Figures 4B, 4C, 4E and 4F represent the scores of the individuals for the gels prepared at 200 $g.kg^{-1}$ under various hydrolysate:CMS ratios. Principal component 1 explained most of the variation (73.55% for the Figures A to C and 74.98% for the Figures D to F) and was related to the gumminess, hardness and stickiness of the gels (Figure 4A). The different gels were also separated according to the principal component 2, which represents 15.46% of the total variation for the Figures A to C and 18.46% of the total variation for the Figures D to F and was related to the cohesiveness. As for gels at 100 $g.kg^{-1}$, the gels at 200 $g.kg^{-1}$ were better separated according to their hardness, stickiness and gumminess than according to their elasticity (Figures 4B and 4C). The gels made with 100% of hydrolysates, G1, G2, T1, T2 and T3 were the firmest (Figure 4E). The gel 100% G3 had, as at 100 $g.kg^{-1}$, the same properties than the gel made with CMS. The addition of hydrolysates at a ratio more than 25% to the CMS increased the hardness and the stickiness of the gels (Figure 4F). At 200 $g.kg^{-1}$, the gels were very different in terms of texture. Consequently, the firmer gels were well separated rendering the other gels formed difficult to distinguish one from each other (Figures 4E and 4F).

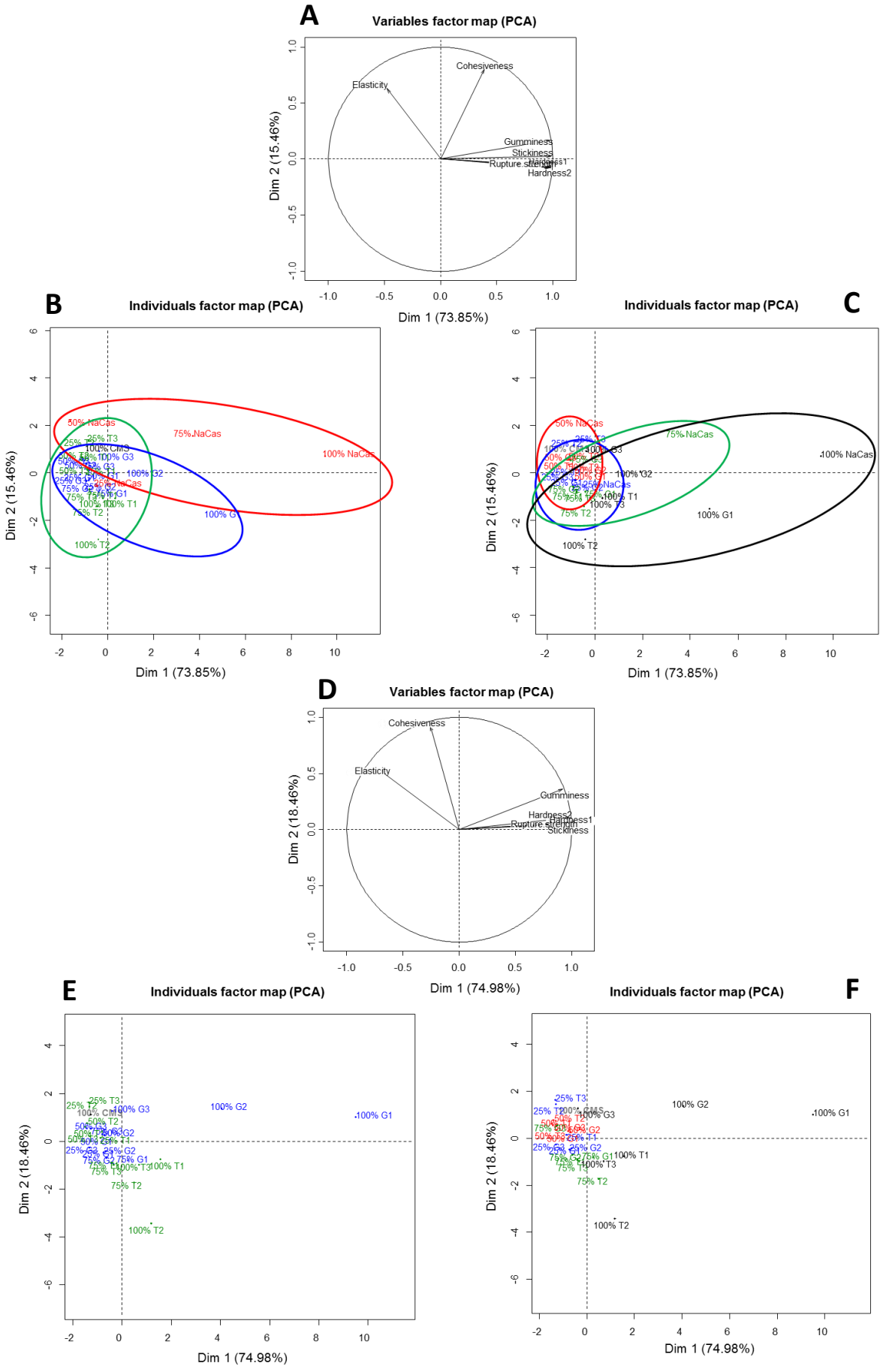

A
Variables factor map (PCA)
Cohesiveness
Elasticity
Gumminess
Stickiness
Hardness2
Dim 2 (15.46%)
Dim 1 (73.85%)
B
Individuals factor map (PCA)
50% NaCas
75% NaCas
100% NaCas
100% G2
100% G1
100% T2
Dim 2 (15.46%)
Dim 1 (73.85%)
C
Individuals factor map (PCA)
50% NaCas
75% NaCas
100% NaCas
100% G2
100% G1
100% T2
Dim 2 (15.46%)
Dim 1 (73.85%)
D
Variables factor map (PCA)
Cohesiveness
Elasticity
Gumminess
Hardness2
Hardness1
Stickiness
Dim 2 (18.46%)
Dim 1 (74.98%)
E
Individuals factor map (PCA)
100% G2
100% G1
100% T2
Dim 2 (18.46%)
Dim 1 (74.98%)
F
Individuals factor map (PCA)
100% G2
100% G1
100% T2
Dim 2 (18.46%)
Dim 1 (74.98%)

Figure 4. Texture profile analysis of gels manufactured at 200 $g.kg^{-1}$ with sodium caseinate (NaCas), hydrolysates of Trypsin (T1, T2 and T3), hydrolysates with Glu-C (G1, G2, G3) at different ratios hydrolysate:CMS (4:0, 3:1, 2:2, 1:3, 0:4). (A) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 73.85% of the total variability and dim 2 = 15.46% of the total variability). (B) Gels are represented according to their texture parameters and are colored according to the type of hydrolysate (red: NaCas, green: Trypsin, blue: Glu-C, black: CMS). (C) Gels are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate). (D) The texture properties are represented on the Principal Component Analysis variables factor map (dim 1 = 74.98% of the total variability and dim 2 = 18.46% of the total variability) without the presence of NaCas. (E) Gels, except gels made with NaCas, are represented according to their texture parameters and are colored according to the type of hydrolysate (green: Trypsin, blue: Glu-C, grey: CMS). (F) Gels, except gels made with NaCas, are represented according to their quantity in hydrolysates (black: 100% hydrolysate, green: 75% hydrolysate, red: 50% hydrolysate, blue: 25% hydrolysate, grey: 100% CMS).

The hydrolysate impacted the gel texture according to the concentration of the system. T1, T2, T3 and G3 had a specific texture at 50 $g.kg^{-1}$ that changed drastically at 100 $g.kg^{-1}$ to stay the same at 200 $g.kg^{-1}$. On the contrary, G1 provides the same texture regardless of the concentration whereas the texture of G2 hydrolysates changed drastically from 50 to 100 and from 100 to 200 $g.kg^{-1}$ from a hard to a cohesive and sticky one.

Specific peptides are involved in the gel structure

The gels at 200 $g.kg^{-1}$ were ultracentrifuged in order to extract peptides which were not involved in the gel structure. The casein micelles and the peptides involved in the gel backbone were trapped in the pellet whereas the peptides not strongly linked to the gel structure were found in the supernatant.

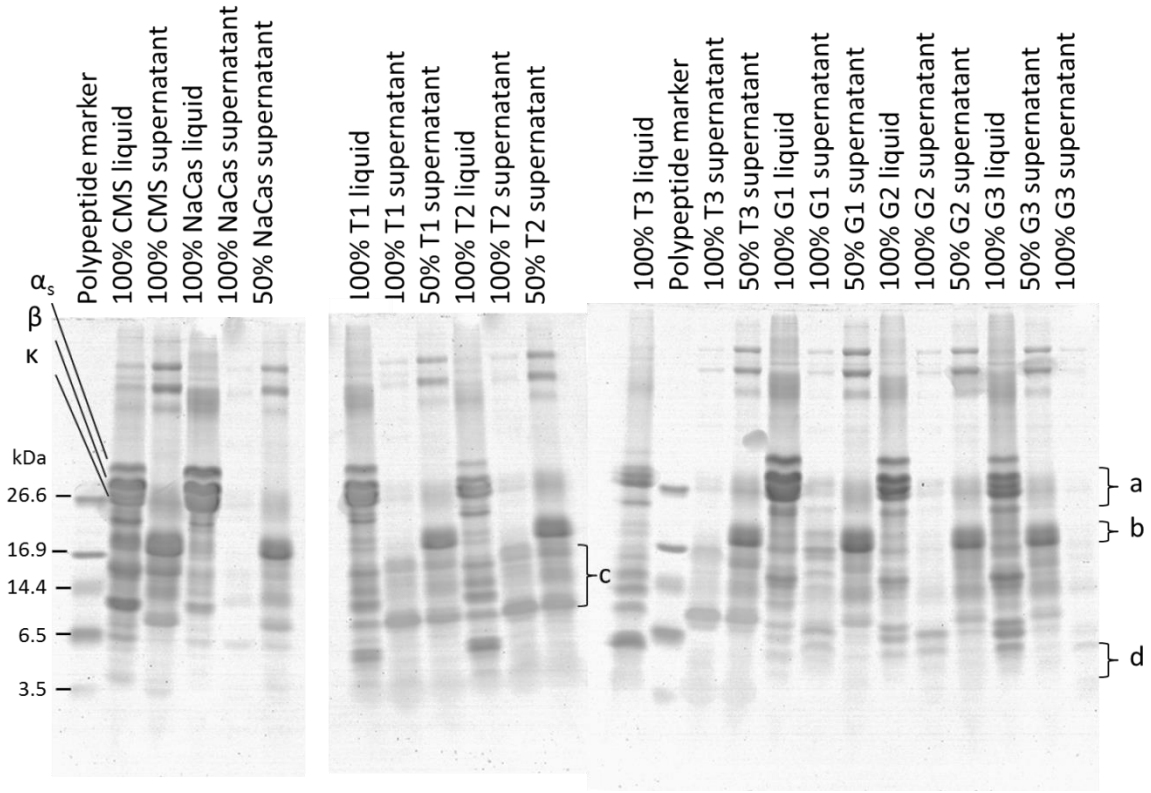


Figure 5. Tris-tricine gel electrophoresis of the hydrolysates before mixing with CMS (100% liquid) and gel supernatants. Gels were made with 100% CMS, 100% NaCas, 100% hydrolysate, 50% NaCas or 50% hydrolysate.

The Figure 5 shows that most of the total caseins participated to the structure of the gels and were present only as traces in the electropherogram of supernatants. Large peptides (MW between 26.6 and 16.9 kDa) or small peptides (MW <6.5 kDa) produced by the hydrolysis with Trypsin whereas only large peptides (MW >6.5 kDa) produced by the hydrolysis with Glu-C were not involved in the gel structure. This was shown by bands having apparent molecular size around 16.9 kDa found in each supernatant containing CMS and referred as band (b). Peptides with molecular weight between 6.5 and 16.9 kDa (c) were found in the supernatants of gels made with 100% hydrolysates of Trypsin. With Glu-C, peptides with a molecular weight around 3.5 kDa were found in the region (d) in the supernatant of the gels, excepted for 100% G1 for which caseins, and peptides with molecular weight from 16.9 to 3.5 kDa were found.

Discussion

Gelation of casein micelles under acid pH conditions has been thoroughly studied and mechanisms that drive the casein network formation mostly elucidated *(11)*. Thus, acid gelation of casein micelles occurred near the pI of the proteins favoring their aggregation *(12)* with little change in their spherical shape and size likely due to the presence of the κ-casein at the particle-particle interface

that keep part of repulsion between the casein micelles *(11)*. This was already observed by electronic microscopy *(13)* and was confirmed in this study by cryo-microscopy of the casein micelles suspensions (Table II). The incorporation of caseinate and hydrolysates in the casein micelle suspensions actually did not change the overall shape of the casein micelles but provide denser and smaller particles. Acidification decreased part of the negative and positive charges of the peptides and this concerned notably those present in T hydrolysates whereas the number of charges at pH 4.6 was shown to increase in the G hydrolysate reinforcing the hydrophilic of G3 hydrolysates (Table I). Such a variety in pI values of the peptides *(6)*, did not inhibit the gel formation at pH 4.6 as shown in Figure 1. And the denser particles observed are in line with the possibility of caseins and peptides to penetrate within the casein micelles and to help reduce electrostatic imbalance that can be provoked, by the complete solubilization of the calcium phosphate nanoclusters on reducing the pH of milk to 4.6 *(3, 14)*. Actually, peptides are polyelectrolytes that can serve as counter ions replacing the calcium and phosphate released during acidification, enhancing protein-protein and protein-peptide repulsion within the gel network, causing a weakening of the gel until charge neutralization finally occurs and the gel again strengthens *(14)*. But, peptides are also able to interact through hydrophobic interactions with the caseins inside the casein micelles and even to link the micelles together as in the case of G2 hydrolysates (Table II). In this case, the hydrophobic regions can expel water and render the casein micelles denser to electrons. Such equilibrium between charge repulsion and hydrophobic attractive force led to various casein micelle network configurations from casein micelles linked together to destruction of the casein micelle structure depending on the type of hydrolysates used. From the best of our knowledge, this was never studied in the literature before. This increase in interactions between peptides and caseins were also reflected in the increase in cohesiveness observed that is usually related to an increase in internal bonding inside structure *(15)* as confirmed by the few amount of peptides released in supernatant after ultracentrifugation (Figure 5).

However, such a discrepancy between hydrolysates at the microstructural scale was not observed in the texture profile analysis. Although adding caseinate and hydrolysate drastically change the casein micelle texture regardless of the concentration used from 50 to 200 $g.kg^{-1}$, the predominant factor impacting the gel texture was the quantity of native caseins present in the caseinate and in the hydrolysates that contained both peptides and residual caseins. Actually, the higher the amount of the native caseins in the CMS was, the firmer the gels were. At 50 $g.kg^{-1}$, the presence of hydrolysates with a high quantity of native caseins such as T1 or G1 increased the hardness of the CMS. Such a fact was already observed by Lamsal et al. *(16)* on soy proteins. When the peptides became as the main components of the hydrolysates (T3) the gels were found more elastic and more cohesive. It was shown in the literature, on the example of whey proteins, that the hydrolysis provides elastic

properties to the gel *(17)*. Nevertheless, the authors highlighted that the types and amounts of aggregating peptides produced are important and research is still needed in this area to better understand the gelation mechanism. Our results were also confirmed by Lamsal et al. *(16)*, who had shown that the hydrolysis involved a decrease in the gel hardness, when the degree of hydrolysis increased. In contrast to previous study *(6)*, there were less differences between the type of hydrolysates T and G in changing the texture properties at pH 4.6 than at pH 7, maybe due to the overall decrease in charge during acidification.

The concentration of the CMS had a very high impact on the gel texture with a behavior that is different at 50 $g.kg^{-1}$ compared to the other two higher concentrations. Actually, the concentration of casein micelles in presence or not of caseinate and hydrolysate under these conditions of acid pH looks like to the states of caseins that were observed during compression of casein micelles by osmotic stress technique *(18)*. The texture of the HS: CMS drastically changed when only 25% of hydrolysates were added at 50 $g.kg^{-1}$ that can be considered as a dilute system in which caseins do not interact each other. In contrast at 100 and 200 $g.kg^{-1}$ only percentage above 50% were able to change the texture from that observed on the CMS alone that can correspond to a regime in which casein micelles interact with their neighbors as well as the caseinate and the peptides present. The dispersions then behave as more coherent solids, that was reflected by an increase in hardness. In that sense, the system became more confined and some crowding effect can be put forward *(19)*.

In this study we showed that limited or controlled enzymatic hydrolysis of caseins could provide various textural functionalities in agreement with other types of hydrolysates *(20, 21)*. Peptides produced either by Trypsin and Glu-C can therefore be used as ingredients to form more or less cohesive gels that can be used in various food products as sauces, yogurts, soups or infant food formulations that require less viscous product mix and weaker gelling properties.

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Notes

The authors declare no competing financial interest.

Abbreviations used

CMS: Casein micelle suspension

NaCas: Sodium caseinate

T1: sodium caseinate hydrolyzed by Trypsin at time 1

T2: sodium caseinate hydrolyzed by Trypsin at time 2

T3: sodium caseinate hydrolyzed by Trypsin at time 3

G1: sodium caseinate hydrolyzed by Trypsin at time 1

G2: sodium caseinate hydrolyzed by Trypsin at time 2

G3: sodium caseinate hydrolyzed by Trypsin at time 3

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Chapter 4: Structural changes of caseins and peptides under a high concentration

From the previous parts of the thesis, we showed that concentration of casein systems had deep influence on the interactions between caseins and between casein and peptides. This can lead to confinement of the proteins and peptides, in such way that could lead to peculiar interactions under very high concentration conditions as those reached in some food products as cheeses. Actually, cheeses contain proteins, from 100 $g.kg^{-1}$ to 500 $g.kg^{-1}$, such as in Cottage Cheese, Mozzarella, Cheddar and Parmigiano Reggiano.

However to reach high concentrations, the solubilization of peptides and casein micelles is not possible by motor agitation, and other techniques have to be used as osmotic pressure technique to concentrate peptides and casein micelles at concentrations higher than 200 $g.kg^{-1}$, in order to mimic cheese.

To conduct this study, we chose only one hydrolysate (T3) to study the impact on visual aspects and on the structural changes of casein and peptides mix at different concentrations. T3 was chosen as it is the hydrolysate which contains mainly peptides and few native caseins in order to focus on peptide behavior.

This chapter is presented as a complementary study which permits to get at first sight information on the behavior or peptides and casein micelles at concentrations higher than 200 $g.kg^{-1}$.

The manipulations were realized by Mabelyn Tan during the training period of her Master 2 and in collaboration with Stéphane Pezennec.

Chapitre 4 : Changements structuraux des caséines et des peptides à hautes concentrations

Dans l'étude précédente nous avons montré que la concentration des systèmes caséiniques a une forte influence sur les interactions entre les caséines et les peptides. Cela peut conduire à un confinement des protéines et des peptides et à des interactions particulières à de très fortes concentrations telles que celles retrouvées dans quelques aliments comme les fromages. Généralement, les fromages contiennent des teneurs en protéines de l'ordre de 100 à 500 $g.kg^{-1}$ comme le fromage blanc, la Mozzarella, le Cheddar ou encore le Parmesan.

Cependant, pour atteindre de telles concentrations, la solubilisation des peptides et des micelles de caséines est impossible par simple agitation moteur. Par conséquent, d'autres techniques ont dû être utilisées telle que la technique de concentration par pression osmotique afin d'atteindre des concentrations supérieures à 200 $g.kg^{-1}$ dans le but de mimer celles d'un fromage.

Afin de mener cette étude, nous avons choisi d'utiliser seulement un des hydrolysats produits : T3. Nous l'avons choisi car c'est un hydrolysat contenant principalement des peptides et peu de caséines natives. Nous avons étudié son impact sur l'aspect visuel et sur les changements structuraux des caséines à différentes concentrations.

Ce chapitre est présenté comme une étude complémentaire qui permet d'obtenir une première étude du comportement des peptides et des caséines à des concentrations supérieures à 200 $g.kg^{-1}$.

Les expériences ont été réalisées par Mabelyn Tan lors de son stage de Master 2 en collaboration avec Stéphane Pezennec.

Introduction

The vast variety of textures in food comes from the complex organization of basic components such as carbohydrates, fats and proteins. Proteins play an important role in food structure, building networks that form microstructures, peculiar texture and give rise to important classes of foods such as fermented food, cheeses, yoghurts, sausages, ice cream (Renard et al., 2006).

Texture of food is a major parameter for consumers and can be changed during the process according to the physico-chemical or biochemical treatments. During fermentation, proteolysis is the main factor that influences the final texture of various food products such as cheese. These changes are mainly due to the breakdown of proteins and the peptides produced are expected to play an important role, since peptides have been shown to have functional properties such as solubility, water-binding, foaming and emulsifying ability (Banach et al., 2013).

It is important to understand which interactions are involved in the formation of caseins-peptides network and the signification of these interactions.

High concentrations are similar to conditions in real food systems. We created a simulation of cheese matrices by examining aqueous casein micelles suspension, peptide solution, as well as a 1:1 mixture of casein with peptides by mass, at protein concentrations of up to 500 $g.kg^{-1}$, which were obtained by reducing their water content using high osmotic pressure.

Protein and peptides exist in suspension as charged colloid particles. The interactions between proteins change according to their concentration. In dilute solutions, electrostatic repulsions and hydrophobic interactions are dominant whereas in concentrated solutions van der Waals and hydrogen bonds, repulsive effect and steric hindrance increase (Saluja and Kalonia, 2008). A protein solution is considered as concentrated when the volume fraction of the solute is at $\phi \geq 0.1$ (Rivas and Minton, 2004). In concentrated system, there are strong intermolecular interactions.

This study aimed to evaluate:

- The feasibility of concentrating peptides and casein micelles using this technique.
- Whether there is a concentration threshold that induces sol-gel transition of peptides and a mixture of peptides and caseins.
- The differences of texture and visual aspect between peptides alone, casein micelles (CM) alone and mix peptides-CM.
- How peptides influence the interactions of casein micelles in a concentrated matrix.

Materials and methods

Preparation of maleate-$CaCl_2$-thimerosal buffer

A maleate-$CaCl_2$-thimerosal buffer of pH 7.1 at a ionic force of 80mM was prepared using 25 mM Maleic acid (Sigma-Aldrich, lot no.107K5432), 2 mM Calcium Chloride.$2H_2O$ (AnalaR, lot no. 12J170011) and MilliQ water, with 0.02% (w/v) of Thimerosal as bacteriostatic agent (Sigma-Aldrich, Lot no. BCBH5232V). 2 M NaOH was used for pH adjustments. This buffer was used for all hydrolysate, mix and casein resuspension solutions.

Solubilisation of casein micelles

Native phosphocaseinate powder, Promilk 852B (IDI SAS, France) with a protein content of 94% (w/w) determined via spectrophotometry. Promilk 852B was dispersed in maleate buffer at 53.2% (w/w) or 10.6% (w/w) to make casein micelle stock solutions of 50 g kg^{-1} or 100 g kg^{-1}, respectively. The dispersion was then agitated with a magnetic stirrer for 3h at ambient temperature, before transfer into a 50°C water bath for another 3h of stirring to allow for complete rehydration.

Trypsin hydrolysis of sodium caseinate

Trypsin is a digestive enzyme with a narrow specificity. Regardless of size and sequence, trypsin hydrolyses proteins at the carboxylic end of lysine and arginine, both of which are basic amino acids. Each tryptic peptide, except the peptide with the carboxyl end of the substrate, should contain either an arginine or a lysine. Unless the carboxyl terminus of the substrate is an arginine or a lysine, the number of peptides produced should be one more than the total number of lysine or arginine. However, there are some factors that modulate the specificity of tryptic action, which leads to “miscleavages” and resulting in the formation of large peptides, also termed as “tryptic cores” (Konigsberg and Steinman, 1977). For instance, aliphatic residues are preferred in the first and second residue extending towards the carboxyl terminus, followed by aromatic and basic residues; proline and acidic side chains, on the other hand, are much less preferred (Tauzin et al., 2003).

Sodium Caseinate (Armor Protéines, France) was determined to contain 87% (w/w) protein, as measured by UV spectrophotometry (UVmc2, Monaco SAFAS, France) at λ = 280 nm using extinction coefficient of 0.81 ml mg-1 cm-1 (Oliva et al., 2001) for caseins as a reference and by Kjeldahl method. It was then dispersed at 10 $g.L^{-1}$ into MilliQ water using motor agitation.

Next, tosyl-phenylalanine-chloromethyl-ketone (TPCK) -treated trypsin (Thermo Scientific Pierce), which is immobilized on agarose beads, was used for the hydrolysis of caseinate to form peptides. The optimum working conditions are at pH 7.5-9.0 and 37° C. In the immobilized form, the enzyme is covalently cross-linked to 4% beaded agarose, which allows for the ease of separation from the

cleavage products, so that the enzyme can be efficiently removed by filtration and the casein digestion can be stopped without heating or adding trypsin inhibitor.

Before each hydrolysis, the activity of the enzyme was measured by spectrophotometry at 247 nm using 0.01 M 4-toluene-sulphonyl-arginine-methyl-ester, TAME (Sigma Aldrich, France), according to the method of Worthington Laboratories, using the UV-mc2 spectrophotometer (Monaco-SAFAS, Monaco, France). Tris-HCl buffer and TAME were mixed in cuvettes according to Table 2 and incubated in the spectrophotometer at 25°C for 3-4 min to reach temperature equilibrium and to establish the blank. Diluted enzyme (100 µg mL-1) was then added and the absorbance at λ = 247 nm (A247) was recorded for 3 min. A247 was determined from the initial linear portion of the curve and the trypsin activity was determined according to the equation below. One unit (1U) was defined as 1 µmol of TAME hydrolyzed per min per mg of protein, at 25°C and at pH 8.1. The trypsin activity in this study was 33.1 $U.mL^{-1}$.Units mL^{-1} enzyme gel. Next, 2.9 L of 10 $g.L^{-1}$ sodium caseinate solution was heated to 37°C before the enzyme was added at 1.157 $U.L^{-1}$, corresponding to the enzyme: substrate ratio of 336 U g-1. The pH of the hydrolysis was maintained at 7.1, using the pH Stat (Mettler Toledo, France), which monitors the rate of protein hydrolysis by automatically titrating the hydrogen ions produced, using NaOH at 1 M as titrant. After 5500 s, the reaction was stopped by filtering the mixture to remove the immobilized enzyme.

Lyophilisation

A thin layer of hydrolyzed peptide solution was poured into stainless steel trays and covered with tissue paper to prevent dust contamination. The solution was then freezed at -20°C and lyophilized (S.G.D. - SERAIL type CS 10-0.8, Argenteuil France). The layer of dried sample obtained was crushed in order to have a homogeneous powder, which was then vacuum sealed and stored in darkness, at ambient temperature, until further use.

Solubilization

The lyophilized peptide powder was then dispersed in maleate buffer at 5.75% (w/w) or 11.5% (w/w) to make peptide stock solutions of 50 $g.kg^{-1}$ or 100 $g.kg^{-1}$ respectively. The dispersion was then agitated with a magnetic stirrer overnight (16 h) at ambient temperature, before it is transferred to a 50°C water bath for another 3 h of mixing to allow for complete rehydration.

Characterization of the tryptic hydrolysate by tandem mass spectrometry

Mass spectrometry (MS) experiments were performed using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a

nanoelectrospray ion source. A preliminary sample concentration step was performed on a nanotrap PepMap 100 (C18, 3 µm, 75 µm Inner Diameter (ID) × 20 mm Length (L)) (Dionex, Amsterdam, Netherlands). Separation was performed on a reverse-phase column PepMap RSLC C18 3 µm, 100 Å (75 µm ID, 150 mm L) (Dionex, Amsterdam, Netherlands) at 35°C, using solvent A (2% v/v acetonitrile, 0.08% v/v formic acid and 0.01% v/v TFA in deionized water) and solvent B (95% v/v acetonitrile, 0.08% v/v formic acid and 0.01% v/v TFA in deionized water). 5 – 35% of solvent B in 67 min and 35 – 85% in 2 min was applied as separation gradient at a flow rate of 0.3 µL/min. 3 µl were injected onto the column corresponding approximately to 60 ng of peptides. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode and a voltage of 2 kV with the help of a Proxeon Nanospray Flex ion source (Thermo Scientific, San Jose, USA). Spectra were recorded in full MS mode and selected in a mass range 250-2000 m/z for MS spectra with a resolution of 70,000 at m/z 200. For each scan, the ten more intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z 200 and the parent ion was subsequently excluded of the analysis during 15 s. The instrument was externally calibrated according to the supplier's procedure.

To identify peptides, all data (MS and MS/MS) were submitted to X! Tandem using the X! Tandem pipeline developed by PAPPSO (Plateforme d'Analyse Protéomique de Paris Sud-Ouest (PAPPSO), INRA, Jouy-en-Josas, France, http://pappso.inra.fr).

The search was performed against a database composed of a homemade database named STLO containing milk and egg proteins and cRAP database allowing excluding list of proteins such a human keratin for example. Database search parameters were specified as follows: unknown enzyme cleavage was used with one missed cleavage and the peptide mass tolerance was set to 10 ppm for MS and 0.05 Da for MS/MS. As caseins are phosphorylated, phosphorylation (corresponding to mass adduct of 79.96633 Da) were chosen as variable modification as well as oxidation of methionine (corresponding to mass adduct of 15.99491 Da). Semi-tryptic peptides were allowed during the "refinement" process of X!tandem. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation. Only peptides containing more than 5 amino acid residues and less than 5500Da can be identified by this methodology.

From the sequence of the peptides were calculated from the protparam tools (Gasteiger et al., 2005) of the ExPASy server (http://www.expasy.org/) (Artimo et al., 2012) their theoretical isoelectric point, their number of positive and negative charges and their hydropathy index according to the method of Kyte and Doolittle (1982).

Preparation of 1:1 mixture

Prior to the equilibration process, equal masses of casein micelle suspension and the peptide solutions were weighed into a plastic tube, and this 1:1 mixture was prepared by gently rotating the tube to mix well, without introducing too much air bubbles.

Osmotic stress technique

From previous trials, it has been determined that peptide samples and casein samples of concentrations over 200 $g.kg^{-1}$ will not be achievable from the dispersion of powder into a solvent. The strategy to obtain highly concentrated samples was therefore through the use of the osmotic stress technique, as described by Bouchoux et al. (2009), where water is forced out of the sample, through a semi-permeable membrane into a solution of higher osmotic pressure, π (Figure 1).

The stock solution of 50 $g.kg^{-1}$ peptide or mixture or casein suspension was placed in a dialysis bag, which was then submerged in a reservoir containing solutions of polyethylene glycol (PEG) that fixed the osmotic pressure of the solution at concentrations that correspond to the required osmotic pressure as described in Table 1. Over time, the chemical potential of water across the membrane reaches equilibrium and thus the osmotic pressure of the sample equals the osmotic pressure of the polymer solution.

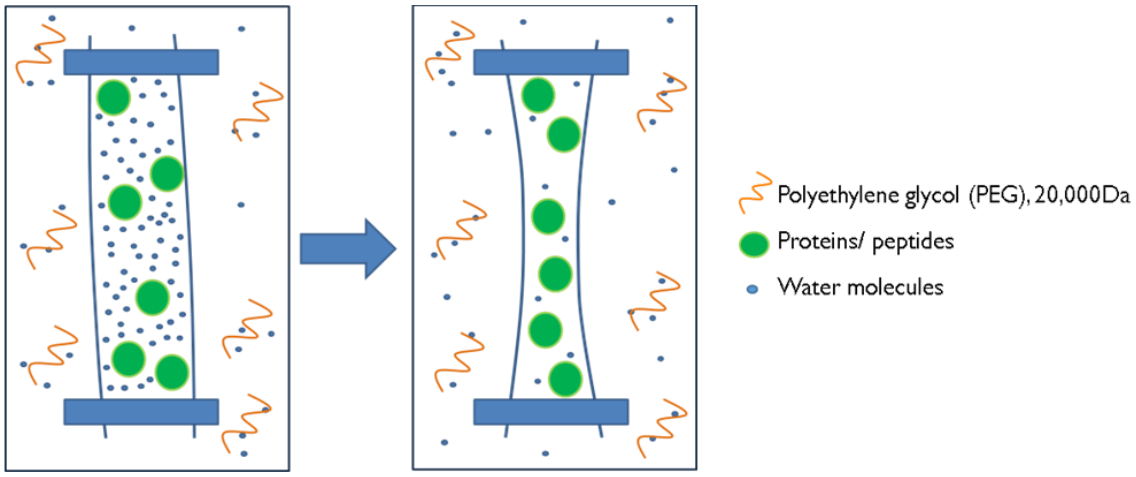


Figure 1. Water molecules from a protein-peptide solution is drawn out of the semi-permeable membrane, into a PEG solution of higher pressure.

Table 1. Stipulated osmotic pressure required to attain expected casein micelle concentrations. Values for peptides and 1:1 mixture were to be determined through experimental results.

Osmotic pressure (Pa)	Osmotic pressure (bar)	PEG concentration % w/w	Expected casein micelles concentration*
1 000 000	10	24.190	~500g kg^{-1}
500 000	5	17.846	~450 kg^{-1}
150 000	1.5	10.350	~400g kg^{-1}
30 000	0.3	4.780	~300g kg^{-1}
8 000	0.08	2.350	~200g kg^{-1}
3 000	0.03	1.288	~100g kg^{-1}

*From Bouchoux et al. (2009)

Figure 2. Set-up dialysis, with duplicate tubes filled with casein-peptide suspensions and placed is a sealed, transparent plastic box with PEG solution of required osmotic pressure.

Table 2. Dialysis protocol.

Batch	Pressure (bar)	Stock Solution (g kg^{-1})	Week 0: Day 0 – 7	Week 1: Day 8 – 14	Week 2: Day 15 – 21	Week 3: Day 21 – 23
A	10	100	Fill tubes with stock solution. To achieve equilibrium against PEG solution: Refill if tubes loses water and becomes flaccid. Remove sample if tubes gain water and becomes turgid.		Day 16: Change of PEG solution for equilibration	Day 23: Remove tubes from PEG bath for analysis.
	1.5	100				
B	0.08	50				
	0.03	50				
C	*1.5**	100				
	0.3	50				
D	5	100				

*Repeated trial for caseins only (duplicate)

Preparation of PEG solutions

Polyethylene glycol of 20 000 Da (Fluka, Switzerland, lot no.BCBJ0611V) was used to prepare solutions. The required concentration (% w/v) for a given π (Pa) was calculated according to the equation below, proposed by (Cohen et al., 2009):

$$\Pi N^{9/5} = \frac{RT}{M_{\mathrm{m}}\bar{V}}\left[\left(\frac{C}{C_N^*}\right) + \alpha\left(\frac{C}{C_N^*}\right)^{9/4}\right]$$

where

- N = number of monomers = 17100/44 (Li, 2014)
- R = ideal gas constant = 8.314 $Pa.m^3.K^{-1}.mol^{-1}$
- T = temperature = (20+273) K
- M_m = molecular mass of monomer = 44 X 10^{-3} $kg.mol^{-1}$
- $\bar{V}$ = partial specific volume of PEG = 0.825 $ml.g^{-1}$
- α = constant = 0.49
- C_N^* = $N^{-(4/5)}$ / $\bar{V}$ = 10,278 $Kg.m^{-3}$

Solutions of PEG at osmotic pressures as described in Table 3 were prepared by dispersing the PEG in 25mM maleate – 2mM $CaCl_2$ – 0.02% thimerosal buffer, pH 7.1, followed by 1 h of stirring at 37°C in a laboratory shaker (THO 500, Gerhardt-Germany).

Dialysis protocol

Cellulose ester dialysis membrane with the smallest molecular weight cut-off (MWCO) available, 100 – 500 Da, were used (Spectrum Laboratories, USA) to concentrate peptide, mixture or casein suspensions. This MWCO was chosen to limit the movement of PEG (20 kDa), caseins (>19 kDa, (Swaisgood, 2003) and especially the small peptides, to a maximum degree, while allowing exchange of water, ions and lactose. The dialysis membrane (tube of 6.4 mm diameter) were first cut into lengths of 12-16 cm and washed in deionized water to remove traces of the preservative sodium azide, before they were filled with stock solution of peptides, casein micelles or the 1:1 mixture of both. Universal closures (nylon) were used to seal the tubes, which were clips that can be reopened, allowing for ease of refilling of the tubes (Figure 2), which was conducted according to a protocol described in Table 2. Subsequently, the PEG solution was filtered through polyethersulfone membrane with 5000 MWCO (Vivaspin 20, Sartorius, Germany) using centrifugation at 8000 *g* to remove as much PEG as possible, then the filtrate was concentrated under vacuum for analysis via reversed-phase chromatography and SDS PAGE in order to examine whether small peptides were

able to pass through the membrane. However, due to interference from PEG, the results obtained were inconclusive.

Despite the relative straightforward concept of the osmotic stress technique, much care was undertaken to avoid leakage and mixing of the samples with the PEG polymer. The dialysis membrane was unable to withstand repeated stress due to its brittle nature, hence the refilling of the bags with sample solution had to be kept to a minimum. This was achieved by using sample stock solution of higher concentration, 100 g Kg^{-1}, when higher total solids was expected in the sample at higher osmotic pressures of 150 000 Pa and 1 000 000 Pa.

Total solids/ Dry matter

The resulting total solids content after equilibration at each osmotic pressure was determined by drying approximately 2 g of sample, which have been well mixed with sand, in a laboratory oven at 102°C for 15 h. The results were reported as an average of the totals solids measurement of duplicate samples.

Volume fraction calculation

The volume fraction of the equilibrated samples was calculated using the total solid content.

Let V be the total volume of buffer, V_e be the volume of water and V_i, c_i and ρ_i respectively being the volume, the concentration and the density of each solute *i* in the buffer solvent (i.e. maleic acid, calcium chloride dihydrate and thimerosal).

$$V = V_e + \sum_i V_i$$

$$V_e = V - \sum_i V_i = V - \sum_i \frac{c_i V}{\rho_i} = V\left(1 - \sum_i \frac{c_i}{\rho_i}\right)$$

The volume fraction of water (ϕ_e) in the solvent is therefore:

$$\phi_e = \frac{V_e}{V} = \frac{V\left(1 - \sum_i \frac{c_i}{\rho_i}\right)}{V} = 1 - \sum_i \frac{c_i}{\rho_i}$$

The mass of each solute m_i in V volume of solvent is therefore:

$$m_i = c_i V = \frac{c_i m_e}{\phi_e \rho_e}$$

- Where m_e is the mass of water that has evaporated from the sample.

The mass of protein m_p is therefore:

$$m_p = m_s - \frac{m_e}{\phi_e \rho_e} \sum_i c_i$$

- Where $m_s$is the mass of solids remaining in the sample.

With $\overline{v}_p$ being the partial specific volume of the protein, its mass concentration c_p is therefore:

$$c_p = \frac{m_p}{V + V_p} = \frac{m_p}{\frac{m_e}{\phi_e \rho_e} + m_p \overline{v}_p}$$

- Where $\overline{v}_p$ of 0.733 cm^3 g^{-1} was used for both caseins and peptides (Morris et al., 2000), in the absence of specific literature data for peptides.

The volume fraction of the protein in the sample is therefore:

$$\phi_p = c_p \bar{v}_p^*$$

- Where the $\bar{v}_p^*$ of 1.377 cm^3 g^{-1} was used for casein micelles and the $\bar{v}_p^*$ of proteins 0.733 cm^3 g^{-1} was used for peptides (Bouchoux et al., 2009).

Protein content by micro-Kjeldhal

Total nitrogen analysis was performed by a micro-Kjeldahl method, using a factor of 6.38 to convert nitrogen concentration to protein concentration in casein equivalents. Mass quantities of 50 – 200 mg were used for the analysis. Sample that were highly concentrated (100 $g.kg^{-1}$ and above) were diluted 10 times in maleate-$CaCl_2$-thiomersal buffer, in order to keep within the limits of the analysis method (100-300 µg nitrogen per titration). Results for the equilibrated samples were reported as the average readings of two duplicate samples, each with duplicate measurements. Results for the stock solutions were reported as average of duplicate measurements of a single sample.

Protein profile by electrophoresis

SDS-PAGE

The hydrolytic effects of the 23 day long dialysis process was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), where the proteins and peptides were incubated in presence of SDS in order to render them all negatively charged that renders the migration independent on their intrinsic electric charge and separation based on the molecular weight of the caseins/peptides.

SDS is an anionic detergent that denatures proteins and binds at a ratio of about 1.4 g SDS per gram of protein, hence it gives an overall negative charge to the polypeptide in proportion to its length. SDS also disrupts hydrogen bonds and hydrophobic interactions, causing the protein molecule to unfold and lose its tertiary and secondary structures. The reducing agent, β-mercaptoethanol was also added to cleave any disulphide bonds that may be present between cysteine residues. Therefore, the denatured and reduced polypeptides are in similar rod-like form with uniform negative charge density. A pore-gradient gel, which has increasingly smaller pores towards the

bottom of the gel, was used for greater resolution of the wide size range of proteins and peptides on a single gel (Amersham Biosciences Inc., 1999).

To prepare a 12 – 18% gradient gels, 11 mL of light- and 11mL of heavy-running gradient gel solutions were prepared according to the Table 5, with 60 µL of ammonium persulphate (10% w/v) mixed into each, and they were then poured into separate compartments of a gradient maker, placed on a magnetic stirrer to ensure adequate agitation of the solutions. Next, 18 µL of the catalyst, Temed (N,N,N',N'-tetramethylethylenediamine), was added into each of the gel solutions to initiate the polymerization of acrylamide with bis (N,N'-methylenebisacrylamide). With a peristaltic pump, the heavy gel solution was allowed to fill the bottom 0.5 cm of the mould at 5 ml min^{-1}, before the valve between the heavy and light gels was open, so that the density gradient would form. A layer of isopropanol (0.5 cm) was added to cover the gel, while polymerization proceeds for the next 30 min.

Once polymerized, the isopropanol was poured out and the top of the gel was rinsed with deionized water, before adding 6 ml of the 4% stacking gel (with 100 µL of APS and 20 µL of Temed added to 15 ml of 4% stacking gel solution as described in Table 5) was layered on top and the wells were formed by inserting a plastic mould at an angle to avoid trapping air bubbles. The completed gels were then loaded into a Protean II xi Cell (BIO RAD, XB0031).

Samples were dissolved in maleate buffer (as described above) to obtain dilute solutions of 5 g L^{-1}. The solutions were then further diluted to 2.5 mg protein ml^{-1} in a sample denaturation buffer (980 µL Tricine sample buffer, BioRad, with 20 µL of β-mercaptoethanol, Sigma). The denaturation and unfolding of the proteins was accelerated by heating the diluted samples at 100°C for 5 min. Twenty µL of denatured samples were loaded into 4% stacking gels and then separated on 12-18% running gels with electrophoresis at 60 V. Low molecular weight (LMW) protein standards (GE Healthcare) ranging from 97 to 14.4 kDa were prepared according to the manufacturer's conditions and polypeptide SDS-PAGE standards (BioRad, 161-0326) ranging from 1 kDa to 27 kDa, were diluted 1:20 in Tris-Tricine sample buffer. Both were heated for 100°C for 5 min, before being loaded (10 µL each per gel). Finally, 2 L of Tris-HCl buffer (0.2 M, pH 8.9) was added to the bottom of the tank, and 250 mL of Tris/Tricine/SDS buffer (BIO RAD) was added to the top compartment.

The generator was turned off when the Coomassie Blue dye runs out the bottom of the gel. As peptides tend to diffuse out of the gels more easily than the proteins, the gels were rinsed with deionized water, then soaked in a fixative solution (10% v/v acetic acid, 40% v/v ethanol and 50% deionized water) for 30min. Next, the gels were stained with Coomassie Blue solution for < 2 h (Biosafe™, BioRad). Next, the gels were de-stained with successive washes of deionized water, every 15 min, to remove all unbound dye in the background until the stained protein bands can be clearly visible. Coomassie Blue binds to proteins in an approximately stoichiometric manner, quantitative comparisons of the protein contents in each band could thus be made, using densitometry.

The gels were kept at 4°C, in a 30% ethanol solution to prevent drying until image scanning was done.

Urea-PAGE

In order to determine the extent of casein degradation, urea-PAGE was performed with a Mini-protean III electrophoresis unit and a 1000/500 power supply (BIO RAD). An 18 % (w/v) continuous polyacrylamine gel (size; thickness) was prepared with a 0.33 M Tris-HCl buffer pH 8.9 containing 4.33 M urea. Samples were dissolved at about 2.5 $mg.mL^{-1}$ in a 0.133M Tris-HCl buffer pH 6.8 containing 9 M urea, 5% (w/v) β-mercaptoethanol and 10% (w/v) glycerol. Next, 7µl of each sample were loaded into the gel slots. A migration buffer containing 0.192 M glycine and 0.025 M Tris was used to fill the cell unit. The electrophoresis was terminated 30 min after the dye front was eluted. Proteins were fixed with 10% (v/v) glacial acetic acid and 40% (v/v) ethanol for 20 min and then stained with Biosafe™ Coomassie stain for 60 min. The gels were then washed with distilled water and stored in distilled water before being scanned.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a measurement of wavelength and intensity of the absorption of IR radiation by molecules due to vibrational resonances of their bonds (Kong and Yu, 2007). We used FTIR to elucidate the changes in protein and peptide structure upon concentration.

The resonance frequency of bond vibrations are affected by the atomic composition, the vibrational modes, and the low-energy interactions with neighbouring atom groups (Barth, 2007). In proteins, the periodic organization of the polypeptide backbone in various types of secondary structures, stabilized by specific hydrogen bonding patterns, gives rise to corresponding specific resonance frequencies of the backbone amide (CONH) groups. They result in the main mid-infrared absorptions of proteins (amide bands) (Kong and Yu, 2007). Infrared spectroscopy thus can be used to monitor conformational changes of proteins. The lateral chains of amino-acid residues also give rise to specific absorptions owing to their chemical structure.

Infrared light absorbance spectrum of each protein/peptide sample was measured in the attenuated total reflection (ATR) mode between 4000 cm-1 and 850 cm-1, with a resolution of 4 cm-1, using the Tensor 27 spectrophotometer (Bruker, France), equipped with a Germanium ATR crystal (mono-reflection) and a mercury-cadmium–telluride detector cooled with liquid nitrogen, and controlled by the OPUS software. Background spectra were recorded for the buffer used to prepare protein stock solutions. Each spectrum was reported from the average result of 128 scans.

ATR is a sample environment setup that allows absorption spectra to be recorded for small-volume liquid, solid or powder samples. As the incident ray of IR light passes through the boundary between

a high-refractive index medium and a low-refractive index medium, the incident ray may achieve total reflection (no transmission), depending on the wavelength and incidence angle. Due to continuity conditions of electromagnetic fields at boundaries, there is a small-depth penetration of the electromagnetic fields in the low-refractive index medium, called an evanescent wave, without energy transmission. A higher refractive index of the second medium at some wavelength close to the boundary may give rise to transmission through the boundary and to energy absorption. This phenomenon is called attenuated total reflection (ATR).The spectra were automatically corrected for water vapor and carbon dioxide absorptions using the OPUS software. To focus on conformational changes, the differences in overall infrared absorbance due to differences in concentration between samples needed to be compensated. In addition, a major drawback of using IR spectroscopy on aqueous solution is that the H – O – H bending vibration of liquid water absorbs strongly around 1643 cm-1, which interferes with the amide I band (Güler et al., 2011). Since the background spectra were recorded using buffer, differences in sample protein concentration resulted in differences in contribution of this liquid water absorption, which also needed to be compensated. We used Extended Multiplicative Scatter Correction (EMSC) for these automatic compensations (Martens and Stark, 1991). EMSC consists in calculating a mean spectrum and then, using linear regression, in expressing each sample spectrum as a linear combination of this mean spectrum and of the liquid water “interference” spectrum. The coefficients estimated by linear regression then allow the water contribution to be subtracted and the overall absorbances to be normalized.

The spectral variability among corrected spectra was then analyzed using principal component analysis (PCA). PCA allows us to summarize a data set with minimum “information” loss, by computing new variables consisting in linear combinations of the initial variables (absorbance at every wavenumbers). These new variables, called principal components, can be represented as spectral patterns. Each spectrum is characterized by its scores on principal components, which can be used to draw “maps” of similarity between spectra. By design, principal components maximize the variances of scores, in such a way that the first few principal components gather the most part of the overall “information” of the data set.

Spectral corrections and statistical computations were performed using the “R” free software (Team, 2005).

Results

Three types of samples: casein micelles, hydrolysate (T3) and the mixture of hydrolysate-casein micelles (T3-CM) suspensions, of an intended ratio of 1:1 (w/w), were subjected to various changes in osmotic pressure from 0.03 to 10 bars, corresponding to the higher pressure values that would result in concentration of casein micelles of over 200 g kg^{-1} as determined by Bouchoux et al. (2009). The aim of this study was to test whether peptides, when added to casein micelles, were able to modify the ability of casein micelle suspension to form gels or solid under conditions of increasing concentration, attained due to increasing osmotic pressures. The peptide and the casein micelle suspension alone were used as controls, subjected to the same osmotic pressures.

Peptide characterization

The peptides produced by trypsin hydrolysis were identified and quantified by tandem mass spectrometry in order to elucidate information on the peptide sequences and their physico-chemical characteristics: size, charge, isoelectric point and average hydropathy scores, which indicated whether a peptide is hydrophilic (negative values) or hydrophobic (positive values).

The sequences of peptides were detected and matched to the casein that they originated from. However, several atypical peptides were found with cleavage sites that did not fully correspond to the specific cleavage action by trypsin. Trypsin cleaves peptide bonds after a lysine or arginine residue. Instead of the trypsin used is TPCK treated, peptides corresponding to the action of chymotrypsin were therefore identified to be present in the enzyme preparation. Cleavages after tyrosine, tryptophan, phenylalanine or leucine were found.

The largest peptide identified was from α_{s1}-casein, residue number 152 – 199, with a molecular weight of 5444 Da. However, there were gaps in the polypeptide peptide chain that were not identified, which may be due to limitations of the tandem mass spectrometry in the identification of large peptide fragments with high extent of phosphorylation, as phosphorylated residues were more difficult to ionize and to identify thereafter. The smallest peptide detected comes from α_{s2} -casein, residue number 200 – 205, and has 745 Da. Similarly, up to now, the detection of peptides is limited to chains of more than 5 amino acid residues. There may be smaller peptides produced that were not detected by this analysis.

From the listed pI values (isoelectric points) and charges, the peptides from κ-casein and α_{s2}-casein have stronger basic character and they also tend to be more positively charged. Peptides from α_{s1} casein were more acidic and were mostly negatively charged. Peptides from β-casein were mostly weakly charged, except for a few highly negatively charged peptides such as the N terminal peptide β-casein (f 1 – 25), with slightly acidic pI values.

The GRAVY number of a protein/ peptides was obtained by taking the average hydropathy value of its constituent amino acid residue as defined by Kyte and Doolittle (1982). This index indicates the hydrophobicity or hydrophilicity on a hydropathy scale, ranging from -2 to +2 for most proteins, with the most positively rated proteins being more hydrophobic (Kyte and Doolittle, 1982), (Bio-Rad Laboratories). β-casein yielded more hydrophobic peptides, which agrees with the observation that it yielded mostly uncharged peptides, while the peptide produced from α_{s1}-, α_{s2}- and κ-caseins were mostly hydrophilic and charged.

Texture and appearance of equilibrated samples

The equilibration process of T3, mixture and casein micelle suspensions using the osmotic pressure technique (Bouchoux et al., 2009) was implemented over a period of three weeks (23 days) for all samples. This time was essential to achieve equilibrium against the PEG solution that determines the final osmotic pressure of the suspension, either by refilling or removing sample depending on the osmotic pressure applied.

The appearance of the samples after 23 days of equilibration showed differences according to sample composition and pressure, as shown in Figure 3. From 0.03 to 1.5 bars, the samples were in liquid form, but with further increase in osmotic pressure, the samples solidified. Generally, the samples tend to be clearer at lower osmotic pressures as they were more dilute, hence turbidity (data not shown) increased as osmotic pressures increased. This trend applied fully to the peptides. However, the casein micelle suspensions tend to lose their turbidity from 5 bars onwards and become clearer as osmotic pressure increased. The mixture behaved in a similar manner as the caseins, although the mixture started with a clear appearance at 0.03 bar, while the casein suspension was cloudy.

As the samples were too small to realize rheological measurements, the texture of the samples was qualitatively appreciated (Figure 4). In terms of apparent texture, at 5 bars, the peptide samples had a texture similar to a water gel and the mixture samples were a soft gel. At 10 bars, the samples were much thicker in consistency than at 5 bars. While the peptide samples had a short, non-stringy texture and did not stick, the mixture samples were sticky, especially at 10 bars. Moreover, duplicate tubes of caseins that were equilibrated separately at 1.5 bars, resulted in different apparent textures, which corresponded to their measured protein content (data not shown). At a protein concentration of 173 $g.kg^{-1}$, the casein sample was a thick, viscous liquid which could be transferred using a pipette, while at a higher concentration of 187 $g.kg^{-1}$, the sample was a soft gel that looked similar to the peptide sample at 10 bars, with a protein content of 298 $g.kg^{-1}$. The peptide sample at 5 bars had the same protein concentration of 187g kg-1, yet the texture was thinner than the casein sample at 1.5 bars. Next at 5 bars, the casein samples formed a solid with a defined form that was much firmer

than the other samples. Fragments of the broken solid did not stick to one another. At 10 bars the solid was firmer, more brittle and was surprisingly clear, in comparison to the original stock suspension of casein micelles, which was milky white. Furthermore, there were color changes in all samples as the osmotic pressure increased from 5 to 10 bars, as they became increasingly yellow instead of being clear or white (Figure 3).

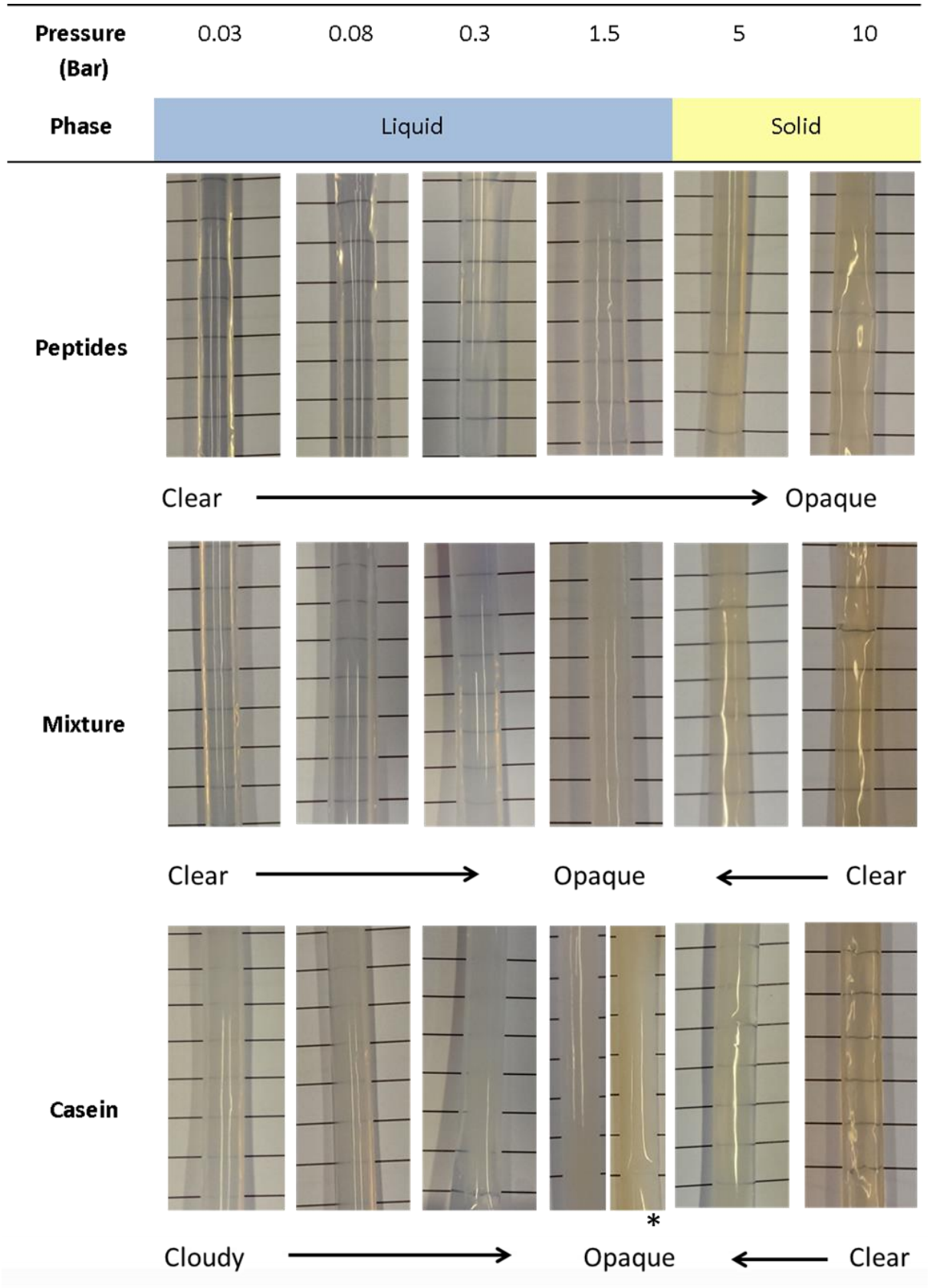


Figure 3. Visual observation of the peptide, mixture and casein suspensions at various pressure values inside the membrane 100-500 Da. * Sample form duplicate trial for casein at 1.5 bar.

Protein concentrations adjustments

As the dialysis membrane was unable to withstand repeated stress due to its brittle nature, the refilling of the bags with sample solution had to be kept to a minimum. This was achieved by using sample stock solution of higher concentration, 100 g kg-1, when higher total solids was expected in the sample at higher osmotic pressures of 1.5 bar and 10 bars, so as to avoid repeated handling of the fragile tubes.

The stock suspensions were prepared to the required concentrations (50 or 100 $g.kg^{-1}$) according to the protein contents of the peptides and the casein powders measured via spectrophotometry, as described in Section 3.2 (Materials and Methods). In order to establish the final concentration of the various suspensions under various osmotic pressures, the amount in nitrogen was determined by Kjeldahl analysis and reported as casein equivalent thereafter. This analysis was also applied to the stock suspensions and revealed that the protein content of the stock suspensions was less than expected. For 50 $g.kg^{-1}$ stock solutions of peptides, mixture and casein suspensions, the measured protein content were 47.46 ± 0.05 $g.kg^{-1}$, 43.86 ± 0.01 $g.kg^{-1}$ and 41.02 ± 0.05 $g.kg^{-1}$ respectively and for the 100 $g.kg^{-1}$ stock solutions of peptides, mixture and casein suspensions, the values were 92.44 ± 0.19 $g.kg^{-1}$, 87.76 ± 0.41 $g.kg^{-1}$ and 81.32 ± 0.36 $g.kg^{-1}$respectively. Therefore, instead of being a mixture of equal masses of peptides and caseins, the mixture a posteriori consisted of peptides and caseins in a “1 : 0.9” mass ratio.

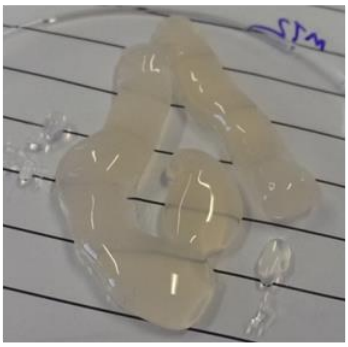
Pressure (Bar)	1.5	5	10
Peptides	NA	 Light water-gel.	 Soft gel.
Mixture	NA	 Soft gel.	 Sticky gel.
Casein	 Soft gel.*	 Solid. Broken fragments do not stick.	 Firm solid. Broken fragments do not stick.

Figure 4. Texture of solid samples at 10 bars and at 5 bars after demolding. * Sample from duplicate trial for casein at 1.5 bar with protein content of 187 $g.kg^{-1}$.

Equation of state and volume fraction

The equation of state (EoS) for casein micelles has already been described by Bouchoux et al. (2009), but the EoS of hydrolysate and of a mixture hydrolysate-caseins has yet been defined. Since by van t'Hoff's law described below, the osmotic pressure in a dilute system is proportional to the molar concentration of its solutes, we can hence put forward that the peptides, which are smaller

molecules than the casein micelles, would exert a higher osmotic pressure inside the membrane, due to a higher molar concentration at the experimental mass ratio of 1: 0.9.

$$\Pi_{vH} = RTc$$

Where R = universal gas constant, 8.314 $Pa.m3.K^{-1}.mol^{-1}$

T = temperature (K)

C = polymer molar concentration (M)

Indeed, the results obtained in Figure 5 confirmed this statement, since peptides equilibrated at a lower protein concentration than casein micelles when subjected to the same osmotic pressure. There was an approximately 9 – 14% difference between the concentration by volume calculated from the total solids data and the concentration by volume calculated from the Kjeldahl analysis that may be attributed to the presence of non-protein dry matter in the sodium caseinate powder and the casein micelles powder. Total solids data was used for the expression of the EoS for comparison with data from Bouchoux et al. (2009). While the van t'Hoff's model fits well with the experimental data for peptides, calculated with an estimated average molar mass of 2600 Da, the behaviour of caseins did not follow the model, calculated with the molar mass of 156 kDa (Bouchoux et al., 2009), nor did the behavior of the mixture, calculated as a weighted average using the mass ratio of peptides: casein micelles of 1:0.9.

Furthermore, it was found that at 0.03 bar to 1.5 bars, the osmotic pressure of the peptide stock solution was greater than that of the PEG solution, hence the sample was being diluted instead of being concentrated. From 0.03 bar to 0.3 bar, the osmotic pressures of the mixture and casein stock solutions were also greater than that of the PEG solution, hence they were also being diluted.

At low osmotic pressures, i.e. < 1.5 bar, the EoS of the mixture was closer to the behavior of the peptides than that of the casein micelles, which was due to the dominant osmotic pressure exerted by the small peptides. This general trend was in agreement with the relative position of the van t'Hoff equations, although the data points for the mixture and the caseins differed greatly from the model, especially at low osmotic pressures of 0.03 and 0.08 bars. As the osmotic pressure increased, the concentrations of the samples would not be expected to vary linearly according to van t'Hoff's law as their protein volume fraction surpassed 0.1 (Rivas and Minton, 2004), where the concentration of the mixture was observed to approach intermediate values between the concentrations of the peptides and the casein samples. In this region of higher protein volume fraction, the intermolecular interactions started to have a significant impact on the osmotic pressure

of the samples, and this phenomenon could be compared to the hard spheres model, which would be discussed in Section 5.

For the same osmotic pressure of 1.5 bars the final concentration greatly varied amongst the samples, from 91 $g.kg^{-1}$ for the peptides to 187 $g.kg^{-1}$ for the casein micelles. In order to reach the same peptide and casein concentration of ~300 g kg^{-1}, an osmotic pressure of 10 bars will be needed for peptides and only 5 bars for casein micelles.

By comparing the EoS with the texture shown in Section 4.2, we can also see that below a threshold of 100 $g.kg^{-1}$, the samples were liquid regardless of the composition of the suspensions (peptides, casein micelles or both), and the samples became a gel or even solid at higher pressures corresponding to concentration over 300 $g.kg^{-1}$.

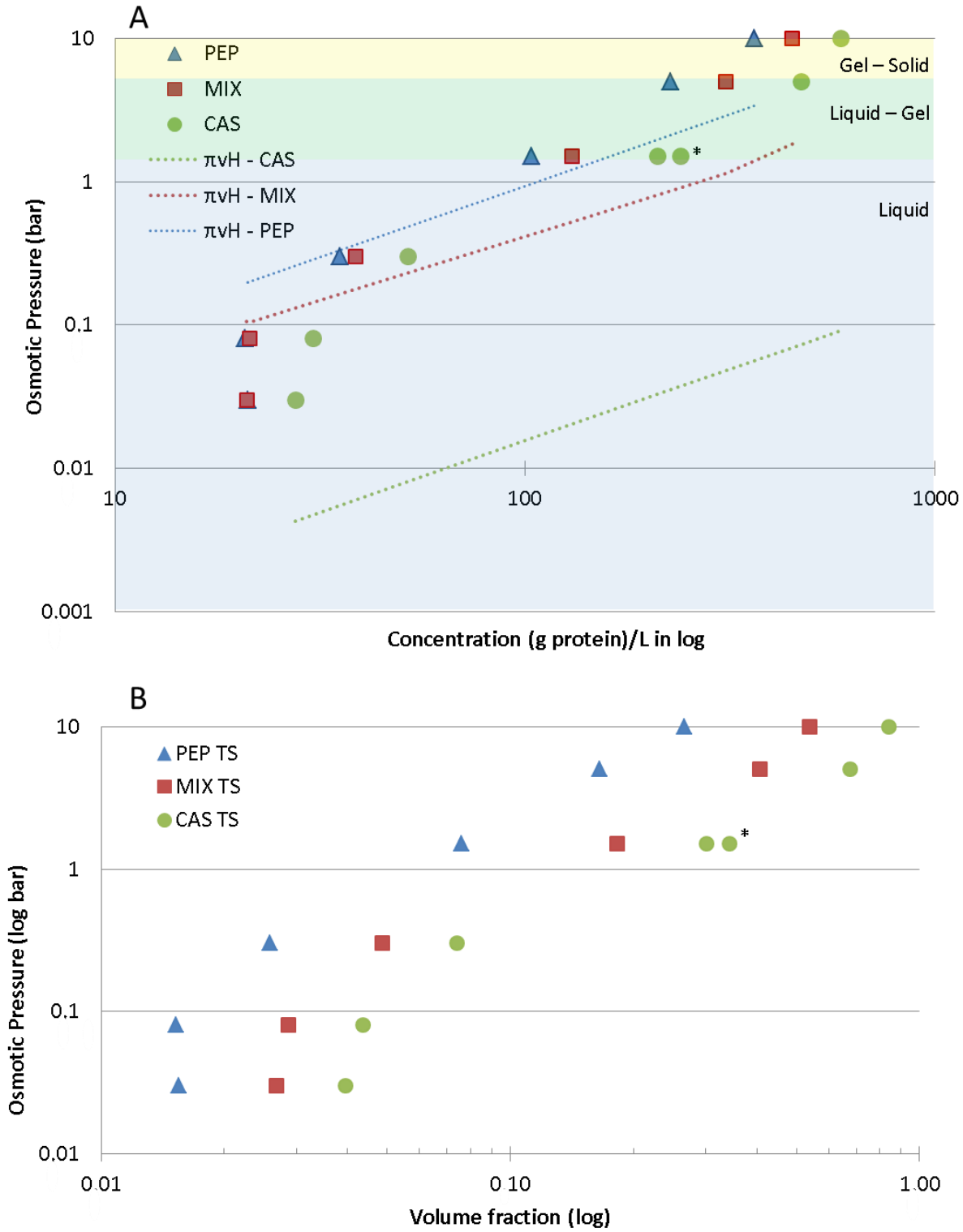


Figure 5. (A) Equation of state between the osmotic pressure and the protein content of the samples, calculated using he specific volume of 0.733 ml.g^{-1}. At 5 bars and at 10 bars, the samples were observed as solids, while at 1.5 bars and below, the samples were found to be in liquid form. (B) Osmotic pressure was also plotted against the volume fraction of the samples, calculated using specific volume of casein micelles at 1.433 ml.g^{-1}, and peptides at 0.733 ml.g^{-1}. The volume fraction of the mixture was taken as a weight average of the peptides and casein, based on the mass ratio of 1:0.9. * A duplicate equilibrated sample was made with caseins at 1.5 bars.

FTIR

The variation of osmotic pressure and hence the concentration of peptides, mixture and caseins reflects not only loss of water but can also be due to changes in their secondary structures, which is related to changes in the interactions between peptides, caseins or both during the equilibration process. FTIR was thus conducted to measure such changes.

The absorbance of the samples was measured on the FTIR spectrophotometer. In order to have a more precise analysis of the overall data, certain spectra were observed with atypical peaks and valleys (outside of the amide I and amide II band region) that were not usually related to protein structure, and were likely to be noise due to interference from contaminants or inadequate background subtractions were removed from the analysis, even though this implied the loss of spectral data on certain samples, i.e. the samples of casein and mixture equilibrated at 0.03 bars. The resulting spectra after removal of outliers were shown in Figure 6. In general, all samples gave two major peaks in the region of the amide I and amide II bands (1700 – 1600 cm^{-1}), which varied in magnitude along with the sample concentration, as this region corresponds to the wavelengths where amide linkages absorb IR radiation, due to the C = O stretch vibrations as well as in-plane bending of the N – H bonds and C – N stretch vibrations (Kong and Yu, 2007). Hence, the higher the concentration, the larger the amplitude of the peaks would be. For more in-depth interpretation of the spectra, corrections on the data were needed nullify differences in magnitude, and also to account for drifts in baseline and interference from water vapor.

Mathematical corrections using extended multiplicative scatter correction (EMSC) were performed, where intensities of absorbance were normalized by expressing each spectrum as a function of the mean so that differences in spectral patterns can be analyzed. The spectral patterns of the samples after EMSC were presented in Figure 7. These minute differences in the form of each spectrum were then analyzed via principal component analysis (PCA). Fluctuations in the baseline was improved (Figure 7) but erratic fluctuations due to interference from water were still visible (e.g. 1900 – 1700 cm^{-1}), and they were also taken into account in the PCA. Equilibrated samples at 5 bars were not analyzed on the spectrophotometer due to time limitations.

According to PCA (Figure 8), 85% of the total variance was represented by three main vectors PC1 to PC33, while PC4 only contributed to 8% of the variance. However, the variance represented by PC1 (38%) was noted to be essentially a correction artifact due to imperfect correction on water concentration in the samples, resulting in the displacement of the amide I band as observed from its loading plot (Figure 8A). As loading plots provide information about the contribution of each original variable to a principal component, the spectral regions corresponding to the protein/peptide structural differences should be readily identifiable according to the peaks and valleys in the loading

pattern (van der Ven et al., 2002). However, the loading pattern on PC1 showed limited correspondence with the major protein absorption bands (Amide I and II: 1700 cm^{-1} – 1600 cm^{-1}), except at 1634 cm^{-1}, assigned to presence of β-sheets (Barth, 2007; Kong and Yu, 2007). Since loading patterns on PC1 revealed relatively limited information on peptide/protein structure differences, the analysis was focused on variance shown on PC2 (28%) and PC3 (19%) only (Figure 8B).

As such, a 2 dimensional PCA map with PC2 and PC3 was plotted (Figure 7C), where the samples were observed to be segregated by composition (casein, mixture and peptides) and pressure (0.03 bars to 10 bars). The peptide samples tend to have higher scores on PC3, while casein micelle samples tend to have lower scores. Next, as pressure increases, the scores on PC2 decrease, therefore the most concentrated samples are on the left side of the map. Hence, we observed that there is a correlation between variances in spectral data, with sample composition as well as sample concentration.

To understand on which parameter, e.g. composition or pressure, the different axes PC2 and PC3 could be related, the mean coordinates on PC2 for each sample were plotted against osmotic pressure or stock suspension concentration as shown in Figure 9A. It was observed that PC2 was affected by osmotic pressure in a manner that was independent of sample composition, since the mean coordinates for all samples decreased in a similar manner as osmotic pressure increased.

Similarly, the mean coordinates on PC3 for the different samples were plotted against osmotic pressure and stock suspension concentration (Figure 9B). It was observed that the coordinates on PC3 varied according to sample composition, with peptides having high positive scores and caseins having negative scores. It was also observed for the stock solutions at 50 $g.kg^{-1}$, the mean coordinate score on PC3 increased after 3 weeks of equilibration.

From the loading plots of PC2 and PC3 in Figure 8B, it was noted that large magnitudes were observed in spectral regions described in Table 3, which indicates the type of conformational changes that occurred, due to the effects of PC2 and PC3.

The negative values of the loading on PC2 at 1626 cm-1, with highly negative PC2 coordinates for the samples at 10 bars indicated a higher proportion of intermolecular β-sheets than in peptide samples. Hence as osmotic pressure increases, the coordinates on PC2 decreases, which implied there was an increase of intermolecular β-sheets in the all samples as osmotic pressure increased. However, it was noted that the PC2 values for the peptides samples was unchanged between 0.03 bars to 0.3 bars.

The positive peak of the loadings on PC3 at 1585 and 1404 cm^{-1}, which corresponds to the absorbance frequency of aspartate residues and free carboxylates (COO-) respectively, implied that variance on PC3 was associated with exposed aspartate residues and the presence of free C terminal extremity of peptides; this correspond with the observation that the peptides had high positive

values on PC3 while caseins had negative values. The increase in the PC3 coordinate values of the stock suspensions at week 0 (S) to week 3 (E) as observed in Figure 9B, also implied that more free peptides were formed as the stock suspension aged over the 3 week incubation period, therefore indicating that variation on PC3 may be linked to casein hydrolysis. It was also observed that there was a decrease in the score of PC3 for caseins from 1.5 to 10 bars, which implied that there were less exposed aspartate residues and free carboxylates present, hence suggesting that caseins were less hydrolyzed at higher concentration.

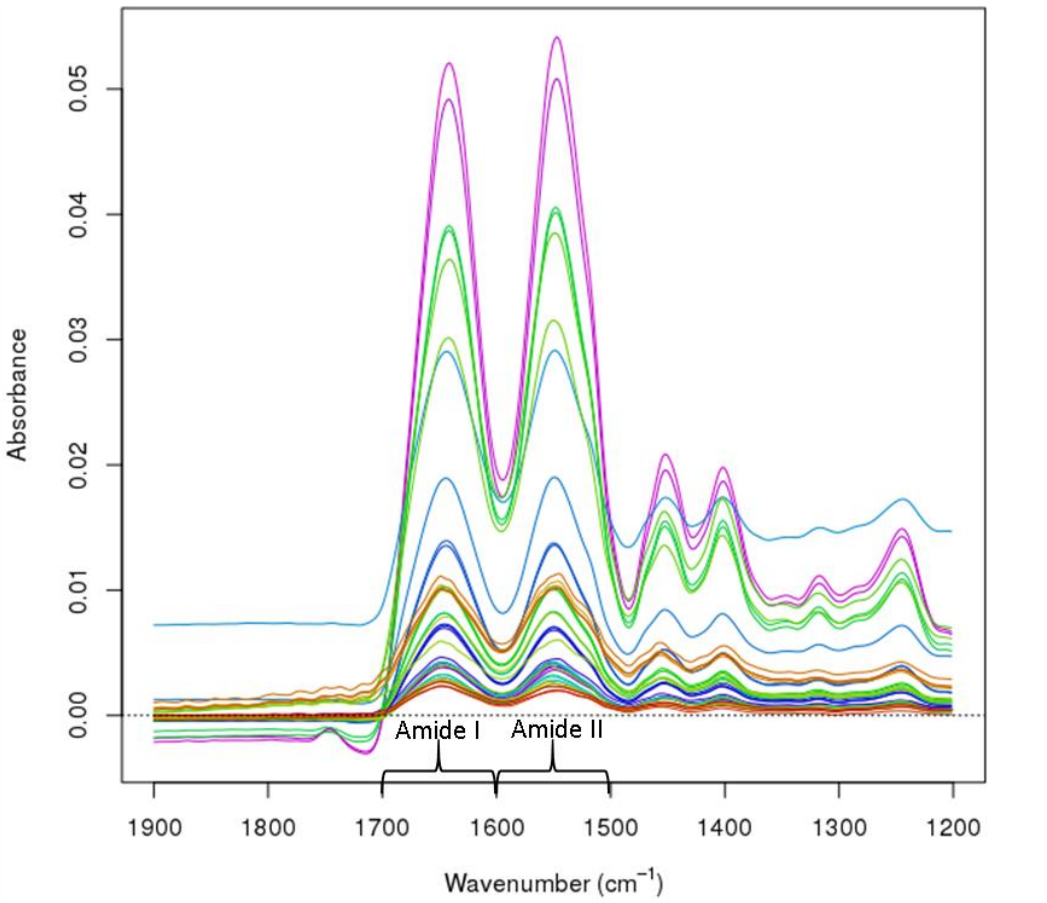


Figure 6. Raw spectra obtained through FTIP spectroscopy on casein, peptide and mixture stock solutions at 50 g.kg^{-1} and 100 g.kg^{-1}, as well as equilibrated samples at different concentrations.

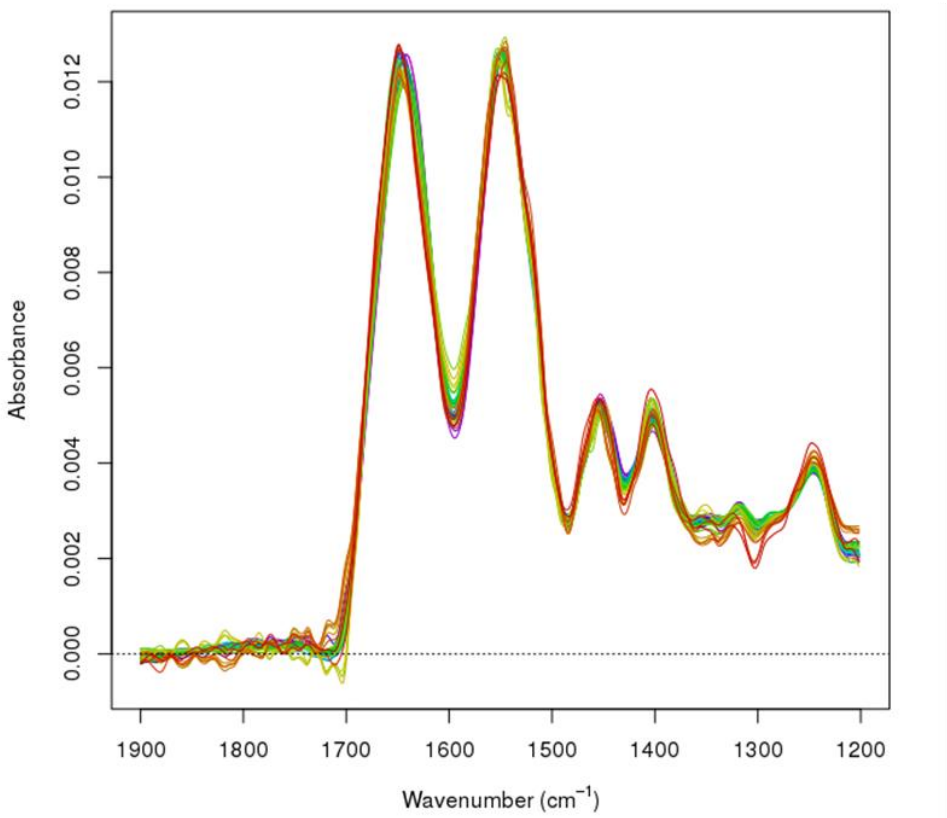


Figure 7. FTIR spectra after normalization *via* EMSC.

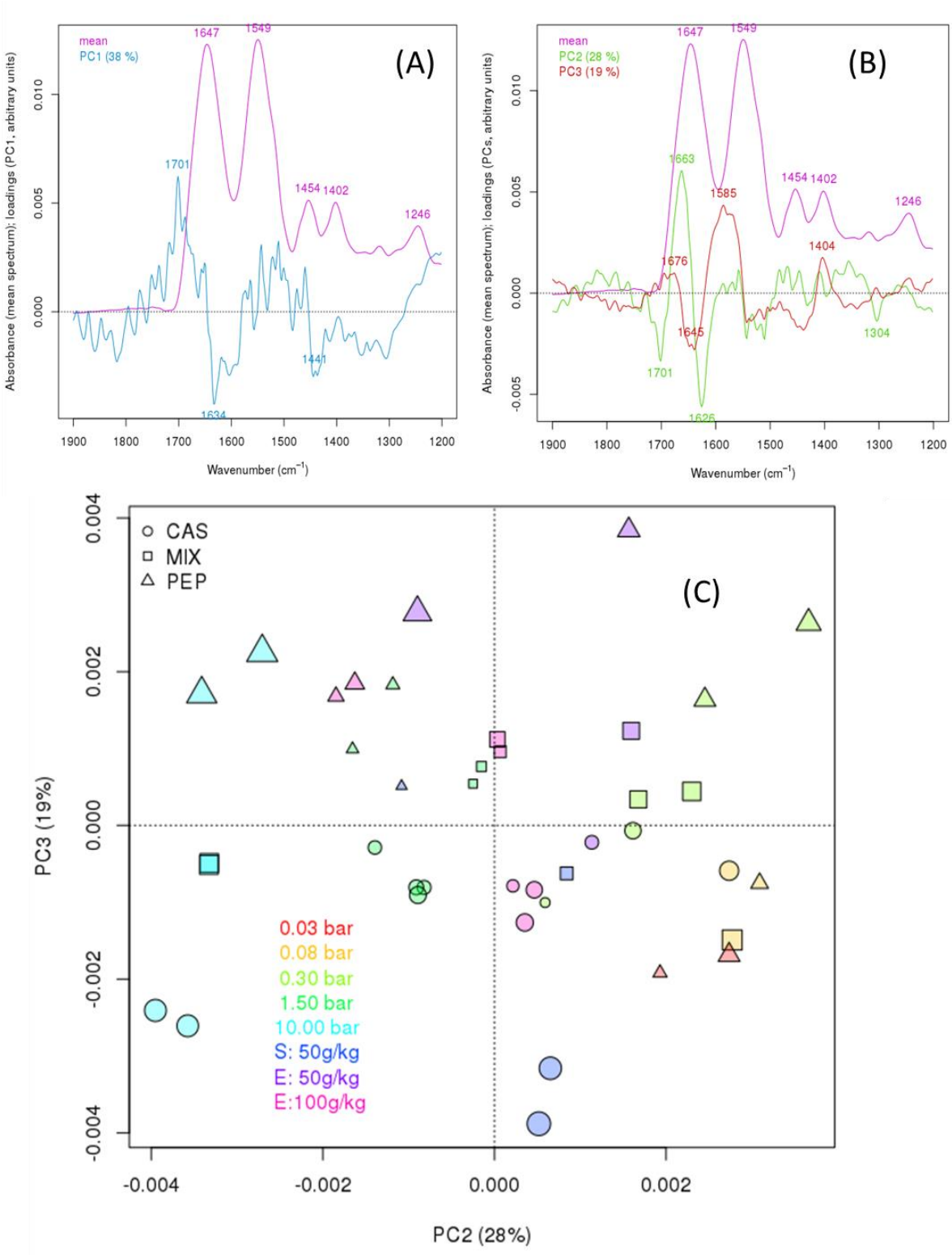


Figure 8. PCA analysis of the peptide, mixture and casein suspension at different concentrations of stock solutions (50 and 100 g.kg^{-1} and under various osmotic pressure A) loading patterns of PC1, B) loading patterns of PC2 and PC3 components and C) PCA map of the scores of the individual samples. Sample compositions were coded by shapes of the coordinate points, while pressure differences were coded by color. Stock solutions at the start (S) and at the end (E) of the equilibration were also analyzed. The size of the coordinate shapes implies the fit of its projection on the chosen axes.

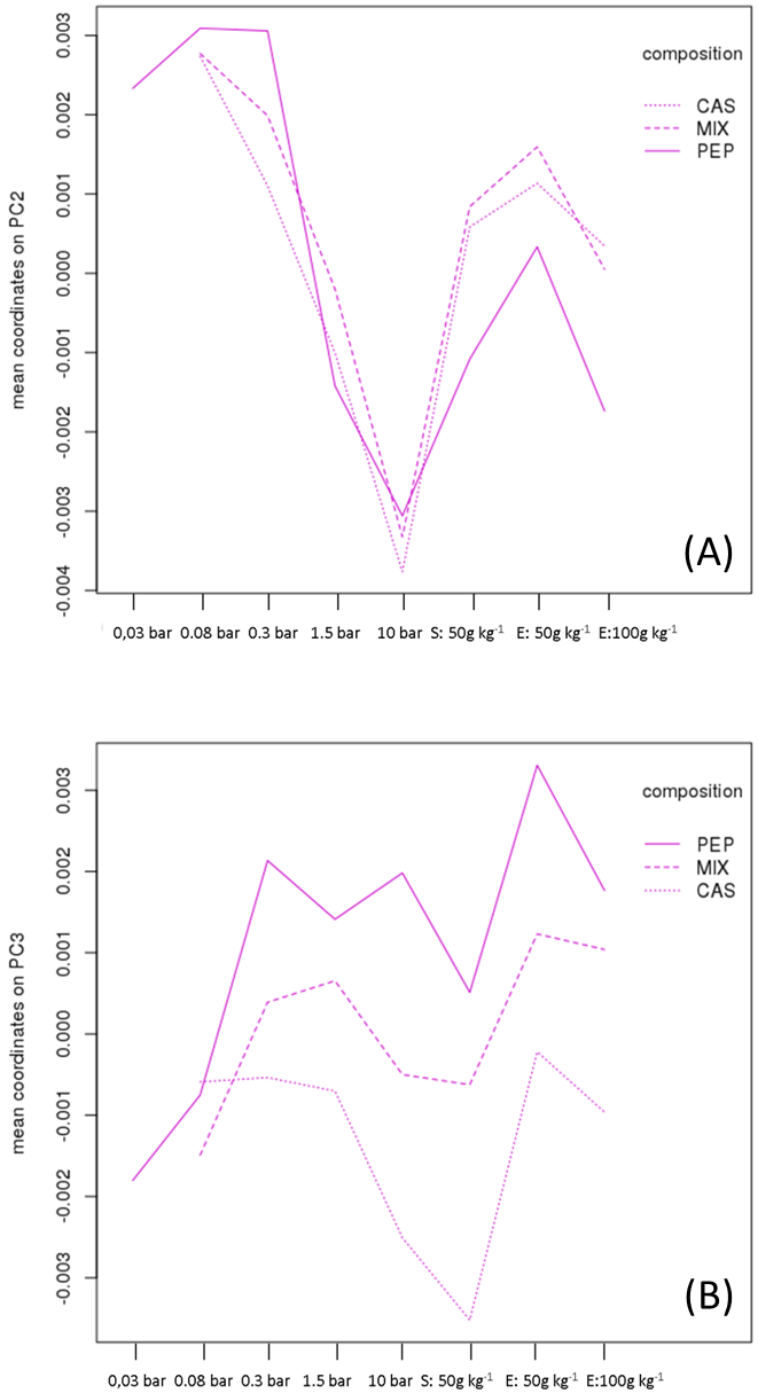


Figure 9. Mean coordinates on (A) PC2 and (B) PC3 axes for peptide, mixture and casein samples according to increasing osmotic pressures; as well as mean coordinates for 50 g.kg-1 stock solutions at week 0 (S) and at week 3 (E) and 100 g.kg^{-1} stock solution at week 3.

Table 3: Frequencies on Figure 13B, corresponding to secondary structures as reported in various references.

Frequency on Figure 12B	Position on plot	Assignment to secondary structure	Reference
1647	Mean	Random	1648 ± 2.0 (Kong and Yu, 2007)
1647	Mean	Disordered	1642– 1657 (Barth, 2007)

			1647 – 1654 (Shivu et al., 2013)
1549	Mean	Amide II	1480 – 1575 (Kong and Yu, 2007)
1454	Mean	Lipids	1464 (Aernouts et al., 2011)
1402	Mean	Free carboxylate	1402 ± 1.0 (Burgardt et al., 2014)
1246	Mean	Random coil	1240 – 1260 (Hu et al., 2008)
1246	Mean	Amide III	1240 (Aernouts et al., 2011)
1663	PC2	3_{10} Helix	1663 ± 3.0 (Kong and Yu, 2007)
1663		turns	1663 – 1695 (Shivu et al., 2013)
1626	PC2	β-sheet	1623– 1641 (Barth, 2007)
			1643 – 1615 (Shivu et al., 2013)
1304	PC2	R-helix	1280 – 1320 (Hu et al., 2008)
1676	PC3	β-Turn	1675 ± 1.0 (Kong and Yu, 2007)
1585	PC3	Aspartate side chain	1584 (Venyaminov and Kalnin, 1990)
1404	PC3	Free carboxylate	1406 (Güler et al., 2011)
			1402 ± 1.0 (Burgardt et al., 2014)
1404	PC3	Carboxylate ion stretching	Around 1400 (van der Ven et al., 2002)

Electrophoresis

In order to check whether the casein micelles underwent hydrolysis during the equilibration process and to what extent, two types of electrophoresis were performed: i) SDS PAGE in Tris-tricine buffer to separate proteins and peptides from 200 to 3.5 kDa (Figure 10) and ii) urea PAGE to evaluate casein degradation (Figure 11).

After running the casein/peptide samples through Tris-tricine SDS PAGE (Figure 10), it was observed that the protein/peptide band profiles of the peptide samples were largely unchanged compared to the stock suspension at week 0 (W0). However, it has been observed that the protein band profile of the mixture and casein stock suspensions have evolved significantly. Protein bands that were present at 35, 30, 28, 23 and 22 kDa in Week 0 and correspond to caseins (W0) became weaker in intensity for the same stock suspension after 23 days of aging (W3). This was also observed in the equilibrated samples, which showed that hydrolysis has taken place in the mixture and the casein stock solutions as aell as the equilibrated samples. However, as SDS PAGE analysis is based on apparent molecular mass, it was impossible to plot casein degradation from this type of analysis, due to co-migration of casein band with peptides produced during incubation period.

New bands as shown in Figure 15 were observed having different apparent molecular masses at the 6.5 kDa mark that were observed in the mixture and casein samples (W3 and equilibrated samples),

but were absent from the peptide samples. This revealed that caseins were hydrolyzed and new peptides were formed over the period of equilibration.

In order to measure the extent of hydrolysis in the samples, urea PAGE was conducted to examine the quantity of caseins that was lost over time. Since the four caseins, α_{s1}, α_{s2}, β and κ, exist in the ratio of 4:1:3.5:1.5 (Dalgleish, 2011), the protein bands at week 0 for β- and α_{s1}-caseins were more intense when compared to that of α_{s2}- and κ-caseins. α_{s1}-casein possesses 5 phosphoserine (PSer) residues and α_{s1}-casein has either 8 or 9 PSer, which is inconsequential to their migration in urea PAGE. On the other hand, as the α_{s2}-caseins are multi-phosphorylated, it exists with different charge densities due to having 10 to 13 PSer (it is also able to form disulphide bonds but not in this case where DTT was added for the electrophoresis analysis). Next, despite mainly having just 1 PSer, κ-casein has different extents of glycosylation (Holt and Roginski, 2001). Therefore multiple bands in the urea PAGE were observed for κ-caseins and α_{s2}-caseins (Figure 10) since they exist in forms with varying charge and size.

From Figure 11, it was apparent that the casein bands in the casein micelles and in the mixture suspensions (gels II to IV) decreased in intensity over the time of equilibration, as quickly as within week 1, especially for β-casein; β-casein have been shown to be more easily hydrolyzed by plasmin than α_{s1}- casein (Holt and Roginski, 2001). This enzyme initially present in milk can actually be linked to the casein micelles through lysine residues and stay within the casein micelle preparation of the CM powder used in this study.

From the intensity of the bands, the average percentage decrease in each casein (κ-, β-, α_{s2}- and α_{s1}-) was calculated and presented in Figure 12. However, due to their small quantities and their multiple bands, κ-casein and α_{s2}-caseins were more difficult to quantify accurately. Moreover, the κ-casein bands were also likely to have been contaminated by peptides that were formed by the hydrolysis of the caseins, likely the β-casein by plasmin, since the κ-casein bands became darker although the amount of casein could not have increased with time.

In the case of the peptides, there were little caseins remaining in the peptide samples from the beginning of the incubation, confirming that the tryptic hydrolysis of caseinate used for their preparation was conducted to more than 90% hydrolysis. However, a lightly colored β-casein band remained visible in week 0, before it then disappeared in week 2 and 3 samples (Figure 10 – I) and it was determined that the β-casein content of the peptides samples dropped to 40% after 3 weeks of equilibration. This observation corresponded with data from the FTIR spectrophotometry, where hydrolysis of the peptides was observed in the stock solutions in week 3.

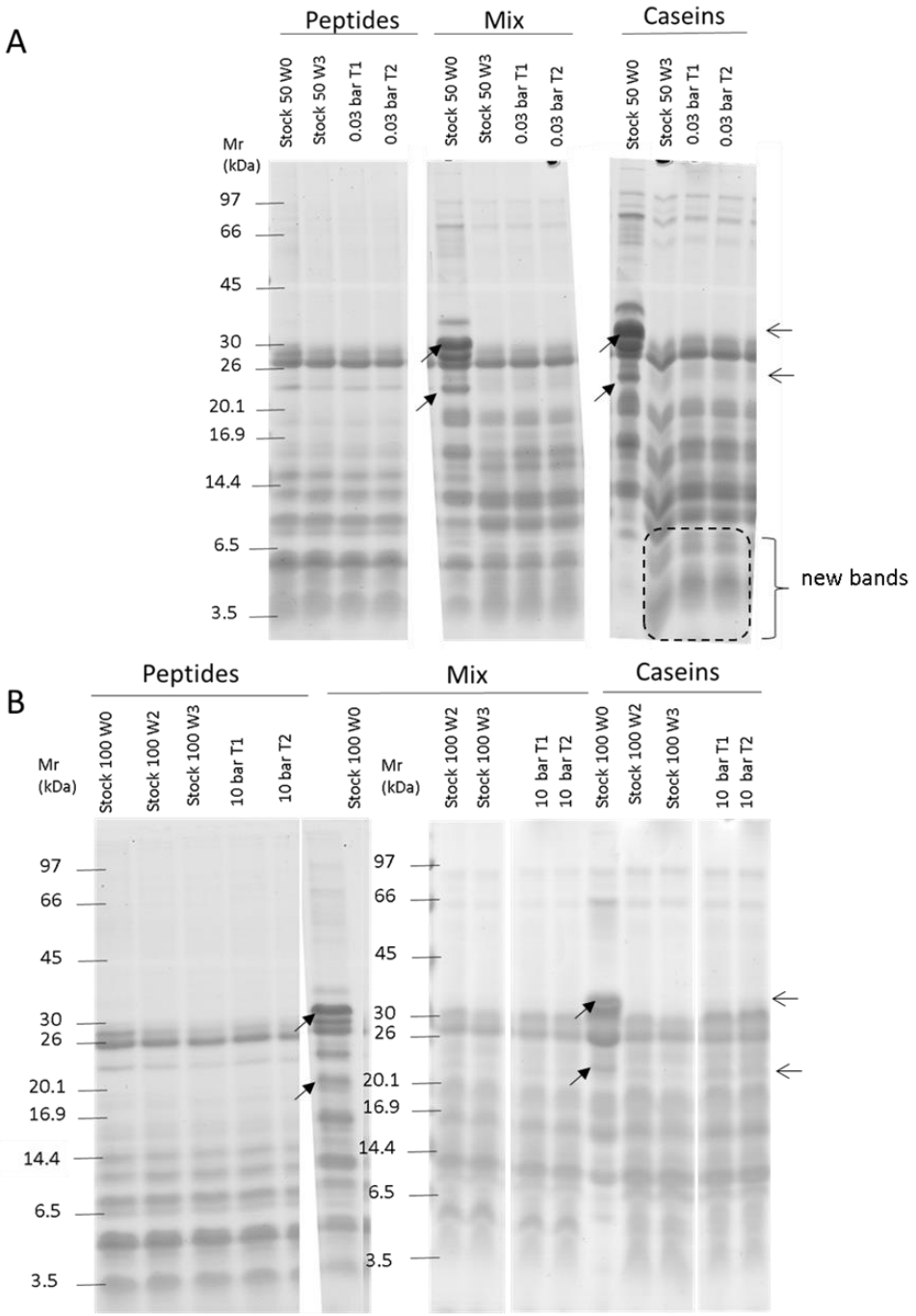


Figure 10. Tris-Tricine SDS-PAGE electrophoregram with a gradient of 12-18% acrylamide of peptide, miwture and casein suspensions at A: 50 $g.kg^{-1}$ stock solution (stock 50) and after concentration by osmotic pressure at 0.03 bar, and B: 100 $g.kg^{-1}$ stock solution (stock 100) and after concentration by osmotic pressure at 10 bar. T1 and T2 were duplicate tubes of the sample. The unusual migration for casein sample, 50 $g.kg^{-1}$, W3, was caused by a crack in the glass panel during electrophoresis. Large narrow represents marked bands originally present on week 0, small narrow represents marked bands that have disappeared.

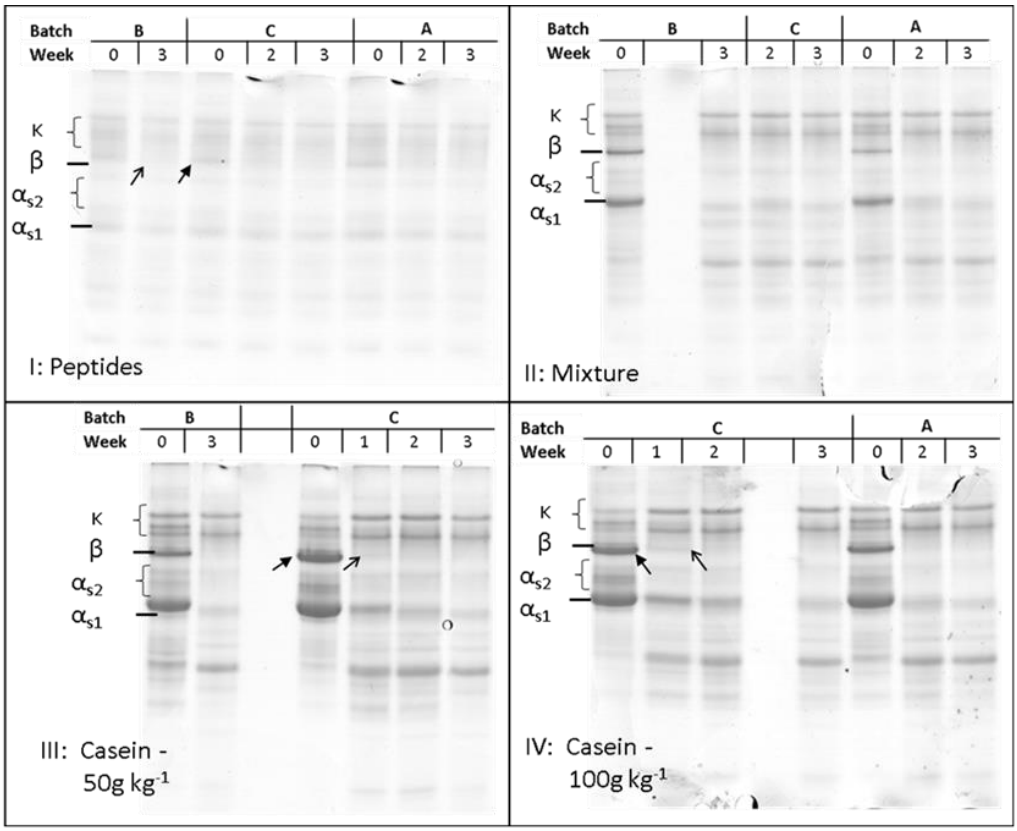


Figure 11. Urea-PAGE electrophoregram at 18% acrylamide of the caseins in the stock solutions and after 1 to 3 weeks of storage at 20°C in 25 mM maleate 2 mM calcium 0.02% (w/w) thimerosal buffer pH 7.1. I: 50 g.kg^{-1} peptides (except batch A at 100 g.kg^{-1}). II: 50 g.-kg-1 mixture (except batch A at 100 g.kg^{-1}). III: 50 g.kg^{-1} casein micelles (except batch A at 100 g.kg^{-1}). IV: 100 g.kg^{-1} casein micelles. Large narrow represents marked bands originally present on week 0, small narrow represents marked bands that have disappeared.

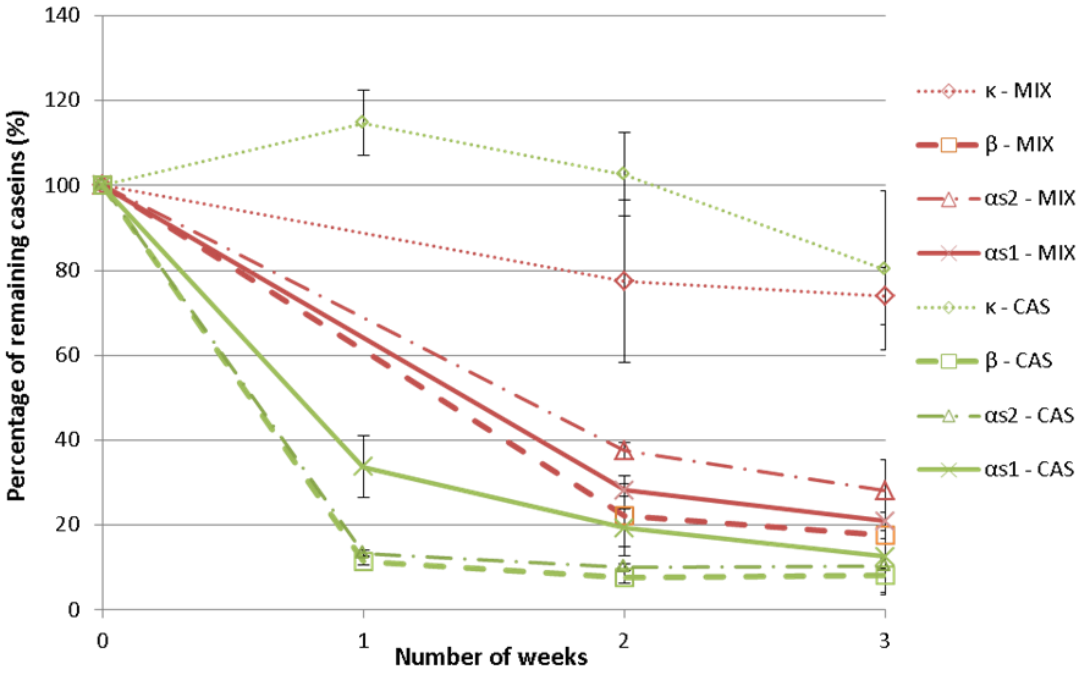
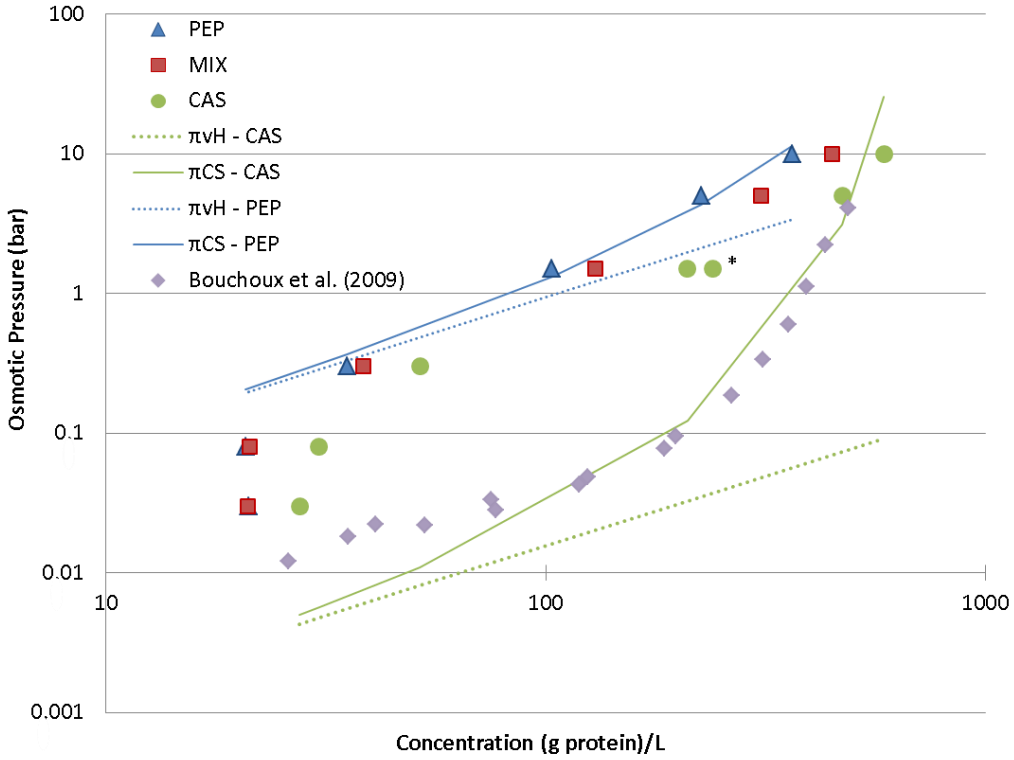


Figure 12. Changes in κ-, β-, α_{s2}- and α_{s1}-casein relative content in the mixture and casein samples over equilibration time.



Discussion

To the best of our knowledge, there were no prior studies done on peptides under high osmotic pressure. In this study, peptide suspension have been successfully concentrated to up to 300 $g.kg^{-1}$ using the osmotic stress technique, along with casein micelle suspensions and a 1:0.9 mixture of both peptides and caseins. This technique made it possible to obtain concentrations above 200 $g.kg^{-1}$, which was not feasible by dispersing peptide or casein micelle powder in a liquid. While highly concentrated casein micelles were previously found to form clear solids at 200 $g.kg^{-1}$ (Bouchoux et al., 2009), it was unprecedented to observe the peptides in the form of a gel at such a concentration. Thus it was interesting to note that at 10 bars, the texture of the mixture of caseins and peptides became sticky, while the peptides and caseins alone did not. The apparent texture of the peptides at 10 bars was gel-like, in contrast to the solid texture of the casein sample (Figure 9). These visual observations are therefore indicative of potential interactions between the casein and the peptides or change in the swelling properties of the casein micelles in presence of peptides (Lacou et al., 2016).

While we showed that the osmotic stress technique was effective in removing water from our peptide stock solution at pressures above 0.3 bars, there was a risk of having small peptides escaping through the pores of the dialysis membrane that needs to be addressed. Having used a dialysis membrane with the smallest MWCO available (100 – 500 Da), it was estimated from the total amount of peptide stock solution added to the dialysis tubes at 10 bars over 23 days, there was minimal difference in the measured protein content and the calculated protein content after the equilibration period, which was estimated to be in range of 0-3%. Therefore, most of the peptides were still contained within the dialysis membrane throughout the equilibration process. Further attempts in analyzing the filtered PEG solution (10 bars) via reversed phase liquid chromatography on C18 column and tris tricine SDS PAGE yield inconclusive results on the presence of small peptides of molecular mass less than 3.5 kDa in the PEG solution.

From the results of this study, we were able to plot for the first time the equation of state for peptides from 0.03 bar to 10 bars (Figure 10). We have found that, at a final pressure of 0.3 bars and below, the stock solution of 50 g kg-1 was in fact at a higher osmotic pressure than the PEG solution, hence the samples were being diluted instead of being concentrated, even though it was indicated that osmotic pressures of 0.5 bar to 1 bar would be sufficiently high to produce a transition from liquid to solid phase by Bouchoux et al. (2009), whose experimental data has been plotted in Figure 13 for comparison with our data. The hard sphere model for peptides and caseins, expressed by the Carnahan Starling equation shown below, were also plotted for comparison.

$$\frac{\Pi}{nkT} = \frac{1 + \phi + \phi^2 - \phi^3}{(1 - \phi)^3}$$

where

- n = number density of spheres
- k = Boltzmann constant = 8.314 Pa.m3.K-1.mol-1
- T = temperature = (20+273) K
- ϕ, volume fraction was calculated in Section 3.4.1, using the specific volume of peptides given as 0.73g cm-3 and the specific volume of caseins given as 1.377 g cm^{-3} (Bouchoux et al., 2009).

Besides sample composition and concentration, which are the variables tested in this study, pH, temperature, as well as solvent composition and ionic strength are other variables that impact non-covalent molecular interactions, and therefore impact the self-assembly behavior of the caseins and peptides (Guy and Voyer, 2012). While minor discrepancies in the solvent composition and ionic strength exist between our data and Bouchoux et al. (2009) (a maleate-calcium chloride-thiomersal buffer was used in this study, while a UF permeate was used in the study of Bouchoux et al., 2009), there was a key difference in the MWCO of the dialysis membrane used, which resulted in a major difference in the composition of the casein sample in each studies after the equilibration period.
According to Bouchoux et al. (2009), hydrolysis of casein was minimal during the dialysis period of 7 days and while longer equilibrium periods of 10 to 50 days were applied to samples at osmotic pressure > 0.05 bars, enzymatic hydrolysis was limited at these conditions due to lower activity in viscous liquids and solids. However, we have concluded that extensive hydrolysis that have taken place in our samples as observed by electrophoresis, although a lower extent of hydrolysis could be inferred for casein samples at 10 bars in our FTIR spectroscopy data (Figure 8B), which were in solid form. Our casein samples were in fact already a mixture of casein fragments with some small peptides of sizes 6.5 kDa and below. Since the casein micelles were no longer intact, thus our experimental data for caseins fell far away from the Carnahan Starling equation (Figure 13), which was calculated using the specific volume of 1.377 g cm-3 and the molecular mass of 156 kDa (Bouchoux et al., 2009). On the other hand, our data was more coherent with the study of Bouchoux et al. (2009) and also with the Carnahan Starling model at higher osmotic pressure of >5 bars, which agrees with Bouchoux's proposition that there was limited hydrolysis due to lower enzyme activity at higher concentrations. In addition, the data points at 0.03 and 0.08 bars appeared to be outliers that fell far away from the van t'Hoff model likely because it was more challenging for the samples at low

osmotic pressures to attain equilibrium by dilution, due to the higher osmotic pressure in the stock solution than the PEG bath, especially while hydrolysis was taking place within the membrane tubes.

Although the equilibration period was longer in our study, i.e. 3 weeks as compared to the 1 week period (for osmotic pressures< 0.05 bar) employed by Bouchoux et al (2009), we observed a rapid rate of hydrolysis of the casein suspension within the first week of equilibration, where the α_{s1}-casein decreased to 34% of its original content at week 0, and the β-casein decreased sharply to 13% of its original content (Figure 12). After 3 weeks, the casein content in the samples decreased to less than 12.6%, 10.3% and 8.1% of the original content of α_{s1}-, α_{s2}- and of β-caseins respectively. The observations on our sample may be attributed to the behavior of casein-derived-peptides more than being attributed to casein micelles alone. This proposition would be supported by the fact that we used the smallest available MWCO of 100-500 Da to retain as much tryptic peptides as possible within the dialysis tubes. Since a much larger MWCO of 12 – 14 kDa was used by Bouchoux et al. (2009), the lower protein concentration attained in this study for the casein micelles at similar osmotic pressures used, implied that our sample composition was likely to contain more peptides, while the plasmin-hydrolyzed peptides in the study by Bouchoux et al. (2009) would have escaped the dialysis membrane. The presence of these peptides exerted a higher osmotic pressure within the dialysis tubes, therefore resulting in less water diffusing from the sample, into the PEG bath, and therefore a lower total protein concentration attained at the end of equilibration than expected. In fact, since the data from Bouchoux et al. (2009) differed from the van t'Hoff's model at low concentrations, this suggest that there were significant hydrolysis in the samples, which resulted in higher molar concentration due to the formation of smaller casein micelles fragments.

Next, from the volume fraction calculated from total solids content (Figure 10B), we now know that much higher osmotic pressures would be required to attain the maximum concentrations of peptides, mixture and caseins. The particle volume fraction required to observed crystalline and glassy states begins from 0.5 (Loveday et al., 2007; Pasquier, 2014). Yet the volume fraction we found at 1.5 bars for the casein soft gel was at $\phi = 0.18$, for a casein concentration of 187 $g.kg^{-1}$, and the most solid-like casein sample at 10 bars had a volume fraction of $\phi = 0.43$, casein concentration of 419 $g.kg^{-1}$. Therefore, it would be necessary to evaluate the peptide and casein interactions at higher osmotic pressure that would result in higher volume fraction, increased interactions due to closer packing and hence the probable formation of solid peptide and mixture samples.

The liquid-gel transition from 0.03 to 1.5 bars and the gel-solid transition from 5 to 10 bars indicated a change in intermolecular interactions regardless of the composition of the different suspensions. This phase change was due to a reduction in water content and an increase in volume fraction, which made it possible for peptides or casein micelles to come closer together and likely to interact with each other at higher concentrations. For instance, there may also be interaction between the

peptides and caseins in the mixture samples as suggested by the peculiar sticky texture only observed in the mixture.

However, the FTIR spectrophotometry did not reveal evidence of interaction between the peptides and caseins. Figure 8A showed that the increase in osmotic pressure resulted in a drop in PC2 coordinates, which implied an increase in aggregation, such as through intermolecular β-sheets; this trend was observed in all 3 samples compositions, hence osmotic pressure resulted in an increased intermolecular interaction in a manner that was roughly independent of sample composition. Therefore, samples at osmotic pressures higher than 10 bars would be required in order to observe whether intermolecular interactions between the casein and peptides can be exerted or not. Additionally, higher osmotic pressure would be required to obtain peptide and mixture samples with similarly high concentrations as the casein samples.

However, it was noted that the PC2 coordinates for the peptides remained independent of osmotic pressure when it was at 0.3 bars and below. This implied that the peptides were too diluted at 0.3 bars and below to be able to interact or aggregate with each other.

FTIR spectrophotometry also confirmed that hydrolysis has taken place in all samples, as seen through electrophoresis, as sharp decreases in α_{s1}, α_{s2} and β- casein content in the mixture and in the casein samples were observed, including for the peptides even though there were little remaining caseins to begin with. In the case of peptides the hydrolysis by trypsin was almost complete and only few caseins remained in the peptide stock solution as expected.

A cocktail of protease inhibitors could have been added to prevent casein hydrolysis by the action of the difference proteases that may be present, though the effect of the cocktail diminishes over time as the inhibitors could be degraded. Other types of inhibitor are chelatants such as EDTA may help inhibit hydrolysis of metalloproteases if present, however due to their small molecular weight, they would help inhibiting some proteases but it can also deplete casein of its calcium and destabilize the casein micelle organization.

Another consideration for the lack of evidence of interaction between peptides and caseins may be the presence of repulsive interaction between the peptides, such as through electrostatic repulsion. From the hydropathy scores of the peptides identified from tandem mass spectrometry, it was identified that most of the peptides were hydrophilic and charged, with β-casein yielding several hydrophobic peptides and there were also a handful of highly hydrophobic peptides that originated from α_{s1}-casein. α_{s1}-casein gave peptides that were mostly net negatively charged, while α_{s2} and κ-casein gave peptides that were mostly net positively charged. The peptides of β-caseins were more amphiphilic as they are hydrophobic, yet several of them carried a net negative charge, except the peptides β-CN (f 1-25) that was highly negatively charged. Hence, a large proportion of the peptides were charged and hydrophilic, there may be significant electrostatic repulsions amongst the

peptides, resulting in a lack of interaction to form tertiary structure and the level of concentration after equilibration may be insufficient to counteract them. As hydrophobic interactions is a key driving force in initiating peptide self-assembly through the formation of β-sheets (Guy and Voyer, 2012), the amount of hydrophobic peptides may be too low in this preparation or the complexity of the hydrolysate too high to induce sufficient interactions between them in contrast to study performed ion rationally designed peptides (Guy and Voyer, 2012; Hauser et al., 2011).This could contribute to explain why the apparent texture of the peptide equilibrated samples was thinner than that of the mixture and of the casein samples.

Conclusion

Having established the method for the concentration of peptides by osmotic pressure, we are now able to plot its equation of state and predict the osmotic pressure required to obtain higher concentrations of peptide samples in the next step of study, where closer packing of the peptides could reveal its mechanism of molecular self-assembly; the peptides remained too diluted with the experimental conditions applied in this study, 10 bars and below, and solid structures were not observable. Only gel peptide samples were observed between the osmotic pressure of 5 – 10 bars. Using the equation of state obtained in this study, the osmotic stress technique opens new ways to study peptide interactions in highly concentrated suspensions that can be reached in fermented milk products such as cheeses.

On the other hand, through electrophoresis, FTIR as well as through van t'Hoff and Carnahan Starling models, we have found that hydrolysis of casein samples takes place rapidly within the first week of equilibration, hence it was a challenge to maintain the integrity of the casein micelles in the time required for the sample to equilibrate to the osmotic pressure required. Furthermore, the casein derived peptides, produced by the hydrolysis, were contained within the membrane of MWCO of 100 – 500 Da, and had a significant impact on the osmotic pressure of the caseins. A solution would be to add protease inhibitors; however there are several limitations, one of which would be the possible degradation of inhibitors over time, which implies that the equilibration duration of the samples may have to be shortened accordingly, perhaps by reducing sample volume and increasing the surface area of exchange.

Once the conditions have been optimized to limit the hydrolysis of caseins, another series of equilibration may be conducted at increased osmotic pressure to attain higher peptide and casein concentrations for the study of interactions between peptides and caseins.

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Chapter 5: Incorporation of defined peptides in semi-hard model cheeses

Proteolysis occurs throughout cheese ripening and provides to the cheese its texture and flavor. The *in situ* proteolysis is partly controlled by the physico-chemical conditions applied during cheese making and ripening and by the main proteolytic agents present and added. However, the proteolysis mechanism is still far from being elucidated to orientate the cheese texture. Consequently, the addition of hydrolysates directly at the beginning or the cheese manufacture is one way to investigate the role of targeted peptides on the evolution of cheese texture.

The aim of this study was to determine how and to what extent the presence of defined peptides changed the texture of model cheeses under different ionic strength conditions.

The hydrolysates T2 and G2 were chosen because of their different behaviors in the complex matrices (chapters 2 and 3). The liquid preconcentrated cheese method was used to manufacture cheeses with these hydrolysates in order to keep peptides within the cheese curd without a draining step and to show the impact of targeted peptides on the model cheese texture. The cheese fabrication was realized in collaboration with Actalia dairy products that are expert on the manufacture of cheeses.

This study showed that the addition of Trypsin and Glu-C hydrolysates during the cheese manufacture impacts the cheese texture until the end of the ripening. Depending on the nature and quantity of peptides present in the hydrolysates, the cheese texture was deeply changed. Moreover, changing the ionic strength had also an impact on the interactions peptides-peptides and/or peptides-caseins involving different textures and techno-functional properties as stretchability.

This approach is used as a first step to understand the role of peptides on the model cheese texture, permitting later to appropriately orientate the textural properties of real cheeses.

The highlights of this chapter are:

- The nature of peptides and their quantity impact the texture of cheeses
- The proteolysis and the texture are dependent on the ionic strength
- The addition of hydrolysates at the beginning of the cheese manufacture could be a way to change texture and to have a more controlled proteolysis during ripening

This chapter is presented under the form of an article which is in preparation for *Journal of Dairy Science*[9].

[9] Lacou, L. et al. Changes in textural properties of model cheeses by adding defined milk-derived peptides in presence or absence of NaCl. In preparation for *Journal of Dairy Science*.

Chapitre 5 : Incorporation de peptides dans des fromages modèles semi-durs

La protéolyse a lieu au cours de l'affinage du fromage et donne au fromage sa texture et saveur. La protéolyse *in situ* est en partie contrôlée par les conditions physico-chimiques appliquées lors de la fabrication et de l'affinage du fromage ainsi que par les principaux agents protéolytiques ajoutés. Cependant, les mécanismes régissant la protéolyse sont toujours loin d'être élucidés en vue de texturer à façon les fromages. Par conséquent, l'addition d'hydrolysats directement au début de la fabrication du fromage est une façon d'étudier le rôle de peptides ciblés sur l'évolution de la texture du fromage.

Le but de cette étude était de déterminer comment et dans quelle mesure la présence de peptides connus change la texture de fromages modèles à des force ioniques différentes.

Les hydrolysats T2 et G2 ont été choisis du fait de leurs comportements différents dans les matrices complexes (chapitres 2 et 3). La méthode de préconcnetration de fromages liquides a été utilisée pour fabriquer des fromages avec ajout de ces hydrolysats afin de garder les peptides au sein du caillé tout en s'affranchissant de l'étape d'égouttage et de démontrer l'impact de peptides ciblés sur la texture de fromages modèles. La fabrication des fromages a été réalisée en collaboration avec Actalia produits laitiers qui sont experts qui possède une grande expertise sur la fabrication de fromages.

Cette étude a montré que l'ajout d'hydrolysats trypsiques et de Glu-C lors de la fabrication des fromages a un impact sur leur texture jusqu'à la fin de l'affinage. Selon la nature et la quantité de peptides présents dans les hydrolysats, la texture des fromages a été profondément modifiée. De plus, la force ionique a aussi eu un impact sur les interactions peptides-peptides et peptides-caséines induisant des textures différentes.

Cette approche est utilisée comme première étape à la compréhension du rôle des peptides sur la texture des fromages modèles, permettant ensuite d'orienter convenablement les textures de fromages réels.

Les points à retenir de ce chapitre sont les suivants:

- La nature de peptides et leur quantité impactent la texture des fromages
- La protéolyse et la texture dépendent de la force ionique de l'aliment
- L'addition d'hydrolysats en début de fabrication des fromages pourrait être une façon de changer la texture et de contrôler la protéolyse lors de l'affinage.

Ce chapitre est présenté sous forme d'article in préparation pour soumission à *Journal of Dairy Science*[10].

[10] Lacou, L. et al. Changes in textural properties of model cheeses by adding defined milk-derived peptides in presence or absence of NaCl. En préparation pour *Journal of Dairy Science*.

Changes in textural properties of model cheeses by adding defined milk-derived peptides in presence or absence of NaCl

In preparation for Journal of Dairy Science

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ABSTRACT

Texture is an important characteristic of cheese in determining consumer acceptability and quality. One of the possibilities to enhance textural properties of cheese is the hydrolysis of caseins. This study aimed to compare the texture of cheeses manufactured with ultrafiltrate retentate (R), sodium caseinate (C), trypsin hydrolysate (T) of Glu-C hydrolysate (G). Cheeses were manufactured using a liquid pre-concentrated cheese according to the microfiltration MMV (Maubois, Mocquot, and Vassal) process with addition of 15% of sodium caseinate or hydrolysates, in presence or absence of NaCl. Cheeses were ripened for 23 days at 12°C. Texture characteristics (hardness, cohesiveness, stickiness, gumminess, elasticity and rupture strength) were determined by Texture Profile Analysis (TPA). The stretchability was measured at each time of ripening. The present study shows that texture attributes were influenced by the presence of peptides, the nature of the hydrolysate, the ionic strength and the time of ripening. The presence of hydrolysate impacted the proteolysis according to the cheese ripening time and induced formation of cheeses that were firmer and less elastic than the control. Cheeses without NaCl addition had good stretchability unlike cheeses containing NaCl which had no stretchability.

ABBREVIATIONS

+: with NaCl addition; -: without NaCl addition; C: Cheese manufactured with sodium caseinate; CFU: Colony Forming Units; D0: Day 0 of ripening; D3: Day 3 of ripening; D13: Day 13 of ripening; D23: Day 23 of ripening; DH: Degree of Hydrolysis; G: Cheese manufactured with Glu-C; MMV: Maubois, Mocquot, Vassal; NaCas, sodium caseinate; NaCl: sodium chloride; NaOH: sodium hydroxide; OPA: o-phthalaldehyde spectrophotometric; PC: Principal Component; PCA: Principal Component Analysis; R: Cheese manufactured with ultrafiltrate retentate; SDS: Sodium Dodecyl Sulphate; SEM: Standard Error of the Mean; T: Cheese manufactured with Trypsin; TCA: Trichloroacetic; TPA: Texture Profile Analysis.

KEYWORDS

Food, stretchability, proteolysis, TPA, salt, hydrolysate, MMV cheese

INTRODUCTION

An increasing proportion of cheeses is used as an ingredient added on several meals as pizza, burgers, lasagna or breads. In most of these applications, physical and rheological characteristics of cheese, such as hardness, elasticity, meltability, stretchability or even color, are important textural and flavor attributes that are strong criteria of choice for consumers.

The vast variety of taste and textures of cheese arises from the complex organization of components such as proteins, lipids and carbohydrates and from their evolution during the ripening. Among them, the caseins, the main milk proteins constitute the backbone of cheese structure and give initial textural properties that are progressively changed by proteolysis that occurs throughout the ripening period (Fedrick and Dulley, 1984; Fox et al., 1995; Fox and McSweeney, 1996). Actually, the extent of proteolysis and the type of peptides produced control and modify the nature and strength of the casein interactions and the casein and casein-derived peptides which in turn have an impact on the final cheese texture (Lucey et al., 2003). Proteolysis involves the concomitant and successive action of proteinases and peptidases from different origins: milk (plasmin), coagulants added during manufacture (rennet or chymosin) and microorganisms (lactic acid bacteria and other ripening flora) (Lucey et al., 2003; Upadhyay et al., 2004). Therefore, several hundreds of peptides are produced throughout ripening time more or less implied in final functional properties of the cheese. Such a complexity renders difficult the elucidation of the proteolysis mechanisms and the development of strategies to produce specific hydrolysis of caseins and to orient functional properties such as meltability, stretchability and firmness.

The texture generally changes markedly in the first weeks of ripening as the hydrolysis of α_{s1}-casein by the rennet into the α_{s1}-casein derived peptide, α_{s1}-I (f 24-199), results in a general weakening of the casein network. This proteolytic step was correlated with changes in the rheological properties of cheese, notably a lower elasticity and a decrease in the force required to fracture the cheese (creamer, Richardson) and in the melting and stretching properties (Borsting et al., 2012; Brickley et al., 2007). Thereafter, the changes in texture are determined mainly by the rate of proteolysis and the other kinds of peptides produced. Thus stretching properties rapidly decreased when level of proteolysis is increasing throughout ripening time (Sadat-Mekmene et al., 2013). However, stretchability was differentially affected according to the types of peptides produced (Lacou et al., 2014; Sadat-Mekmene et al., 2013). Therefore, knowing which kinds of peptides can be associated with defined functional properties will allow orientating the way they are produced and hence orientate the functional properties.

The aim of this study is to show how incorporation of peptides with peculiar physico-chemical characteristics can impact the texture of cheese. For this, casein hydrolysates were produced by

using two enzymes with known specificities, Trypsin and protease from Staphylococcus aureus V-8 (endoproteinase Glu-C) and were reincorporated into milk retentate by using the liquid pre-concentrated cheese method (MMV) (Maubois et al., 1969). MMV cheese making process was used to promote casein concentration by membrane filtration as well as whey removal before coagulation step and to avoid the draining step and the withdrawal of the added peptides. The texture, proteolysis, microbial development and physico-chemical characteristics of cheeses were followed according to the ripening time. Differences between cheeses could mainly be explained in terms of the composition and physico-chemical characteristics of the reincorporated peptides.

MATERIALS AND METHODS

Sodium caseinate sample

Sodium caseinate (NaCas) powder (Armor-Protéines, France) contained 87% (w/w) of proteins. The protein content was determined by both Kjeldhal method and UV spectrophotometry (UVmc2, Monaco SAFA, France) at λ = 280 nm using extinction coefficient of 0.81 ml. mg^{-1}. cm^{-1} (Oliva et al., 2001). The caseinate contained only minor amount of whey proteins as shown by SDS-PAGE electrophoresis (protocol described in the part "Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-page Analysis" of this article). This powder was dispersed in ultrafiltrate permeate or in water using motor agitation in order to obtain a solution at 10 g of caseins.kg^{-1}.

Enzyme digestion

NaCas was hydrolyzed by two immobilized enzymes with different specificities: Trypsin and Glu-C, according to the protocol of Lacou et al. (2016). Trypsin (T) and Glu-C (G) hydrolysates present different degree of hydrolysis (DH): 4.5% with Trypsin and 1.8% with Glu-C.

Cheese making

Two cheese-making experiments were carried out following the MMV process (Fig. 1). The raw milk was pasteurized at 72°C during 20 secondes, skimmed and microfiltered on a membrane with pore size of 1.4 µm. The fat:protein (1:1) content was standardized by adding heat-treated cream (120°C for 20 s).The standardized pasteurized skim milk was than ultrafiltrated on a mineral membrane with pore size of 0.02 µm, a surface of 1.8 m^2 (Membralox, Pall Exekia, France) at 50°C during 2.5 hours (1 hour up to a concentration factor of 3 and diafiltered with 1 diavolume during 1.5 hours up to a concentration factor of 6). The protein content of the milk retentate was concentrated six times

compared to the original milk protein content and the lactose was partly removed by diafiltration. The lactose was removed in order to mimic the conditions of traditional cheeses that have cell lysis of *Lactococcus lactis* during ripening due to lactose starvation to liberate the internal proteolytic enzymes. Then, 1.5% of NaCl was added or not to the milk retentate at 50°C. 15% of hydrolysate or NaCas (w/w), suspended in ultrafiltrate permeate, were added to this retentate kept at 50°C. After, the mix was cooled at 30°C and both *Lactococcus lactis* starter *(*MM100, *Danisco*®, France) and chymosin (CHY-MAX Plus, Chr. Hansen A/S, Denmark) were added to the mix. This mix was incubated at 30°C until both coagulation and lactic fermentation were complete (around 20 hours). Cheeses were vacuum-packed and were ripened at 12°C and withdrawn for further analyses at 3 days (D3), 13 days (D13) and 23 days (D23).

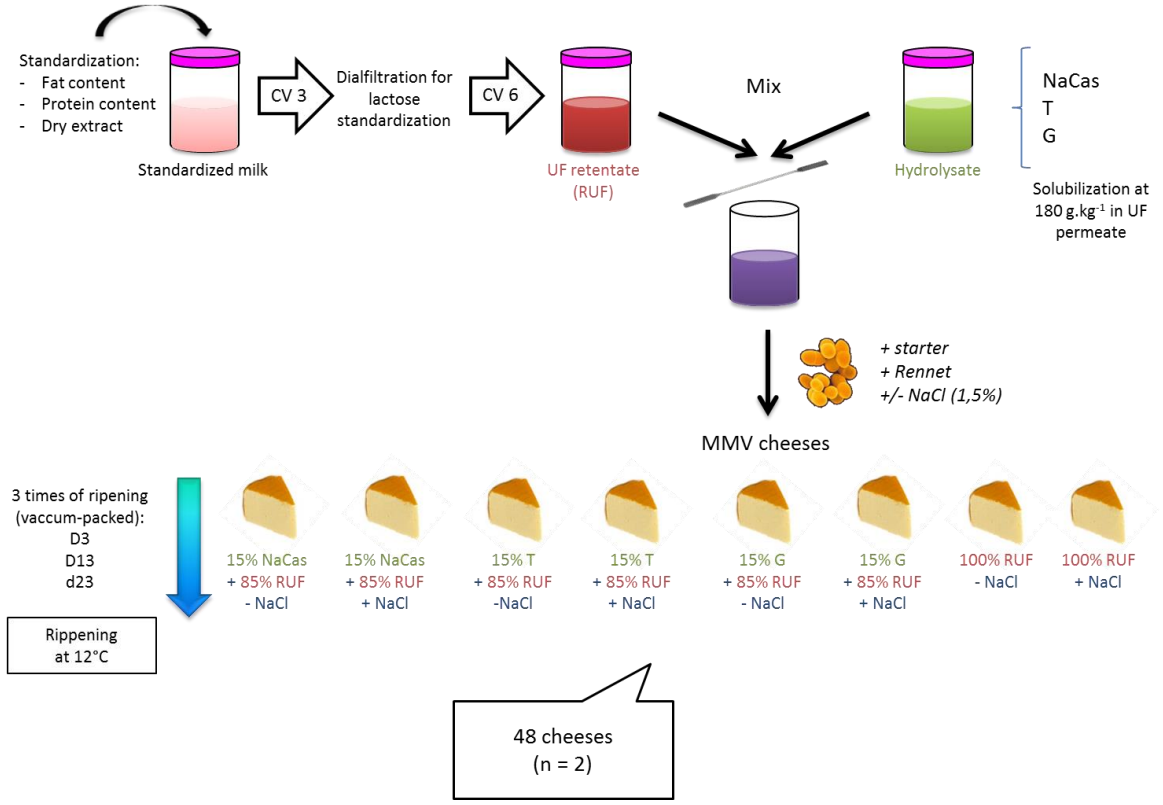

Fig 1. Manufacture of MMV cheeses with addition of hydrolysates (Tryptic or Glu-C hydrolysates) or sodium caseinate (NaCas).

Hereafter, the cheeses manufactured using only retentate, or with addition of caseinate, tryptic hydrolysate and Glu-C hydrolysate that are designated as R, C, T, and G cheeses respectively. Moreover the salting of the cheeses are noted + with NaCl and – without.

Cheese Analysis

pH was measured directly in the middle of cheeses using a pH meter (WTW pH 197i, Geotech, USA). MMV cheeses were analyzed for moisture (IDF ISO 5537:2004, 2004), fat content (Heiss, 1961) and dry matter (DM) (ISO 2920:2004 IDF 58: 2004).

Lactococcus lactis enumeration

Under a sterile atmosphere, one gram of grated MMV cheese at D3, D13 and D23 was dispersed in10 mL of sterile sodium citrate 20g.L^{-1} in a bag and crushed in a pendular blender Mixwel^{+} (Awel, France) for 120 s. From this suspension, the enumeration of the *Lactococcus Lactis* flora cell counts was made extemporaneously, by successive series of dilution in tryptone water in microwells according the method of Baron et al. (2006) and plate count on M17 medium (Terzaghi and Sandine, 1975) at 30°C for 48h . The citrate suspension was then frozen and conserved at -20°C until used.

o-phthaldialdehyde (OPA)

Proteolysis was measured in triplicates using the OPA method of Church et al.(1983) with methionine as a standard and adapted to microplate by Darrouzet-Nardi et al. (2013). Citrate suspension of cheeses were thawed in a water bath at 30°C for 15 min, precipitated with HCl 1N at pH 4.6 or with trichloroacetic acid 12 % (w/v) final concentration. Then, samples were centrifuged at 3500 *g* for 10 min at 25°C before the supernatants were diluted at a ratio 1:5 with sodium tetraborate 0.1 M pH 9.5 for each sampling except initial time samples (ratio 1:2). The results were expressed as mmol methionine equivalent. L^{-1}.

Protein profile by electrophoresis: Tris-Tricine/SDS-PAGE and Urea-PAGE Analysis

The extent of casein degradation was evaluated by Urea-PAGE (14% of acrylamide). The pH 4.6 precipitates obtained from the OPA sample preparation were resuspended at the original volume with urea 8.75 M and condition of electrophoretic migration were used.

The peptide pattern was analyzed by SDS-PAGE as described by Schägger and von Jagow (1987) using Protean II system (16 × 16 × 0.1 cm; BioRad, France) with SDS/Tris/Tricine buffer and a concentration gradient from 12 to 18% of acrylamide according to Sadat-Mekmene et al. (2011). All gels were scanned by LabScan (GE Healthcare, France) and analyzed by ImageQuant software (GE Healthcare, France), band volumes were estimated by the net area parameter.

Texture Profile Analysis (TPA)

MMV cheeses were cut into cylindrical samples (20 mm thick and 20 mm diameter) using a cookie cutter and a wire cutter, and equilibrated at 20°C for at least 1 hour prior to testing. A two-bite

compression test was performed using the texturometer Lloyd instrument (Ametek, France) with a 100 kN load cell. A 50% compression was used and crosshead speed was 60 $mm.min^{-1}$. Data collection was done using the Nexigen Plus software and TPA parameters (hardness 1 for the first compression, hardness 2 for the second compression, cohesiveness, adhesiveness, stickiness, adhesive force, elasticity and gumminess) were determined in duplicate from the texture profile curve as described by Bourne (1978).

Cheese Stretchability

Cheese stretchability was assayed by a method involving vertical traction of the cheese melted at 82°C according to Richoux et al. (Richoux et al., 2001). The length (mm) of strands of heated cheese was measured at the breaking point of the stretched strand.

Statistical analysis

Two independent sets of cheeses were manufactured and, for each data measured, 2 or 3 determinations per sample were done according to the protocol used.

To link cheese stretchability and cheese texture to a specific hydrolysate, a methodology based on multivariate exploratory analyses, such as Principal Component Analysis was performed using FactoMineR, an R package (Team, 2005). One PCA was performed to distribute the cheeses according to their biochemical and microbiological analyses and to their texture parameters. The first two PCs were retained as the totality of the inertia explained by both of them, represented a large part of the total inertia.

For all experiments, significant differences in time and hydrolysate type were tested using a nonparametric analysis of repeated measures with the “f1.ld.f1” function of the package “nparLD” (Noguchi et al., 2012) in R 3.2.2 (Team, 2005). In case of a significant fixed effect, the package “nparcomp” (Konietschke et al., 2014) was used to test multiple comparisons. To analyze the interaction effects, a linear mixed effect model with a random intercept on experiments to take into account the repeated measures was performed and followed by function “difflsmeans” of the package “lmerTest” (Kuznetsova et al., 2013).

RESULTS

Chemical Analyses and Viability of Starters

The composition of the cheeses (Table I) fitted well with the data already published on MMV Saint Paulin – type cheeses (Goudedranche et al., 1986). A slight difference was noted between final composition of the cheeses made with ultrafiltrate retentate (control R) and the cheeses made with

NaCas or hydrolysates regarding the following parameters: dry matter (DM), moisture in the non-fat substance (MNFS), fat in dry matter (FDM) and pH (Table I). The control cheese had a slightly higher DM value than the other cheeses (*P<0.001*). The presence of NaCl involved a decrease in DM of all the cheeses, both at D3 and D13 (*P<0.001*). The DM and FDM were higher for R cheeses than for the others and for cheeses without NaCl than with NaCl whereas these values did not varied according to the time of ripening (*P<0.001*). The cultivability of *Lactococcus lactis*, used as starter, reached ~9.5 $\log_{10}$ colony forming units $.g^{-1}$ cheese (CFU) at D3. The cultivability continuously decreased thereafter until D23, to reach around 9 $\log_{10}$ $CFU.g^{-1}$ cheese. A significant difference (*P<0.05*) was shown according to the ripening time between D3 and D13. A decrease of 90% of the *Lactococcus lactis* population was actually observed during this ripening period. Adversely, no significant difference of *Lactococcus lactis* cultivability was observed regardless of the types of cheeses containing or not caseinate and hydrolysates, i.e. R, C, T and G cheeses or the presence or absence of NaCl.

Extent of proteolysis

The proteolysis, including the proteolysis *in situ* and the peptides added in the cheeses with the hydrolysates, in all cheeses increased significantly according to the ripening time for soluble nitrogen compounds at both pH 4.6 and after TCA precipitation (*P<0.001*) (Table I). With or without NaCl, the proteolysis in cheeses was higher for cheeses manufactured using tryptic hydrolysates than for the other cheeses (*P<0.001*), in agreement with the initial composition of the hydrolysates (Lacou et al., 2016), regardless of the time of ripening and of the precipitation method used (*P<0.001*) (Table I). The NaCl addition weakly changed the overall proteolysis extent in cheeses except for the R and T cheeses, in which the proteolysis extent of R+ and T+ cheese was significantly higher than that of respectively R- and T- cheeses (*P<0.001*) for the pH 4.6 precipitation at each time of ripening whereas the proteolysis in the T+ was significantly higher than T- for the TCA precipitation (*P<0.001*) only at D23. The proteolysis indices were twice higher for soluble pH 4.6 nitrogen compounds compared to the TCA soluble ones. The precipitation at pH 4.6 led to the precipitation of caseins and large peptides, as α_{s1}-I or γ-CN peptides from β-caseins, while the other peptides were present in the soluble fraction (Upadhyay et al., 2004). In the case of the precipitation with TCA, caseins, whey proteins and large and medium peptides were precipitated while smaller peptides, from 2 to 20 amino acids, were soluble (Yvon et al., 1989).

From the statistical analysis of data obtained with the precipitation by both pH 4.6 and TCA, the interactions between the ripening time and the type of cheese were significantly different (*P<0.001*). The proteolysis was higher for the cheeses manufactured with Trypsin hydrolysate (with or without NaCl) than for the other cheeses regardless of neither the time of ripening nor the process of precipitation (*P<0.001*) (Table I). Comparing the cheese T- to the cheese T+, the proteolysis in T+ was

significantly higher than in T- (*P<0.001*), for the precipitation at pH 4.6 at every times of ripening, whereas the proteolysis in the T+ was significantly higher than T- for the precipitation with TCA (*P<0.001*) only at D23.

The presence of NaCl increased the proteolysis extent of pH 4.6 soluble components (*P<0.05*) except for R cheese at D23 and G cheese at D13. For TCA precipitation, the presence of salts influenced significantly the proteolysis in all cheeses (*P<0.05*), except for C and T from D3 to D13. Contrary to T cheeses, the cheeses without NaCl had a higher index of proteolysis in the soluble TCA fraction than the cheeses with NaCl.

Primary proteolysis of caseins, estimated by urea-PAGE, is also shown in Table I. α_{s1}, α_{s2} and β-caseins were slightly or even not hydrolyzed during the ripening time. No significant difference was observed between the cheeses, according to the type of added hydrolysates and to the presence or absence of NaCl.

Table I. Composition and proteolysis of cheese during ripening.

Cheese		R-	R+	C-	C+	T-	T+	G-	G+
Dry matter (DM) (%)	D3	$46.6_{\pm0.0}^{a}$	$44.4_{\pm0.0}^{d}$	$43.6_{\pm0.0}^{b}$	$41.5_{\pm0.2}^{e}$	$44.2_{\pm0.3}^{c}$	$43.7_{\pm0.2}^{f}$	$44.3_{\pm0.3}^{c}$	$41.4_{\pm0.2}^{e}$
	D13	$44.3_{\pm0.1}^{g}$	$45.2_{\pm0.0}^{j}$	$43.2_{\pm0.2}^{h}$	$40.7_{\pm0.0}^{k}$	$43.5_{\pm0.1}^{i}$	$43.1_{\pm0.1}^{l}$	$43.6_{\pm0.0}^{i}$	$40.7_{\pm0.1}^{k}$
FDM (%)	D3	$47.2_{\pm0.0}^{a}$	$45.1_{\pm0.0}^{b}$	$43.5_{\pm0.0}^{d}$	$40.9_{\pm0.2}^{e}$	$44.1_{\pm0.3}^{c}$	$42.4_{\pm0.2}^{f}$	$44.0_{\pm0.3}^{c}$	$42.3_{\pm0.2}^{f}$
	D13	$47.4_{\pm0.1}^{a}$	$44.2_{\pm0.0}^{g}$	$44.0_{\pm0.2}^{h}$	$41.7_{\pm0.0}^{i}$	$43.7_{\pm0.01}^{h}$	$41.8_{\pm0.6}^{i}$	$44.2_{\pm0.0}^{hc}$	$41.8_{\pm0.1}^{i}$
MNFS (%)	D3	$68.49_{\pm0.05}^{a}$	$69.54_{\pm0.01}^{a}$	$69.58_{\pm0.03}^{a}$	$70.46_{\pm0.27}^{a}$	$69.33_{\pm0.32}^{a}$	$69.10_{\pm0.24}^{a}$	$69.12_{\pm0.42}^{a}$	$71.08_{\pm0.24}^{a}$
	D13	$70.48_{\pm0.16}^{a}$	$68.49_{\pm0.01}^{a}$	$88.60_{\pm25.96}^{b}$	$71.41_{\pm0.04}^{a}$	$69.79_{\pm0.08}^{a}$	$69.43_{\pm0.07}^{a}$	$70.09_{\pm0.00}^{a}$	$71.48_{\pm0.08}^{a}$
pH	D3	5.10	5.34	5.22	5.28	5.24	5.30	5.23	5.29
Lactococcus lactis cultivability (log CFU.g^{-1} cheese)	D3	9.85^{a}	$9.53_{\pm0.32}^{a}$	$9.69_{\pm0.11}^{a}$	$9.56_{\pm0.12}^{a}$	$9.55_{\pm0.01}^{a}$	$9.47_{\pm0.07}^{a}$	$9.68_{\pm0.14}^{a}$	9.46^{a}
	D13	$9.46_{\pm0.3}^{b}$	$9.05_{\pm0.13}^{b}$	$9.29_{\pm0.07}^{b}$	$9.20_{\pm0.06}^{b}$	$9.02_{\pm0.33}^{b}$	$8.98_{\pm0.3}^{b}$	$8.74_{\pm0.34}^{b}$	$9.08_{\pm0.48}^{b}$
	D23	$9.35_{\pm0.36}^{b}$	$8.86_{\pm0.43}^{b}$	$8.96_{\pm0.19}^{b}$	$9.17_{\pm0.01}^{b}$	$8.93_{\pm0.19}^{b}$	$8.77_{\pm0.004}^{b}$	$8.74_{\pm0.18}^{b}$	$8.78_{\pm0.05}^{b}$
Proteolysis indices for soluble components at pH 4.6 (eq. mM of methionine)	D0	$22.10_{\pm1.56}^{a}$		$18.35_{\pm0.46}^{b}$		$29.27_{\pm0.79}^{c}$		$24.28_{\pm1.09}^{d}$	
	D3	$25.68_{\pm2.55}^{i}$	$30.39_{\pm1.81}^{e}$	$24.20_{\pm0.41}^{j}$	$26.02_{\pm0.5}^{f}$	$35.19_{\pm0.81}^{k}$	$38.89_{\pm1.18}^{g}$	$28.48_{\pm0.58}^{l}$	$26.7_{\pm0.63}^{h}$
	D13	$33.20_{\pm1.31}^{mo}$	$33.93_{\pm1.11}^{mo}$	$31.92_{\pm0.54}^{o}$	$34.74_{\pm1.01}^{m}$	$44.28_{\pm0.93}^{p}$	$47.33_{\pm0.53}^{n}$	$34.06_{\pm0.71}^{o}$	$34.97_{\pm2.06}^{o}$
	D23	$45.00_{\pm1.77}^{t}$	$43.76_{\pm3.19}^{q}$	$43.60_{\pm0.96}^{u}$	$47.09_{\pm1.69}^{qs}$	$53.26_{\pm1.22}^{v}$	$58.96_{\pm1.95}^{r}$	$43.33_{\pm1.17}^{x}$	$41.68_{\pm0.93}^{s}$
Proteolysis indices for soluble components after precipitation with TCA 12%(w/w) (eq. mM of methionine)	D0					$1.08_{\pm0.05}$		0.66	
	D3	$10.01_{\pm1.28}^{a}$	$7.57_{\pm0.69}^{c}$	$8.86_{\pm1.08}^{a}$	$7.28_{\pm1.68}^{c}$	$16.31_{\pm0.89}^{b}$	$18.00_{\pm1.13}^{c}$	$10.36_{\pm1.12}^{d}$	$7.58_{\pm1.75}^{a}$
	D13	$17.04_{\pm2.07}^{e}$	$11.88_{\pm0.61}^{g}$	$15.17_{\pm2.07}^{e}$	$14.22_{\pm1.10}^{h}$	$24.17_{\pm1.75}^{f}$	$25.52_{\pm0.82}^{i}$	$15.71_{\pm1.75}^{e}$	$12.93_{\pm1.80}^{h}$
	D23	$23.86_{\pm1.67}^{j}$	$19.09_{\pm1.59}^{l}$	$22.48_{\pm1.68}^{j}$	$25.65_{\pm1.55}^{m}$	$30.32_{\pm1.21}^{k}$	$35.23_{\pm1.09}^{n}$	$21.43_{\pm2.42}^{j}$	$15.63_{\pm4.29}^{o}$
Native α_{s1}-casein (%)	D3	$109_{\pm0}^{a}$	$83_{\pm0}^{a}$	$85_{\pm20}^{a}$	$73_{\pm18}^{a}$	$106_{\pm33}^{a}$	$87_{\pm11}^{a}$	$106_{\pm0}^{a}$	$87_{\pm1}^{a}$
	D13	$91_{\pm53}^{a}$	$112_{\pm21}^{a}$	$67_{\pm24}^{a}$	$102_{\pm4}^{a}$	$98_{\pm44}^{a}$	$98_{\pm30}^{a}$	$81_{\pm36}^{a}$	$98_{\pm7}^{a}$
	D23	$88_{\pm12}^{a}$	$107_{\pm16}^{a}$	$94_{\pm14}^{a}$	$98_{\pm5}^{a}$	$95_{\pm34}^{a}$	$98_{\pm18}^{a}$	$96_{\pm38}^{a}$	$98_{\pm39}^{a}$
Native α_{s2}-casein (%)	D3	$108_{\pm0}^{a}$	$96_{\pm12}^{a}$	$109_{\pm4}^{a}$	$95_{\pm6}^{a}$	$100_{\pm20}^{a}$	$93_{\pm5}^{a}$	$111_{\pm0}^{a}$	$93_{\pm15}^{a}$
	D13	$96_{\pm16}^{a}$	$114_{\pm11}^{a}$	$103_{\pm31}^{a}$	$109_{\pm6}^{a}$	$106_{\pm2}^{a}$	$101_{\pm14}^{a}$	$99_{\pm18}^{a}$	$107_{\pm12}^{a}$
	D23	$109_{\pm7}^{a}$	$113_{\pm4}^{a}$	$114_{\pm7}^{a}$	$116_{\pm18}^{a}$	$129_{\pm1}^{a}$	$114_{\pm3}^{a}$	$119_{\pm7}^{a}$	$109_{\pm11}^{a}$
Native β-casein (%)	D3	$109_{\pm0}^{a}$	$83_{\pm17}^{a}$	$92_{\pm24}^{a}$	$89_{\pm3}^{a}$	$100_{\pm32}^{a}$	$91_{\pm16}^{a}$	$106_{\pm0}^{a}$	$78_{\pm5}^{a}$
	D13	$89_{\pm28}^{a}$	$103_{\pm15}^{a}$	$85_{\pm25}^{a}$	$101_{\pm19}^{a}$	$97_{\pm22}^{a}$	$92_{\pm30}^{a}$	$89_{\pm23}^{a}$	$87_{\pm8}^{a}$

	D23	$84_{\pm5}{}^{a}$	$87_{\pm8}{}^{a}$	$103_{\pm3}{}^{a}$	$94_{\pm3}{}^{a}$	$110_{\pm1}{}^{a}$	$93_{\pm4}{}^{a}$	$105_{\pm2}{}^{a}$	$95_{\pm5}{}^{a}$

Data shown are the mean value of the two determinations ± standard deviation
Ripening time: D3, day 13; D13, day 13; D23, day 23
DM=Dry matter; FDM = fat-in-DM; MNFS = moisture in nonfat substances
[ab] different letters = significant differences (*P<0.05*)
(R), control concentrate milk retentate cheese; retentate cheese with (C), sodium caseinate, (G) hydrolysate G or (T), hydrolysate T; (+) with or (-) without NaCl.

Peptide profile

The peptides with an apparent molecular weight ranged from 3 to 26 kDa present in water soluble extract of cheese using ultrafiltrate retentate, sodium caseinate, hydrolysate G or hydrolysate T were separated by Tris/Tricine SDS-PAGE (Figure 2). The main bands were common to all cheeses, exerting various intensities. Ripened cheeses present a band "c" at around 15 kDa that was more intense than that present in the cheeses at D0. Significant differences in peptide profile were shown for the peptides from 14 to 3 kDa (bands d, e, f, g). These bands were only present for the cheeses made with added Trypsin and Glu-C hydrolysates. No great differences were observed between the peptide profiles of the cheeses with or without NaCl except in terms of intensity as exemplified by bands "h" that were clearly less intense in cheeses ripened without salt.

Regarding the band corresponding to proteins over 25 kDa, we confirmed a slight degradation of the caseins, bands that are comprised between 35 and 26 kDa due to their migration above their effective molecular weight when phosphoserine residues are present within the casein sequence. The bands "a" which is the BSA and "b" have similar intensities in all cheeses. It appears that in T+ and G+ cheeses additional bands were present over 30 kDa reflecting the release of intracellular bacterial proteins during cell lysis (Gagnaire et al., 2004), in agreement with the higher decrease in cultivability previously observed.

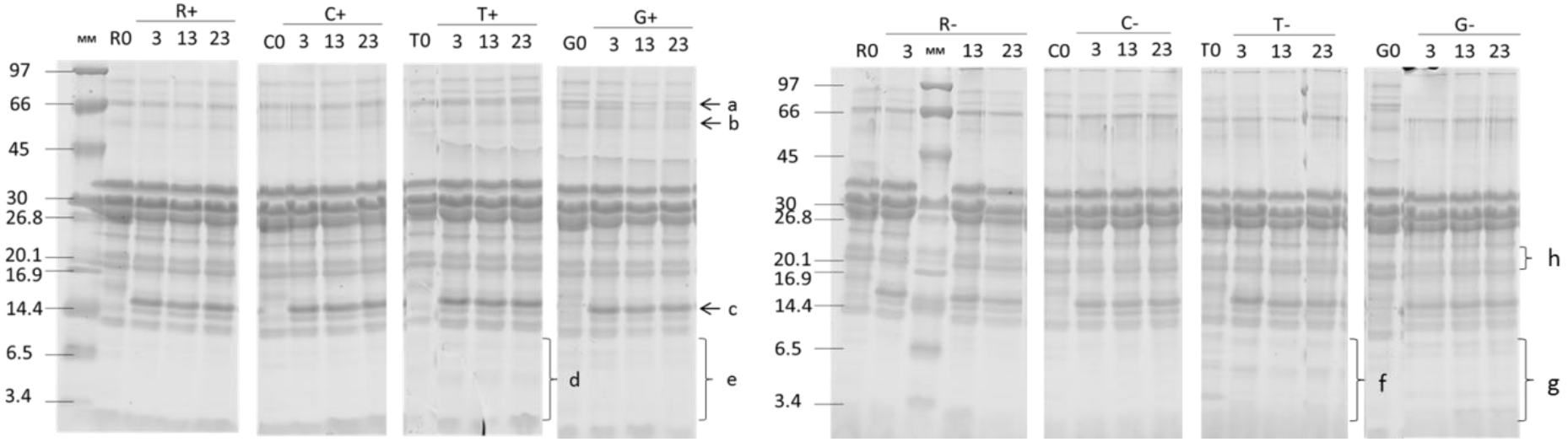

Fig. 2. Tris/tricine SDS-PAGE electrophoregrams of water- soluble extract of MMV cheeses manufactured using ultrafiltrate retentate (R), sodium caseinate (C), hydrolysate G (G) or hydrolysate T (T), with NaCl (+) or not (-). MM = molecular mass marker. 0, 3, 13 and 23 represent the day of ripening. Bands a and b were highly present in sample G+ and the band c is more intense in the ripened cheeses d, e, f and g band present only in T^+ G^+ and T^- G^- cheeses respectively.

Syneresis was observed at the surface of all cheeses except for the cheese T- at D23. The profile of proteins and peptides found in the syneresis were analyzed by Tris-tricine SDS-PAGE and the same profile of peptides and proteins (whey proteins, α_{s1}- and β- caseins) was found regardless of the type of hydrolysate added to the cheese and the presence or absence of NaCl (Figure 3). There were more proteins than peptides between 20 to 3 kDa that were evacuated in the expelled whey and consequently did not participate to the cheese structure. These peptides were particularly presented in the syneresis of T and G cheeses compared to the syneresis of R and C cheeses. Therefore, the presence of these peptides was only due to the presence of the hydrolysates in the cheeses and not to the *in situ* proteolysis.

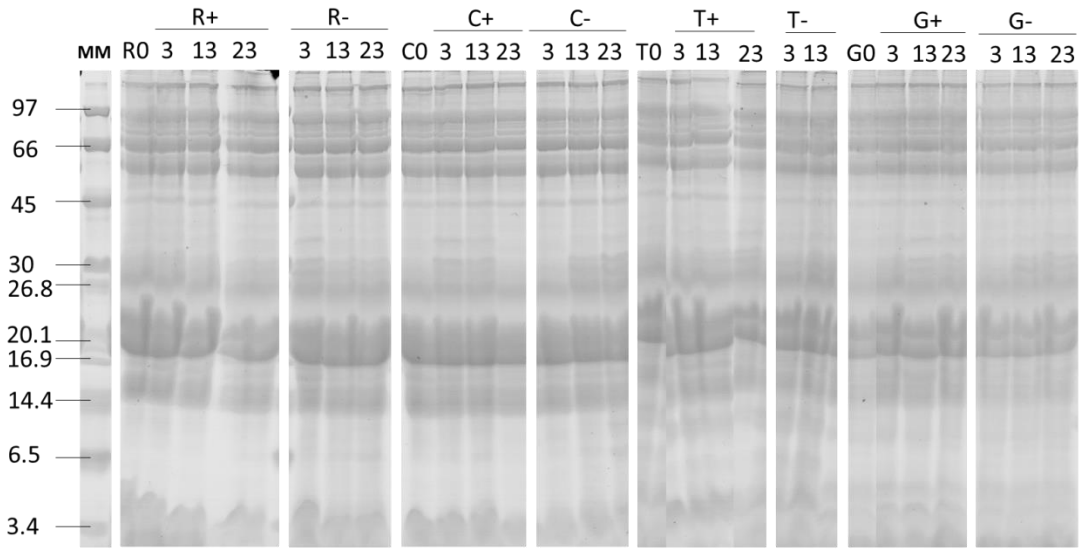

Fig. 3. Tris/tricine SDS-PAGE electrophoregrams of the syneresis of MMV cheeses manufactured using ultrafiltrate retentate (R), sodium caseinate (C), hydrolysate G (G) or hydrolysate T (T), with NaCl (+) or not (-). MM = molecular mass marker. 0, 3, 13 and 23 represent the day of ripening.

Dynamic of stretchability of Cheese during ripening

The stretchability of cheeses according to the time of ripening was shown in Fig. 4. When the cheeses were salted, no strands were observed (data not shown) in contrast to the non-salted cheeses R-, C-, T- and G- which showed variable stretchability according to both the time of ripening and the type of cheese. Thus, at D3, three cheeses, R-, C- and T- formed strands around 100 mm while no strands were observed for G-. At D13, the stretchability was maximal for all cheeses. The strand length was around 450 mm for R- and C- cheeses and around 320 mm for T- and G-. The strand length of R- and C- cheeses decreased at D23 to reach a value of 350 mm, while the strand length of G- cheeses remained constant and the T- cheeses was not stretchable anymore.

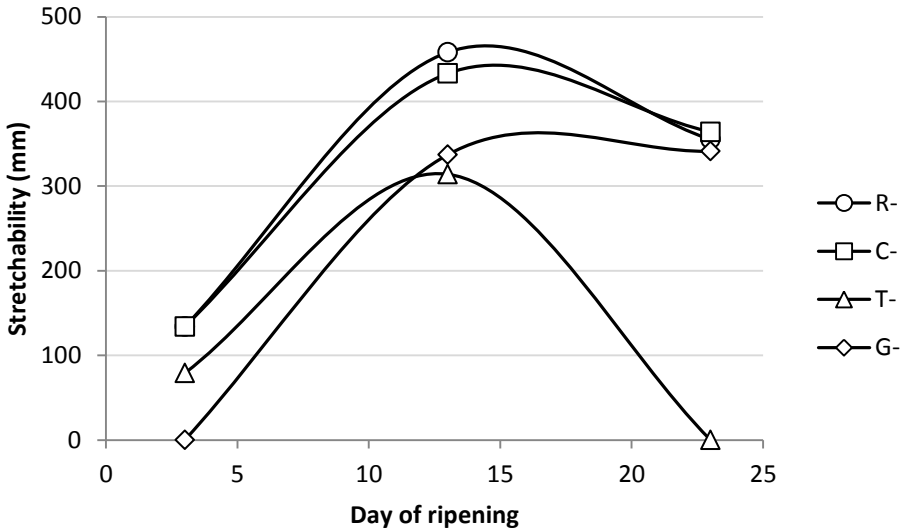

Fig. 4. Changes in stretchability during ripening of MMV cheese manufactured without NaCl (-) using ultrafiltrate retentate (R) as a control, and with 15 % (w/w) sodium caseinate (C), hydrolysate G (G) and hydrolysate T (T).

Texture analysis of cheeses

The parameters of cheese texture, cohesiveness, hardness at the first (hardness 1) and at the second (hardness 2) compression, stickiness, rupture strength, gumminess and elasticity are shown on the Figures 5 to 10. From the ANOVA-type test, it was observed that the elasticity, the stickiness and the hardness at the second compression were dependent from the interaction “type of hydrolysate used - time of ripening”. Concerning the hardness at the first compression, the gumminess and the rupture strength, simple effects were detected. These parameters were only dependent of time and of type of hydrolysate used but no interaction between these conditions existed. The Figures 5 to 10 showed the variations of these texture parameters according to the time of ripening and the type of hydrolysate used whereas the Table II presents the statistical analysis results of the comparison of cheeses with or without NaCl.

The cohesiveness is showed on the Figure 5 and represents the strength of internal bonds making up the body of the product. The cohesiveness value of around 0.4 showed the weak cohesiveness of all cheeses whereas no difference exists between the cheese cohesiveness regardless the addition of hydrolysate or not, the type of hydrolysate used, the presence or not of NaCl or the time of ripening. This could indicate damage of the internal bonds between caseins and thus a higher tendency to fracture under stress. Most of cheeses prepared showed weak resistance to disintegration due to compression. Neither the presence of peptides nor the presence of native individual caseins was remedied this default.

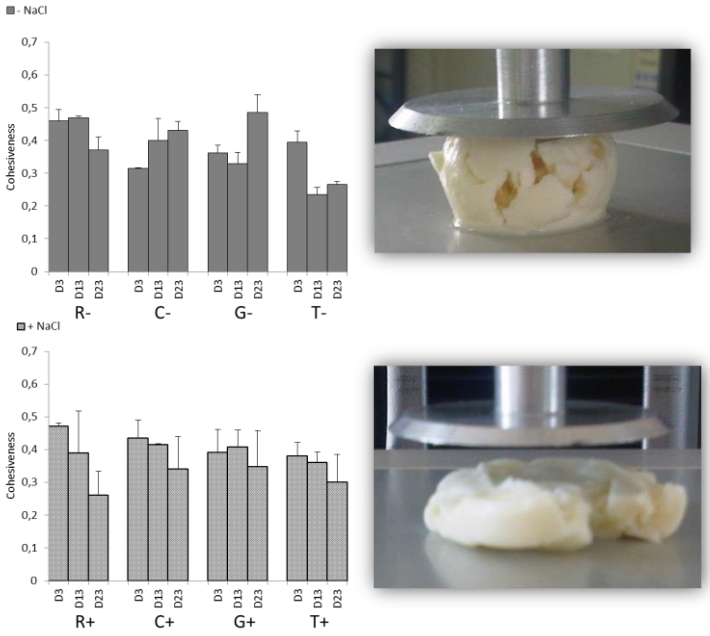

Fig. 5. Cohesiveness of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R), used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented. The photographs show the weak cohesiveness of the cheeses.

Hardness is defined as the force necessary to attain a given deformation (Szczesniak, 2002; Uprit and Mishra, 2004). During the ripening time, the hardness 1 decreased significantly (*P<0.001*) for all cheeses from D3 to D13 and stayed at the same value from D13 to D23, except for (i) C+ for which the hardness decreased from D3 to D13 and increased from D13 to D23 and for (ii) G+ for which the hardness stayed identical during the ripening (Figure 6A). The hardness at the second compression was weaker than the hardness at the first compression for all cheeses. However, the hardness 2 followed the same pattern than the hardness 1 during the ripening time, except for T+ for which the hardness 2 stayed at the same value during the ripening time (Figure 6B). The hardness 2 decreased significantly (*P<0.001*) for all cheeses except for (i) C+ for which the hardness decreased significantly from D3 to D13 (*P<0.001*) and increased significantly from D13 to D23 (*P<0.05*) and for (ii) G+ and T+ for which the hardness stayed the same during the ripening. The presence of hydrolysate impacted the hardness of cheeses too. The hardness 1 of cheeses made with hydrolysates was higher than the hardness of cheese control without NaCl. At D3, the hardness 1 of C- and G- was significantly higher than the hardness of the control R- (*P<0.001*). However, at D13 and D23, the hardness 1 was identical for the control than for C- and G- cheeses. Throughout ripening time, the hardness 1 of T- was significantly higher than the hardness of the control R- (*P<0.001*). The only one difference between C+ cheese and the control R+ cheese was found at D23. At D23, the hardness 1 of C+ was higher than

the hardness 1 of R+ (*P<0.001*). No significant difference was found between the hardness 1 of cheeses manufactured with G+ and R+. For T+, the hardness 1 was higher than for R+ at each time of ripening. The highest hardness 2 was found for T- and the weaker for R-, at D3. No difference was shown between the hardness 2 values of the cheeses manufactured with C- and the control R-, regardless of the ripening time. The hardness 2 was significantly higher for cheese made with G-, T- and R-, at D3 (*P<0.01, 0.01* and *0.05* respectively). However, at D23, no difference was observed between the hardness 2 of G-, T- and the control R-. The hardness 2 of C+ at D3 was higher than R+ (*P<0.01*). It was the same phenomenon at D23 (*P<0.05*) but no difference was established between these 2 cheeses at D13. The hardness 2 of G+ and T+ cheeses were the same than for R+. The hardness at the first compression was higher than the hardness at the second compression, in good correlation with the cohesiveness value which was around 0.4. As the presence of hydrolysate, the presence of NaCl had an important impact on the cheese texture. At D3, the cheese with NaCas addition had the same hardness 1 with or without NaCl. The hardness 1 of R cheese increased with the addition of NaCl whereas the hardness 1 of cheeses with hydrolysates decreased (*P<0.001*). At D13, control cheese had the same hardness 1 with or without NaCl whereas the hardness of C, G and T cheese decreased (*P<0.001*). At D23, cheeses made with Glu-C and Trypsin hydrolysates had the same hardness whereas R and C cheeses made with the addition of NaCl had a weaker hardness 1 than cheeses without NaCl (*P<0.001*). At D3, the control cheeses and the NaCas had the same hardness 2 with or without NaCl whereas cheeses made with hydrolysates T and G had a sharp decline of hardness 2 with the addition of NaCl (*P<0.01* and P<0.001 respectively). At D13, only cheeses made with Trypsin hydrolysate had a strong decrease of hardness 2 with the addition of NaCl (*P<0.05*). At D23, the hardness 2 of each cheese was the same with or without NaCl.

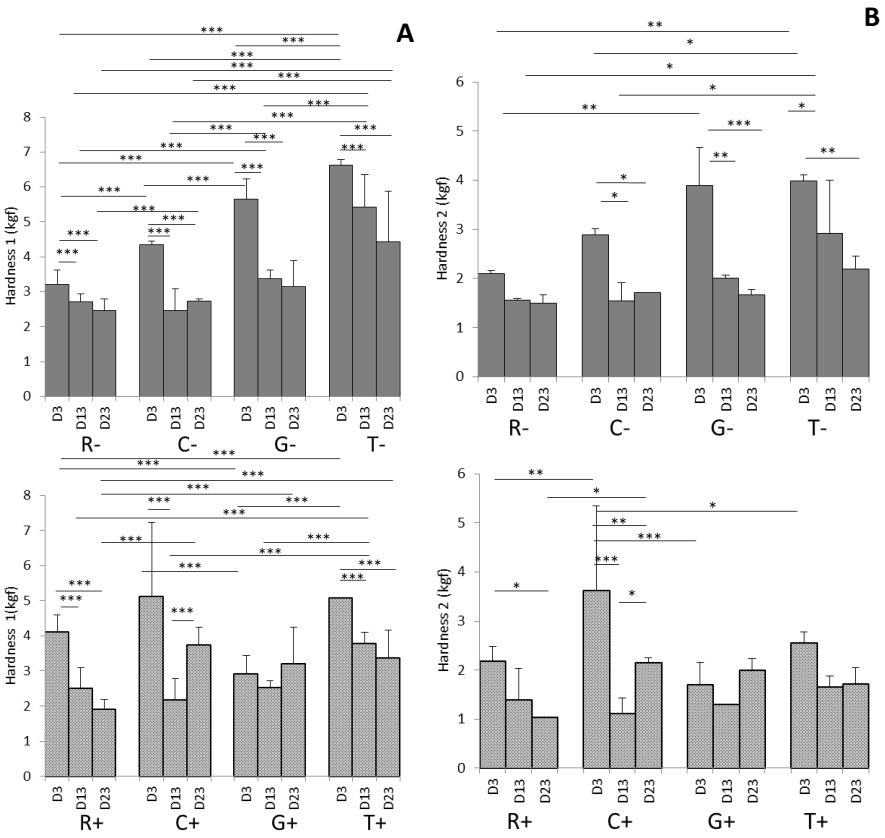

Fig. 6. Hardness at the first compression (hardness 1, Figure 6A) and hardness at the second compression (hardness 2, Figure 6B) of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R) used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented; * *P<0.05*; ** *P< 0.01*; *** *P<0.001*.

The stickiness values are presented on the Figure 7. The stickiness of cheese was higher when the hydrolysates were reincorporated into the cheeses than for the control cheese and the cheese with NaCas, in absence of NaCl. The stickiness was the same for R- and C- cheeses throughout ripening. The stickiness was higher for G- and T- cheeses (*P<0.05* and *0.001* respectively) than the stickiness of the control R-, at D3. At D13 and D23, the stickiness was higher than R- only for T- cheese. With NaCl, the stickiness at D3 was weaker than R+ except for T+, which had the same value than R+. The Figure 7 shows that the stickiness decreased significantly for each cheese (*P<0.01*) from D3 to D13 and stay at the same value from D13 to D23, except C+ and G+ for which the stickiness firstly decreases and then increased significantly (*P<0.05*). At D13, no difference between the stickiness of all cheeses was observed. At D23, the stickiness of C+ and G+ cheeses was higher than R+ (*P<0.01*) whereas it was the same value for T+. At D3, the control cheese and the cheese made with Trypsin hydrolysate addition had the same stickiness whereas the cheeses made with NaCas and Glu-C hydrolysate had a weaker stickiness with NaCl than without NaCl (*P<0.05* and *0.001* respectively). At D13, the addition of NaCl had no impact on the stickiness of the cheese control and the cheese made with NaCas.

However, the addition of NaCl involved a decrease of the stickiness of T and G cheeses (*P<0.001* and *0.05* respectively). At D23, the NaCl had an impact only on the stickiness of cheese made with NaCas. The stickiness increased with the presence of NaCl.

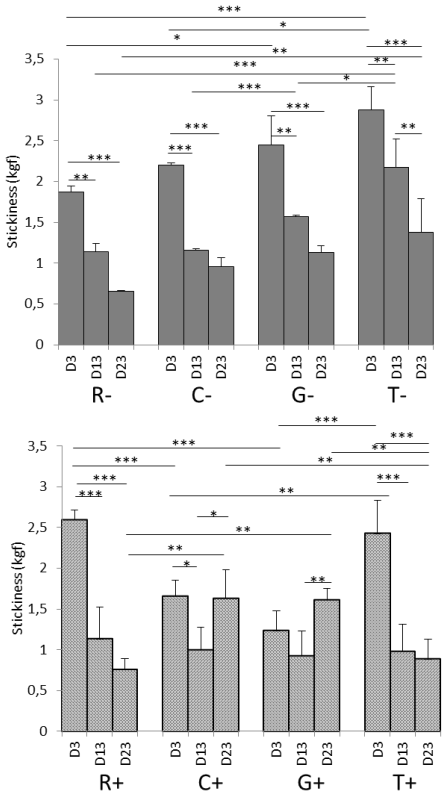

Fig. 7. Stickiness of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R) used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented; * *P<0.05*; ** *P< 0.01*; *** *P<0.001*.

The rupture strength represents a nominal stress developed in a material at rupture. The rupture strength followed the same pattern as the hardness according to the ripening time (Figure 8). More the hardness was more the rupture strength was. The rupture strength was correlated with the hardness. At D3, control cheese and cheeses made with hydrolysates had a weaker rupture strength with NaCl than without (*P<0.001*). At D13, NaCl had no influence on the rupture strength value of the cheeses. At D23, the R cheese had a lower rupture strength with NaCl than without (*P<0.001*) whereas the C cheese had a higher rupture strength with NaCl than without (*P<0.001*).

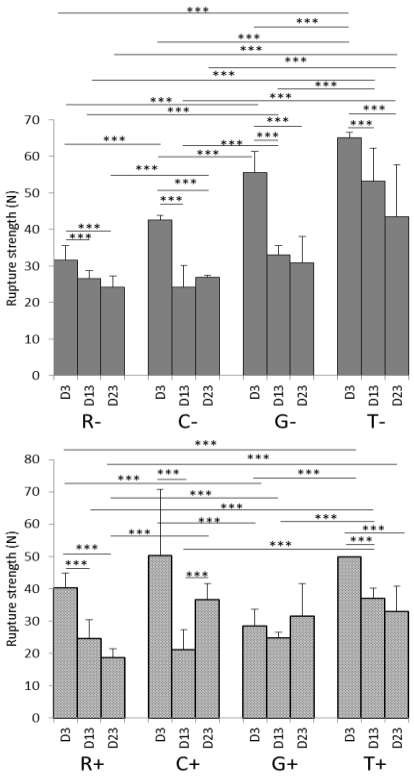

Fig. 8. Rupture strength of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R) used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented; * *P<0.05*; ** *P< 0.01*; *** *P<0.001*.

The gumminess depends on the hardness of the product and is the energy required to disintegrate a semi-solid food product to a state ready for swallowing (Fox et al., 2004). The gumminess values of each cheese are presented on the Figure 9. The gumminess of all cheeses decreased significantly (*P<0.001*) from D3 to D13 and stayed at the same value from D13 to D23, except for the control R- and R+ for which the gumminess stayed at a value of around 8 kgf. One exception was observable. The gumminess of the T+ cheese increased significantly (*P<0.001*) from D3 to D13 and stayed at the same value from D13 to D23. At D3, the gumminess was higher for the cheeses containing hydrolysates and NaCas than for the control, regardless the presence or not of NaCl (*P<0.05*) except for G+ which has the same value than the control. At D13 and D23, the gumminess of only T- was higher than R- (*P<0.001*) but not this of C- and G-. At D13 and in presence of NaCl, the presence of T hydrolysate led to an increase of the gumminess compared to the control (*P<0.001*). At D23, this increase was only valid for T+ (*P<0.001*) but not for G+. At D3, the gumminess of C increased with the presence of NaCl (*P<0.001*) whereas the control stayed at the same value. However, the gumminess of G and T cheeses decreased with the addition of NaCl. At D13, the cheeses manufactured with hydrolysates G and the control had a weaker gumminess with NaCl than without (*P<0.001*) whereas

the T cheese had a higher gumminess (*P<0.001*). At D23, the control cheese and the T cheese had a weaker gumminess with NaCl than without (*P<0.001*).

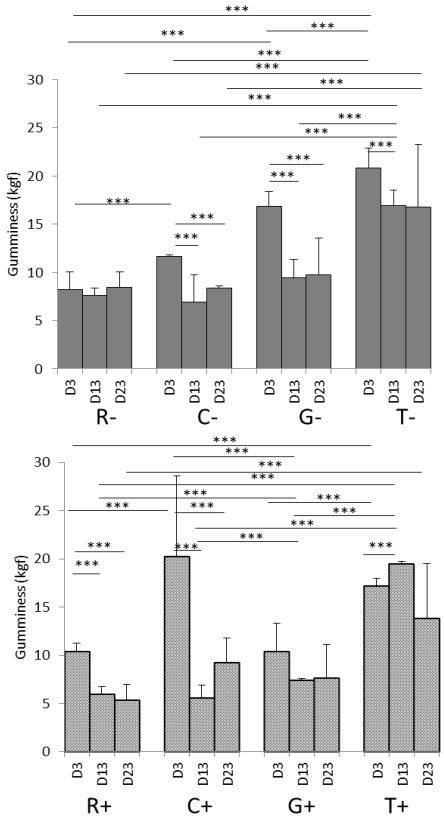

Fig. 9. Gumminess of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R) used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented; * *P<0.05*; ** *P< 0.01*; *** *P<0.001*.

The elasticity is the rate at which a deformed material returns to its original form after the deforming force is removed (Civille and Szczesniak, 1973). The elasticity of cheeses increased significantly from D3 to D13 (*P<0.05*) and stayed at the same value from D13 to D23, except for: (i) T+ for which the elasticity had the same value from D3 to D23 (*P<0.01*), (ii) C+ for which the elasticity increased significantly from D3 to D13 (*P<0.001*) and decreased significantly from D13 to D23 (*P<0.01*) and (iii) G+ for which the elasticity did not change during the ripening time. Without NaCl, the elasticity decreased when trypsin hydrolysate was added to the cheese, regardless of the time of hydrolysis (*P<0.05*). For the other days of ripening and the other cheeses, there was no difference concerning their elasticity. With NaCl, the elasticity at the beginning of the ripening was higher than R+ only for G+. The elasticity of T+ at D13 became weaker than the elasticity of R+ whereas at D23, the elasticity of all cheeses was weaker than the R+ cheese (*P<0.001*). At D3 and D13, the elasticity of G cheese increased with the presence of NaCl (*P<0.05*).

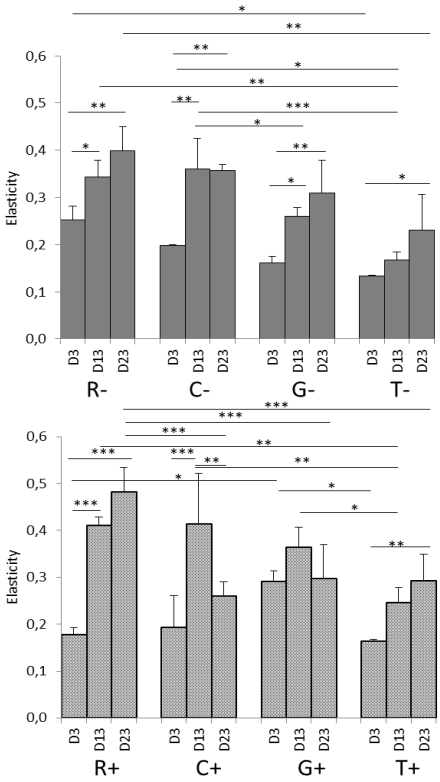

Fig. 10. Elasticity of cheeses at 3 (D3), 13 (D13) and 23 (D23) days of ripening. Cheeses are manufactured with ultrafiltrate retentate (R) used as a control, and with 15% (w/w) sodium caseinate (C), Trypsin hydrolysate (T) and Glu-C hydrolysate (G), with (+ in light grey) or without (- in dark grey) NaCl. The mean and SEM are represented; * *P<0.05*; ** *P< 0.01*; *** *P<0.001*.

Table II: Comparison of cheeses containing NaCl (+) with cheeses without NaCl (-).

Cheese + /- NaCl	Hardness 1			Hardness 2			Stickiness			Rupture strength (N)			Gumminess			Elasticity		
	D3	D13	D23	D3	D13	D23	D3	D13	D23	D3	D13	D23	D3	D13	D23	D3	D13	D23
C+/-			0 ***				0.04*		0.00114**			0 ***	0 ***					
G+/-	0 ***	0 ***		0 ***			0.0001***	0.0155*		0 ***			0 ***	0 ***		0.012*	0.038*	
R+/-	0 ***		0 ***				0.0073**	0.0073**		0 ***		0 ***		0 ***				
T+/-	0 ***	0 ***		0.009**	0.019*			0.0001***		0 ***			0 ***	0 ***				

Principal Component Analysis on MMV Cheeses

Principal component analysis allowed us to summarize the information in two dimensions, accounting 65.65% of the total variation. Figure 11A shows a correlation circle of the variables and Figure 11B represents the scores of the individuals. Principal component 1 explained most of the variation (41.66%) and was related to cheese texture (Figure 11B). The different cheeses were separated according to the principal component 2, which represents 23.93% of the total variation and was related to the cheese aging (Fig 11B). The young cheeses scored negatively and were correlated with the cohesiveness (Figure 11B). The old cheeses scored positively and were well correlated with the proteolysis (Figure 11A). Cheeses made with Trypsin hydrolysate, with or without NaCl, were well separated from the other cheeses and were correlated with a high proteolysis, a high gumminess and a high rupture strength. A linear trajectory of cheese evolution was found for T cheeses whereas for the others the evolution changed during 13 and 23 days of ripening.

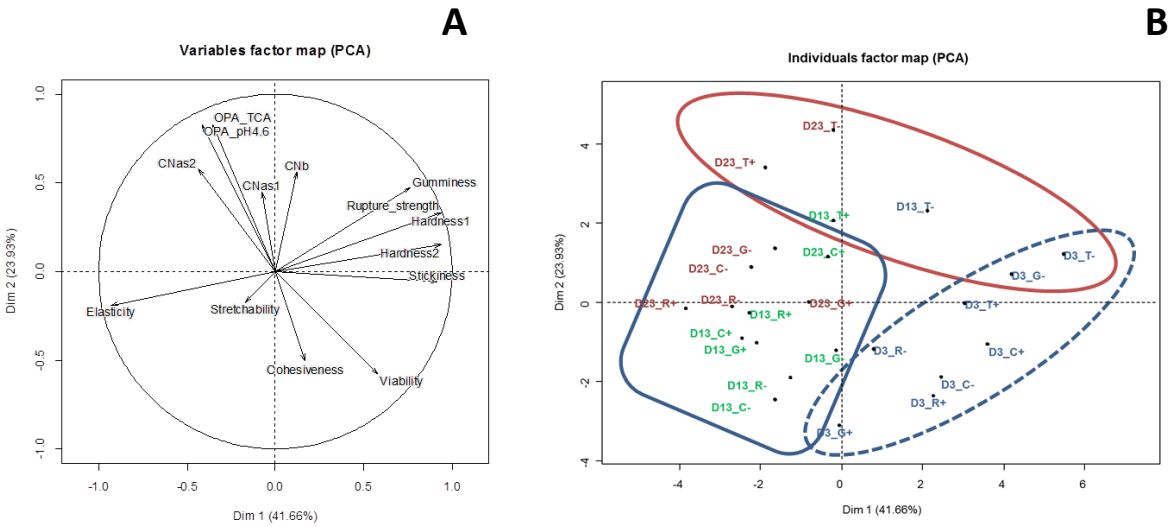

Fig. 11. Characteristics of cheeses manufactured with retentate (R), sodium caseinate (C), hydrolysate produced from Trypsin (T) and hydrolysate produced from Glu-C (G), with (+) or without (-) NaCl at day 3 (D3), day 13 (D13) and day 23 (D23) of ripening. A: The characteristics are represented on the Principal Component Analysis variables factor map (dim 1 = 41.66% of the total variability and dim 2 = 23.93% of the total variability). B: Cheeses are represented as individuals and viability, residual percentage of α_{s1}-casein (CNas1), residual percentage of α_{s2}-casein (CNas2), residual percentage of β-casein (CNb), parameters of texture profile analysis proteolysis (OPA_TCA; OPA_pH4.6) and stretchability.

DISCUSSION

Cheese is a continuous process of proteolysis involving several enzymes from milk, added as coagulant and added with the microbial flora. In previous studies, the nature of the peptides produced has shown to deeply influence the changes in the textural and techno-functional properties. Thus, hydrolysis of caseins increased softening and melt but decreased stretch of cheese (Lucey et al., 2003). The case of α_{s1}-casein-derived peptide, α_{s1}-CN(f24-199) is a well-known example of the cheese softening during ripening of various soft, semi hard and hard cheese varieties (Upadhyay et al., 2004). The production of hydrophobic peptides of various length were also shown to participate in stretching properties of Emmental cheeses (Lacou et al., 2014; Richoux et al., 2009; Sadat-Mekmene et al., 2013).

There is considerable information on proteolysis in several cheese varieties but there is still limited knowledge on the mechanisms by which enzymatic hydrolysis of casein influences texture (Gagnaire et al., 2001). Actually, the huge number of peptides produced in cheese throughout ripening still raises the question of their impact in the texture and techno-functional properties of cheese. As an *in situ* hydrolysis is difficult to control, the addition of hydrolysates directly at the beginning of the cheese making was preferentially used in our study. Therefore, we chose to reincorporate defined groups of peptides arising from the action of Trypsin and Glu-C on sodium caseinate in order to be able to control the nature and the quantity of the added peptides. In that sense, the choice of cheese making type was crucial to keep the peptides within the casein network and that is the reason why the liquid pre-cheese method (MMV) was selected. The usual draining step was actually replaced by casein micelles pre-concentration step by membrane filtration prior to cheese making and the addition of NaCl directly in the cheese milk and not on its surface was possible.

The replacement of 15% of casein by Trypsin and Glu-C hydrolysates in the ultrafiltrate retentate, was clearly sufficient for modifying the texture of the cheese and the stretching properties. The addition of casein hydrolysates also differently affects the proteolysis. The DH of Trypsin and Glu-C hydrolysates was mild and reached 4.5 and 2 % respectively in order to obtain hydrolysates with both native proteins and mainly large peptides, as it is known that extensive hydrolysis providing a high content of free amino-acids and oligopeptides led to weaken textural and techno-functional properties (Singh and Dalgleish, 1998). The hydrolysates produced initially with Trypsin or with Glu-C were different in terms of both composition and structure (Lacou et al., chapters 2 and 3), due to the cleavage sites after lysine and arginine residues for Trypsin and after aspartic or glutamic residues for Glu-C. They provided a different peptide profile of the cheeses as shown by electrophoresis (Figure 2). Among the peptides produced, there were phosphorylated peptides with longer length chain and

peptides with terminal end positively charged in presence of Trypsin whereas peptides were negatively charged in presence of Glu-C. Such an exposure of phosphoryl groups renders caseins more electronegative and can modified functional properties of cheeses (Medina et al., 1992) as observed in terms of hardness, elasticity and stickiness between the T cheeses and G cheeses. This could also have changed the microstructure of cheese which was already shown different in presence of native caseins or phosphorylated peptides (Li-Chan and Nakai, 1989). Such a microstructure with expanded casein micelles was observed on gels in which the Trypsin and Glu-C hydrolysates were added (Lacou et al., 2016). Caric and Kalab (Caric and Kalab, 1993) also showed that the presence of peptides disturbed the compact and continuous casein matrix and could explain why the hardness decreased according to the time of ripening in this study.

As the number of glutamine residues is greater in caseins than that of the lysine and arginine ones, the size of the peptides would have been smaller in the Glu-C hydrolysates if the activity of the enzyme were equivalent to that of the Trypsin. However, the content in residual caseins were higher in Glu-C hydrolysates, explaining partly why the G cheeses were closer to the control and the caseinate supplemented cheeses. We can also supposed that the proteolytic activity of the starter was more influenced by the presence of peptides from both hydrolysates and NaCl than from the caseinate and control, by having a higher lysis of cell during ripening as shown by protein bands of molecular weight higher than 30 kDa that appears during ripening time in T+ and G+ cheeses and not in other cheeses. However, the higher initial content in peptides from Trypsin hydrolysates was sufficient enough to clearly differentiate T cheeses from the G cheeses in terms of proteolysis indices in the respective cheeses.

MMV cheeses manufactured with Trypsin hydrolysate showed a more intense proteolysis than the other cheeses in presence or in absence of NaCl, and they were also qualitatively different by releasing higher large peptides exemplified by twice higher soluble peptides in the pH 4.6 soluble fraction compared to the 12% TCA soluble one. Doubling the values of pH 4.6 soluble fraction during ripening was in agreement with previous studies on Saint Paulin-type cheeses (Kfoury et al., 1989). Peptides originating from the hydrolysis by Trypsin seem to contain higher well-exposed groups hydrolysable by the starter and the rennet. Moreover, the presence of NaCl changed the ionic strength of the cheese as the repulsive forces between peptides are diminished and in turn could have improved the proteolysis. Moreover, the T cheeses had also a complete different texture at the end of the ripening. Actually, at D3, all cheeses were heterogeneous as shown by their projection on the PCA map in terms of proteolysis extent and textural properties. Two groups were clearly distinguishable: the one with cheeses containing Trypsin hydrolysate and the others containing Glu-C hydrolysate, caseinate or the control cheese. Cheeses made with T, with or without NaCl, had a

higher proteolysis and were highly sticky, firm and had a high gumminess value whereas they were relatively inelastic. R, C and G cheeses had lower extent of proteolysis than T cheese and were correlated with a high elasticity, a weak firmness and a high cohesiveness. Without NaCl addition, the hardness of cheeses increased when hydrolysates were present compared to the control cheese. This could be due not only to the increase in peptide content which allowed more peptide-protein or peptide-peptide interactions and formed a firmer protein network but mainly due to the type of the peptides present that led to differential texture properties. Such various textural behaviors were also showed on other studies containing different types of hydrolysates. Thus, addition of whey protein hydrolysates decreased the hardness of processed cheese (Mihulova et al., 2013) while increase in proteolysis by exogenous commercial proteases during Cheddar cheese ripening was correlated with increase in hardness and adhesiveness (Lin et al., 1987) and with a decrease in cohesiveness and springiness in raw goat cheeses (Delgado et al., 2011). Dave and Shah (1998) showed that the supplementation of yoghurt with hydrolysate of caseins renders yoghurt as firmer as the control and with a regular network containing smaller pores than the control. This was due to the generation of peptides with lower molecular weight than caseins which can penetrate into the matrix, declined its firmness due to weakening of protein-protein interactions (Dave and Shah, 1998; Dimitreli and Thomareis, 2007).

Although the behavior of the cheese varied with the addition of NaCl in cheeses, the overall texture was slightly changed in contrast to the stretching properties that were completely cancelled out in cheese with NaCl. This can be due to the direct addition of NaCl in retentate prior to cheese making. Besides changing the ionic strength, NaCl changed mineral balance in the casein micelle network by replacing the calcium ions that interact both with phosphoserine residues of casein and inorganic phosphate. Therefore, this weakened the interactions between caseins and phosphate nanoclusters that are the cross-linker of the casein micelle structure and increased the hydration of the caseins (Gaucheron, 2005). Neither the addition of caseinate or hydrolysate was able to counteract the withdrawal of calcium and to keep the caseins linking to each other enough to form strands. This is quite different with other cheeses for which the salting is performed by brining and the diffusion of salt progressive in cheeses and that the stretching was not so affected by salting (Guinee, 2004).

The NaCl content also influenced the elasticity only for G cheeses that was increased when salt were added and gumminess of C, G and T cheeses at D3, R, G and T cheese at D13 and R at D23. The addition of NaCl increased the gumminess of C and G cheeses at D3 and T cheese at D13. The addition of NaCl has changed the balance between the electrostatically repulsive forces and the hydrophobic attractive forces. At low ionic strength, casein molecules were individualized with

electrostatic repulsions between themselves (HadjSadok et al., 2008). High sodium concentration leads to a decrease in electrostatic repulsions between caseins, by shielding their negative charges.

CONCLUSION

In this study, we showed that the nature of peptides and their quantity greatly impact the hardness, the stickiness, the gumminess, the rupture strength and the elasticity of cheeses. This impact was dependent on the ionic strength and certainly on the way the NaCl is added during cheese making process. We also underlined the importance of rethinking proteolysis phenomenon according to the cheese process and the manufacture conditions. In the light of the actual knowledge, the addition of hydrolysates at the beginning of the cheese manufacture could be a promise and beneficial way to change texture and to have a more controlled proteolysis during ripening. Varying the content of peptides would be able to provide information on the limits of proteolysis extent needed to enhance textural and techno-functional properties of cheeses. Moreover, a sensorial study could be established in order to get the flavor perception of the peptide production.

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Part IV - General Discussion & Perspectives

Highly concentrated-protein systems are fundamental elements of life encountered in cellular phenomena since thousands of years that can also be extended to food products and the food transformation process. Nevertheless, the interest for the fundamental aspects of the interactions between proteins under high concentration conditions is relatively recent and there is a substantial increase in the number of published paper on this topic for 10 years. One possible way, developed in this thesis, to improve some functionalities of proteins in dilute systems as well as in concentrated ones is the modification of the protein structures by enzymatic hydrolysis that further impacts the texture and the microstructure of the food systems.

This thesis can be seen as an exploratory study on various scales of dairy matrix complexity. The objective was to deepen knowledge on how defined groups of peptides were able to modify casein micelles organization and their textural properties. In a long term view, enlarging the potential use of peptides as functionalized ingredients is expected. The strategy adopted in this work was to incorporate targeted peptides in different dairy systems, from dilute and model systems to highly concentrated and real food product, in order to be able to modulate the rheology, the texture and the microstructure of all these systems under different pH conditions.

As discussed in the review, peptides are present in food under the form of hydrolysates containing both peptides and native proteins. Regardless of the functional properties studied, it is still difficult and sometimes even impossible to conclude that changes in functional properties are only due to either (i) the nature of the peptides produced and the way they interact together, or (ii) to the presence of both residual native proteins and peptides and to their respective interactions in the hydrolysate. And the present work attempted to clarify this point. Actually, it is therefore crucial to have information on the type of peptides present, their quantity and their physico-chemical characteristics, in order to be able to know how and to what extent peptides can change the functional properties of proteins.

The main results of this thesis were summarized in the Table 1.

Table 1. Highlights of each chapter of this thesis.

Chapters	**Main results**
Chapter 1 An *in silico* approach to establish a relationship between a techno-functional property of a dairy-matrix and a peptide profile.	- Proteolysis was strongly correlated with the stretchability of Emmental cheese. - The type of protease affected the intensity of the cheese stretchability. - Peptide identification explained the modulation of the cheese stretchability. - A group of peptide was related to a macroscopic property rather than a unique peptide.
Chapters 2 & 3 Properties of milk peptides in liquid or gel casein matrices	- Slightly modifying the time of hydrolysis of caseins or the nature of peptides produced led to a great variation and a high diversification of the rheological properties. - Modification of the quantity of peptides in the hydrolysate impacted the microstructure and the gel formation rates. - High implication of peptides in the reinforcement of the structuration of the matrices up to a limit beyond which the rheological properties were worsened depending on: (i) the type of peptides produced, (ii) their proportion in the hydrolysate and hence in the matrices and (iii) the overall concentration of the dairy systems in which hydrolysates were added. - A non-linearity of the phenomena was observed as interactions existed between the nature of peptides, their proportion and the concentration of the dairy systems used. - Smaller and denser casein micelles observed at the microscale. - Incorporation of hydrolysates drastically change the casein micelle texture by decreasing gel hardness regardless of the concentration used. - There were few differences between the type of hydrolysates T and G in changing the texture properties at pH 4.6. - Predominant factor impacting the gel texture was the

	quantity of residual native caseins present in the caseinate.
Chapter 4 Structural changes of caseins and peptides under a high concentration	- First establishment of the equation of state for peptides and the mix peptide:casein micelles at ratio 1:1 for one tryptic hydrolysate T3. - Gel peptide samples were observed in the range 5 – 10 bars of the osmotic pressure. - The osmotic stress technique permits to study peptide interactions in highly concentrated suspensions reached in fermented milk products such as cheeses. - Hydrolysis of casein samples takes place within the first week of equilibration, hence it was a challenge to keep the integrity of the casein micelles during the time required for the sample to equilibrate to the requested osmotic pressure. - The equation of state of casein-derived peptides showed that osmotic pressure of peptides was largely higher than that observed for the caseins micelles.
Chapter 5 Incorporation of defined peptides in semi-hard model cheeses	- The nature of peptides and their quantity greatly impacted the hardness, stickiness, gumminess, rupture strength and elasticity of cheeses. - The presence of NaCl changed the cheese texture and namely the stretching capability, and increased indirectly the proteolysis indices by inducing higher bacterial cell lysis and release of active intracellular peptidases in cheese. - The addition of hydrolysate at the beginning of the cheese manufacture could be a promise and beneficial way to change texture during ripening of pre-concentrated MMV cheeses.

This thesis projects was a multi-level challenging task. First, it involved multidisciplinary approaches and at multi-scales from molecular to microscopic and macroscopic experiments and from model systems to food products. Second, among the issues raised in the introduction section, there were three main questions that were addressed throughout the different chapters as detailed below.

First question – part a: would it be possible to relate *in silico* the physico-chemical characteristics of peptides and one peculiar techno-functional property of food product?

The *in silico* approach was actually a relevant tool to link a peptide profile to a textural property of a cheese. This approach permitted to confirm that the degradation of caseins during the cheese ripening was responsible for the modulation of the cheese texture (Lucey et al., 2003). This method showed that a profile of peptides was linked to a macroscopic property rather than to one particular peptide. The peptides which were selected had very different physico-chemical characteristics and contained random coils or α-helix structures. The innovative approach was that this work was based on a food product and not on a model dairy system, in order to face the overall complexity of the system and to exhibit the most prominent factors influencing the appearance of a specific texture property, in the present case the stretching property according to the ripening time. Moreover, a combination of statistical methods and the identification of peptides in the food product, which is hardly seen in the literature, had permitted to show the direct correlation between some peptide characteristics and the stretchability of cheese.

This first step helped us finding ways to select peptides that can relate groups of peptides and peculiar textural properties and such an approach can be applied to other matrices and other kinds of functional properties.

However, this study had some limits since the mass spectrometry method of identification used to initially collect the data was based on the presence/absence of the peptide in the sample. Such a qualitative difference between samples did not permit to put weight on each peptide and to quantitatively relate peculiar characteristics to the given cheese texture. New methods of quantification was applied in the analysis of the samples by mass spectrometry performed thereafter.

First question – part b: would it be possible, in turn, to tailor hydrolysate(s) that can be used to modify texture of a complex casein matrix?

Following the *in silico* study, peptides with defined physico-chemical characteristics were selected and two enzymes were chosen to efficiently produce similar ones. The production step needed to adapt hydrolysis conditions from small-sized (few milliliters) to scale up the production mode to liters ans this was successfully obtained.

Second question: what is the impact of the physico-chemical and structural characteristics of these designed peptides on the rheological and the structural properties of highly concentrated casein matrices in a liquid or gel form?

The characterization of these peptides indicated that when caseins were hydrolyzed, α-helices were formed as shown in the *in silico* study and in the literature on single peptides experiments as reviewed in the bibliographic part of the manuscript. Such a α-helix formation was observed in the hydrolysates alone and they were kept when the peptides were mixed with casein micelles suspensions conferring slight but significant changes in secondary structure of the overall mixed dairy system. Consequently, the hydrolysis of unstructured proteins can generate some conformational reorganization and even a certain structuration of the peptides altogether and in the presence of other proteins.

These peptides were identified and deeply characterized in terms of distribution of peptides and residual casein in the hydrolysates and the types of peptides present within; the amount of peptide reincorporation in the casein micelle suspensions was controlled, contrary to many studies used a degree of hydrolysis as exemplified in the review. We hypothesized that among the peptides produced those having predominant characteristics, highlighted in clusters based on their respective mass spectrometry intensity, corresponded to peptides highly involved in the conformational and /or textural changes observed. This does not preclude that peptides present in small quantity were able to change the food system texture. Actually, ionization of peptides in the mass spectrometer is not always associated with the amount of the peptides present.

The incorporation of these peptides in casein micelles suspensions at different concentrations, different hydrolysate:casein micelle suspension ratios and different pH generated changes in rheology and texture of these suspensions in a more or less unexpected manner. In some cases, only 25% of peptides were needed to change the characteristics of the mixed systems whereas in other cases depending on the nature of hydrolysate and the pH used, more than 75% of peptides were required.

Interactions were highlighted between the nature of peptides, their quantity, the ratio hydrolysate:casein micelles and the time of hydrolysis, rendering non-linear the consequences observed in the rheology or texture of systems under various conditions of concentrations. Moreover, the systems were complex as they contained a high concentration of hydrolysates, caseins and many peptides, rendering difficult to find the contribution of one particular factor to the modification of the rheological properties.

Third question: are the same phenomena found from a model solution to a concentrated matrix including food products, as exemplified in a cheese model system?

Real cheeses are much more heterogeneous systems than model systems and had very varied microstructures. It was shown that the impacts observed in model systems were non-linear as for

real food products. Nevertheless, we showed that only 15% of hydrolysates added at the beginning of the cheese production were required to induce changes of the final texture. The peptides present in cheeses seem to change the overall texture profile in a similar way throughout the ripening time for the T hydrolysates and differentially for the G hydrolysates, providing two completely different cheeses.

Interest of this work for the academic and industrial plans

The results obtained in this thesis may interest the milk sector but also the domain of food science.

This project contains, at least, two scientific and technological fallouts. Firstly, it permits to determine what kinds of peptides are of interest and offers clues to control the *in situ* production of functional peptides in terms of quality and quantity. This control is however at the present time difficult to be definitely reached. It can be exerted either by adding specific exogenous enzymes able to selectively produce the targeted peptides or by using bacterial starters with peculiar proteolytic equipment. In the first case the cost of the enzyme and the risk of withdrawal of the enzyme is rather high and in the second case, it is difficult to predict the types of peptides that can be produced as well as the environmental conditions in the product able to induce lysis and to release desired or not peptidases Secondly, generic knowledge was obtained on the interactions between peptides and caseins that are the basis of the techno-functional properties by identification of the molecular, physico-chemical and structural factors involved in. Focus has been paid on (i) the composition in caseins and peptides and their organization in milk matrices with different structures and (ii) on the relationship between the nature of peptides produced during the process of transformation such as the ripening time and their role in the acquisition of these properties.

On the industrial and technological plans, new technologies of preparation of milk matrices with specific techno-functional properties would be developed from the results presented here, either from traditional fabrications of milk products or by creating new ones or new ingredients.

Perspectives

In the next stages of this study, it will be interesting to study more deeply the interactions between the peptides and the native caseins. By depleting the peptides-casein matrices with various solvents, chelating and chaotropic agents it would be possible to selectively extract peptides from the matrices and to determine which peptide preferentially interact with caseins. As an example, the extraction of peptides trapped in the gels by Ethylenediaminetetraacetic acid (EDTA) or urea could be used. The EDTA chelates the calcium ions inducing dissociation of casein micelles. Consequently, the peptide-casein matrices could be mixed with EDTA, centrifuged and the supernatant could be analyzed by

SDS-PAGE electrophoresis in order to show the electrostatic interactions. Urea or Sodium Dodecyl Sulfate (SDS) are able to disrupt the hydrophobic interactions. Additionally, it is possible to study the specific role of hydrophobic interactions by changing the temperature of the system in order to increase or decrease these interactions. Such an approach can also be used to determine whether the types of interacting peptides changed according to pH and whether a saturation of certain sites could be reached that could partly explain the non-linear phenomena observed.

It will be interesting to establish how to orientate the rheology and texture through the peptide production. In our study, many parameters changed in the systems: the nature of hydrolysate, the time of hydrolysis, the ratio hydrolysate:casein micelles, the quantity of peptides, the pH and the concentration of the systems, which interacted themselves rendering difficult the understanding or the mechanisms involved. Therefore, it will be interesting to simplify the casein-peptide systems and to select peptides with the same characteristics to show how one characteristic can affect or not the rheology and texture of the mix peptides:caseins. Such a simplification could be obtained by separation and purification of peptides according to their size by gel filtration, their charges by ion exchange chromatography, their hydrophobicity by preparative reverse phase chromatography or their content in phosphoserine residues by selective precipitation, considering as a non-required condition to know from which type of casein they came from. Another simplification lies in the casein part by using purified casein as substrate to produce peptides and to determine whether one type of casein can preferentially produce peptides of peculiar interest.

Having established for the first time the method for the concentration of peptides by osmotic pressure, it clearly appeared that higher concentrations of pressure have to be found to reach closer packing of the peptides that could reveal mechanism of molecular peptide self-assembly under liquid-solid phase transition. Using the equation of state obtained in this study, the osmotic stress technique opens new ways to study peptide interactions in highly concentrated suspensions that can be reached in fermented milk products such as cheeses.

Finally, the hydrolysis should be realized *in situ*, directly in the model systems and in the food systems at different concentrations, in order to show if a controlled hydrolysis has the same impact than the addition of hydrolysates. Moreover, in some food products, as yoghurt, the addition of hydrolysates is easier than in cheese, in which the addition of hydrolysates is only possible at the beginning of the production. Consequently, some new concept of cheese manufacture should be investigated further.

In the case of the cheese experiments no bitterness was observed when the 15 % hydrolysates were added (data not shown). However, other studies with sensorial tests have to be done to estimate which amount of peptides can be incorporated to improve the textural properties while keeping the appropriate organoleptic quality. In conclusion, to improve the cheese-making properties and final cheese quality, it is suggested that future research have to go on further towards control of the *in situ* hydrolysis.

Thesis outputs – Valorisations, formations, comités de thèse et de pilotage

Oral presentations

Lacou, L., Pezennec, S., Lê, S., Gagnaire, V., Relationship between a techno-functional property of cheese and milk protein derived peptides: an *in silico* approach. 15th Food Colloids conference, from 13 to 16 April 2014, Karlsruhe (Germany).

Lacou, L., Famelart, M.H., Lê, S., Jardin, J., Briard-Bion, V., Pezennec, S., Gagnaire, V., Rheological properties of milk derived peptides in concentrated matrix of caseins. The 7th International Symposium on Food Rheology and Structure(ISFRS 2015), from 7 to 11 June 2015, Zurich (Switzerland).

Poster with 5 minutes of presentation

Lacou, L., Richoux, R., Gagnaire, V., A review on functional properties of peptides: from dilute solutions to food products**.** The 17th world congress of food science and technology (IUFoST), from 17 to 21 August 2014, Montreal (Canada).

Posters

Lacou, L., Famelart, M.H., Lê, S., Jardin, J., Briard-Bion, V., Perreira Caldas Silveira, A., Pezennec, S., Gagnaire, V., Rheological properties of milk derived peptides in concentrated matrix of caseins. The 12th International Congress on Engineering and Food (ICEF12), from 14 to 18 June 2015, Quebec (Canada).

Lacou, L., Famelart, M.H., Lê, S., Jardin, J., Briard-Bion, V., Pezennec, S., Gagnaire, V., Rheological properties of milk derived peptides in concentrated matrix of caseins. The International Dairy Federation World Dairy Summit (FIL-IDF), from 20 to 24 September 2015, Vilnius (Lituania) – **Best poster award: third place.**

Articles

Lacou, L., Lê, S., Pezennec, S., Gagnaire Soumet, V. (2015). An *in silico* approach to highlight relationships between a techno-functional property of a dairy matrix and a peptide profile. Colloids and Surfaces A Physicochemical and Engineering Aspects, 475, 44-54. DOI: 10.1016/j.colsurfa.2014.10.052.

Lacou, L., Famelart, M.H., Cauty, C., Kolotuev, I., Jardin, J., Briard-Bion, V., Lê, S., Pezennec, S., Gagnaire. V., Rheological and structural properties of milk peptides in high concentrated casein matrices. In writing process.

Lacou, L., V. Lechevalier, Jardin, J., Briard-Bion, V., Lê, S., Pezennec, S., Gagnaire. V. Textural and structural properties of milk peptides in high-concentrated casein matrices. In writing process.

Lacou, L., Richoux, R., Frogerais, L., Murdin, M., Gagnaire, V., Textural properties of model cheese containing milk peptides. In writing process.

Review

Lacou, L., Léonil, J., Gagnaire, V. (2016). Functional properties of peptides: from dilute solutions to food products. Food Hydrocolloids, 57, 187-199. DOI: 10.1016/j.foodhyd.2016.01.028.

Lélia LACOU

Bourse de thèse région Bretagne
région Pays de Loire - Bba
•
2013-2016

UMR INRA - Agrocampus Ouest
Science et Technologie
du Lait et de l'Œuf

Equipe MICRO
Microstructure fromagère
et Bactéries

Mots-clés
Peptide
Protéine
Structure
Protéolyse
Viscoélasticité

Les peptides: quelles caractéristiques physico-chimiques et structurelles modulent la viscoélasticité des matrices laitières riches en caséines?

Contexte socio-économique

Forte concurrence entre industriels de la filière agroalimentaire

↓

Nécessité d'innover

↓

Développement de nouvelles formulations

↓

Maintien des propriétés texturantes de l'aliment

Contexte scientifique

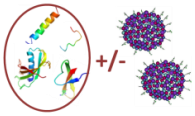
- Les protéines possèdent des fonctionnalités particulières: gélifier, former et stabiliser des émulsions et/ou des mousses, former des fibres, etc.
- Une hydrolyse limitée des caséines favorise certaines de ces fonctionnalités selon les caractéristiques physico-chimiques et structurelles des peptides produits.
- Dans le contexte laitier, une corrélation positive entre la présence de peptides hydrophobes contenant plus de 20 acides aminés et le filant de l'emmental (incluant des propriétés viscoélastiques à chaud) a été établie.

Question de recherche

Quelles interactions caséines - peptides modulent les propriétés viscoélastiques des matrices laitières?

Approche *in silico* → Production d'hydrolysats contenant les peptides cibles avec/sans les caséines intactes → Validation du rôle des peptides dans la viscoélasticité des matrices laitières riches en caséines

Via l'exemple du filant de l'emmental: sélection de peptides cibles



Ajout d'hydrolysats

Solution de caséines diluée — *Caséines concentrées* — *Gel acide de caséines*

Matrices de complexité croissante

- **Etude rhéologique des matrices** (avec ou sans hydrolysats)
- **Etude microscopique des matrices**: comprendre l'organisation des caséines et des peptides
- **Etude moléculaire**: identifier les interactions entre peptides et caséines

Résultats attendus

1/ Prédire *in silico* quels sont les peptides qui participent au filant de l'emmental et déterminer leurs caractéristiques physico-chimiques et structurelles.

2/ Valider l'étude *in silico* via la production de ces peptides cibles et leur ajout en matrices laitières riches en caséines.

3/ Comprendre l'organisation des caséines et des peptides et leurs interactions dans chacune des matrices laitières.

Perspectives

- Produire les peptides cibles *in situ*: dans une matrice laitière riche en caséines puis dans un fromage.
- Diriger l'hydrolyse *in situ* afin d'obtenir la texture alimentaire désirée.
- Identifier le mécanisme d'assemblage des peptides et des protéines qui module la viscoélasticité d'une matrice.

Financeurs

Collaborateurs

A review on functional properties of peptides: from dilute solutions to food products

Lélia Lacou[1,2], Romain Richoux[3], Valérie Gagnaire[1,2]

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BACKROUND AND OBJECTIVES

- **Partial hydrolysis improves functional properties** of proteins, especially solubility, emulsion and foaming capacity.
- Many data explain the relationships between structure and functional properties of peptides in dilute solutions. It is difficult to apprehend these relationships in food products (i.e. cheese, meat, bake products) because:
  - ✓ Food products have a complex composition
  - ✓ Involvement of many interactions and diffusion-limited reactions
  - ✓ The action of proteases and their kinetics are complexes in such concentrated media
- The **challenge** of food scientists is to control the peptide production *in situ* to modulate their functional properties in food products.

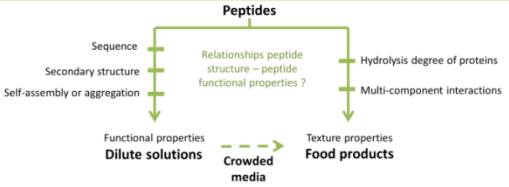

Fig 1. Relationships between peptides and their functional properties in (i) dilute solutions and in (ii) food products.

This review summarizes the main ways of possible improvement to transpose the relationships between peptide structure and peptide functional properties from dilute solutions to food products.

PEPTIDES IN DILUTE SOLUTIONS

❖ Sequence has a key role on the secondary structure of peptides and on their supramolecular structure but doesn't always explain their final functional properties.

		Structure II in dilute solution			Nano-structure	Gel formation		
Peptide sequence	Modifications	Acidic pH	Neutral pH	Basic pH	pH 10	pH 2	pH 6	pH 10
LIVTQTMK	Original milk peptide	Random coil	Random coil	β-sheet		-	-	+
LLIVTQTMK	Increase of the N terminal hydrophobicity	β-sheet	β-sheet	β-sheet		+	+	-
LIVTQTMR	Change of chain pKa	Random coil	Random coil	β-sheet		-	-	+
AcLIVTQTMK	N terminal blocked	β-sheet	β-sheet	β-sheet		-	+	+

Fig 2. Impact of peptide structures on their gelation capacity. Adapted from Guy & Voyer (2012).

Size, **hydrophobicity** and **charge periodicity of peptides** are **main characteristics** to induce **self-assembly** (Subbalakshmi et al., 2012; Chen, 2005; Loo et al., 2012) and consequently to induce **functional properties**.

PEPTIDES IN DILUTE SOLUTIONS AND IN FOOD PRODUCTS

❖ If relationships between peptide structure and their functional properties begin to be established, these relationships are not clearly established in food products yet .

Neutrase casein hydrolysate, with upper functional properties in dilute solutions, is incorporated into wheat bread as potential functional ingredients.

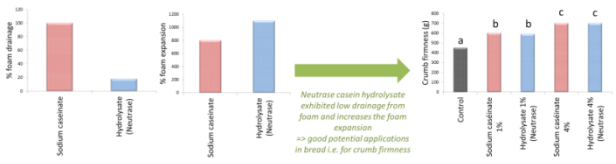

Fig 3. Effect of casein hydrolysis on foam drainage and foam expansion in dilute solution and on crumb firmness of bread. Data in each column annotated by the same letter denote mean values that are not significantly different (P<0.05). Adapted from Crowley et al. (2002).

The Neutrase casein hydrolysate, that contains **high molecular weight peptides**, had **worse potential applications in** the development of protein-enriched **wheat bread** than the control bread.

PEPTIDES IN FOOD PRODUCTS

Complex and variable composition changes:
- the macromolecular structure of the matrix
- the conditions for which the peptides self-assemble or aggregate, and consequently the final functionality of the peptides inside the food matrix.

Protein hydrolysates are either produced *in situ* or added as an ingredient in the food product, leading to more or less controlled final properties.

Peptide structure - function in food products

The enzyme specificity, the protein nature and the hydrolysis degree of protein impact the functional properties of hydrolysates.

The studies on food products are more empirical and less mechanistic than the studies on dilute solutions. Most of publications established relationships between: a degree of hydrolysis of protein and their functional properties (Sodini et al., 2005; Hemantha Kumar et al., 2001;)

Few publications established relationships between peptide profile and texture of a food product (Sadat-Mekmene et al., 2013, Borsting et al., 2012; Brickley et al., 2007; Creamer & Olson, 1982).

What is known in the literature

There is a huge gap between the knowledge acquired on the self-assembly of proteins and peptides in dilute solutions, where mechanisms were well apprehended, and the knowledge in food products.

Hydrolysis degree of protein is an insufficient parameter to establish relationships between peptide production and the enhancement of protein functional properties.
There is a lack of peptide identification in many studies.

What is lacking in the literature

Fig 4. Food product is more complex than dilute solution. In most publications on food products, the action of enzyme was studied. To establish relationships between peptide structure and peptide functional properties, it is necessary to take an interest on peptides themselves and on their physico-chemical and structural characteristics.

CONCLUSIONS

- The knowledge on **relationships peptide structure – peptide functional property** are established in **dilute solutions** but **not in a real food product**. Responsible agents of functional properties are difficult to define in food products so much the composition is complex. Direct correlation between functional properties in dilute solutions and those observed in the food products is still a challenge.
- Research should be developed on more **concentrated systems**, such as **crowded media**, to close this gap.
- Advances in **identification and structure determination methods of peptides** in food products will help (i) to bring a **comprehensive view of the food product** and (ii) to understand **how peptides can modulate food texture**.

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RHEOLOGICAL AND STRUCTURAL PROPERTIES OF MILK PEPTIDES IN HIGH-CONCENTRATED CASEIN MATRICES

Lélia Lacou[1,2], Marie-Hélène Famelart[1,2], Chantal Cauty[1,2], Irina Kolotuev[3,4], Julien Jardin[1,2], Valérie Briard-Bion[1,2], Arlan Caldas Pereira Silveira[1,2,5,6], Sébastien Lê[7], Stéphane Pezennec[1,2], Valérie Gagnaire[1,2]

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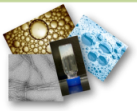
[4]IGDR, UMR6290 University of Rennes 1, 2, F-35043 Rennes, France [5]Universidade Federal de Viçosa, BR-36570 Viçosa, Brazil [6]Scholarship student by CNPq - Brazil
[7]AGROCAMPUS-OUEST, Laboratoire de Mathématiques Appliquées Agrocampus, France

BACKGROUND AND OBJECTIVES

- Partial hydrolysis improves functional properties of proteins.
  Luo et al., 2014; Banach et al., 2013; Hitomi et al., 2012

  ⇒ Is it due to peptides ?
  ⇒ What kind of peptides are involved ?



- Most information comes from the study of dilute solutions whose composition and protein concentration differ greatly from most food products.

Peptides are produced during the milk processing
⇓
Interactions matrice - peptides ?
⇓
Textural changes ?

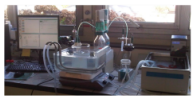
- In complex media, **peptides are usually not identified, hence** and it's difficult to determine how the functional properties of proteins are changed due to their hydrolysis.

i. Does hydrolysis of proteins modify the rheological properties of highly concentrated dairy suspensions?

ii. What interactions are involved?

MATERIALS AND METHODS

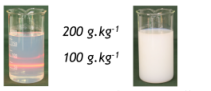
1 Hydrolysis of sodium caseinate followed by pH-stat



Trypsin: T1 T2 T3 — *Time of hydrolysis ↗ Quantity of peptides ↗*
Glu-C: G1 G2 G3 — *Time of hydrolysis ↗ Quantity of peptides ↗*

Hydrolysates	T1	T2	T3	G1	G2	G3
Degree of hydrolysis	2%	4%	5%	1%	3%	4%

2 Preparation of peptide suspension and casein micelle suspension



Hydrolysate Suspension (HS) — Casein micelle Suspension (CMS)

3 Mixing of peptides and caseins at different concentrations

pH 7 and 20°C

4 Rheological measurements

5 Electron microscopy

RESULTS

Gel formation at 200 g.kg-1

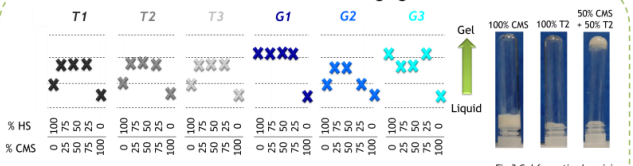


Fig.1 Diagram of properties of CMS +/- HS at 200 g.kg-1, pH 7 and 20°C.

Fig.2 Gel formation by mixing T2 and CMS at 200 g.kg-1, pH 7 and 20°C.

- Differences in viscosity of HS - CMS were observed according to the type of hydrolysate used.
- A highly viscous fluid appeared in presence of hydrolysates in CMS.

Rheological properties

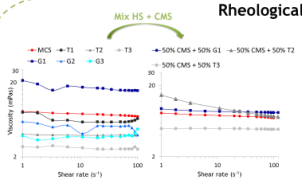


Fig.3 Shear rate dependence of apparent viscosity for CMS, HS and mix CMS-HS at 100 g.kg-1 and pH 7.

- Viscosity of G > viscosity of T
- Viscosity of HS ↘ as hydrolysis ↗
- Viscosity of mix > viscosity HS
- Viscosity of mix < viscosity CMS except T2
- Viscosity of T2 < viscosity of CMS < viscosity of T2 + CMS

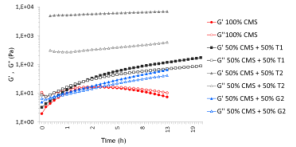


Fig.4 Time dependence of the elastic (G') and loss (G'') moduli for CMS and CMS + HS at 200 g.kg-1and pH 7.

- CMS = liquid at 20°C
- T1 + CMS = became a gel with time
- T2 + CMS = elastic gel
- G2 = gel

Microstructure of casein micelle matrices

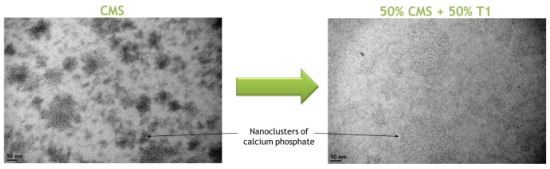


Fig. 5 Electron micrograph of CMS and CMS+ T1 at 200 g.kg-1, pH 7 and 20°C.

- The casein micelles acquired a more expanded structure when peptides were added.
- Nanoclusters of calcium phosphate were still visible.

CONCLUSIONS

- These results showed that peptides modified the microstructure and the rheological behavior of casein micelle suspension, even at low peptide concentrations.
- Differences of rheological properties were highlighted according to the type of hydrolysate used, showing that certain peptides interacted with casein micelles leading to gel formation.

This study will be published in Food Hydrocolloids

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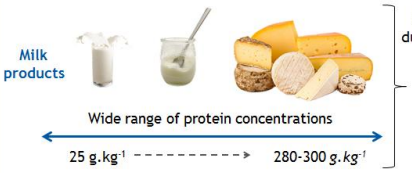
Rheological and structural properties of milk peptides in high-concentrated casein matrices

Lélia Lacou[1,2], Marie-Hélène Famelart[1,2], Chantal Cauty[1,2], Irina Kolotuev[3,4], Julien Jardin[1,2], Valérie Briard-Bion[1,2], Stéphane Pezennec[1,2], Valérie Gagnaire[1,2]

[1]INRA, UMR 1253, F-35000 Rennes, France [2]AGROCAMPUS-OUEST, UMR1253, F-35000 Rennes, France
[3]MRic-TEM, UMS3480 Microscopy Rennes Imaging Center, F-35043 Rennes, France [4]IGDR, UMR6290 University of Rennes 1, 2, F-35043 Rennes, France

Background and objectives

- Partial hydrolysis improves functional properties of proteins.
  Luo et al., 2014; Banach et al., 2013; Hitomi et al., 2012
  ⇒ Is it due to the presence of peptides or due to both proteins and peptides?
  ⇒ How does the nature of peptides change the functional properties of proteins ?



- In complex media, the concentration of peptides and proteins is higher than in dilute solutions and it's difficult to determine how the functional properties of proteins are changed due to their hydrolysis.

i. Does hydrolysis of proteins modify the microstructure of highly concentrated dairy suspensions?

ii. What are the consequences on the rheological properties?

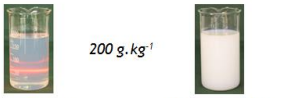
Materials and Methods

Hydrolysis of sodium caseinate (NaCas) followed by pH-stat

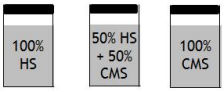
Trypsin: T1 T2 T3 — *Time of hydrolysis ↗ Quantity of peptides ↗*
Glu-C: G1 G2 G3 — *Time of hydrolysis ↗ Quantity of peptides ↗*

Hydrolysates	Degree of hydrolysis
T1	2%
T2	4%
T3	5%
G1	1%
G2	3%
G3	4%

Preparation of peptide suspension and casein micelle suspension



Mixing of HS, NaCas and CMS at different concentrations



pH 7 and 20°C

Electron microscopy

Rheological measurements

Results

Fig.1 Gel formation by mixing HS and CMS at different ratio, at 200 g.kg-1, pH 7 and 20°C.

- Differences in viscosity of HS were observed according to the type of hydrolysate used.
- A highly viscous fluid appeared in presence of hydrolysates in CMS for both HS types used.

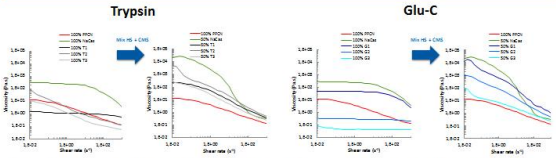


Fig.2 Shear rate dependence of apparent viscosity for CMS, HS and mix CMS-HS at 100 g.kg-1 and pH 7.

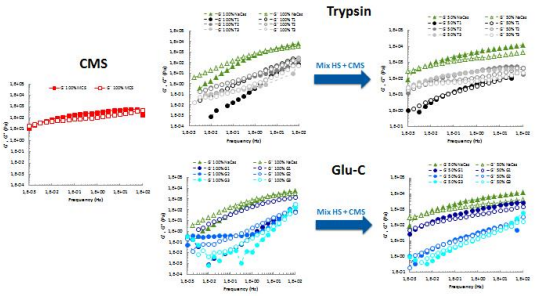


Fig.3 Frequency dependence of the elastic (G') and loss (G'') moduli for CMS and CMS + HS at 200 g.kg-1and pH 7.

- Viscosity of HS ↘ as hydrolysis time ↗
- Viscosity of mix > viscosity HS > viscosity of CMS
- Rheological behavior (viscosity, G' and G") of CMS is modified with the addition of all HS

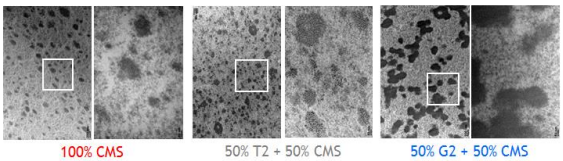


Fig. 4 Electron micrograph of CMS, CMS + T2 and CMS + G2 at 200 g.kg-1, pH 7 and 20°C.

- With T2: the casein micelles acquired a more expanded and disrupted structure.
- With G2: the casein micelles have a structure more compact and aggregated.

Conclusions

- These results showed that all peptides produced with Trypsin and Glu-C modified the microstructure and the rheological behavior of casein micelle suspension.
- Differences in rheological properties and in microstructure were highlighted according to the type of hydrolysate used, showing that certain peptides interacted with casein micelles leading to various gel formation.

Acknowledgments

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LÉLIA LACOU

for Poster Presentation

"RHEOLOGICAL AND STRUCTURAL PROPERTIES OF MILK PEPTIDES IN HIGH-CONCENTRATED CASEIN MATRICES"

Chair of the Dairy Science and Technology Conference Scientific Committee

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Abstract

Food products are matrices of complex and varied composition, among which proteins are the main molecules possessing various functionalities, i.e. foam and emulsion stabilisation, gelation, solubilisation of some components. They led to various final textural properties of the food products. Modification of proteins by mild enzymatic hydrolysis, allowed improving some of the functionalities and in turn changing the structural and rheological properties of the food product. Protein-derived Peptides derived are present in a huge number of food products either by in situ production or by incorporation as an ingredient during food formulation. Questions remains i) whether some general rules can be put forward or not to explain the building-up of the interactions between peptides and between peptides and proteins and ii) the subsequent reorganization of the food matrices and namely dairy ones that has to be done to induce new or modified functional properties in various dairy matrices. The objective of this thesis was to determine how and to what extent casein-derived peptides are able to modify the texture, the rheology and the microstructure of highly-concentrated casein matrices.

We showed i) what kinds of structural and textural changes occurred at various scales from microscopic to macroscopic ones, when we varied the type of peptides produced, their percentage in the dairy matrices and the concentration of the matrices they were incorporated in; ii) interactions between peptides and caseins and the non-linearity of the phenomena explaining some adversely evolutions of the rheological properties of the casein matrices observed.

On the basis of this work, peptides can be considered as building blocks with defined physico-chemical properties that can be chosen to be reincorporated into various dairy matrices of increasing composition and complexity.

Keyword: Peptides, caseins, functional properties, texture, hydrolysis, cheese, concentrated systems, dilute systems

Résumé

Les aliments sont des matrices de composition complexe et variée au sein desquelles les protéines possèdent diverses fonctionnalités, pour stabiliser des mousses ou des émulsions, gélifier, solubiliser certains composés menant à différentes textures finales du produit. Une modification de ces protéines par hydrolyse enzymatique ménagée permet d'améliorer certaines de ces fonctionnalités et donc de changer les propriétés rhéologiques et structurales du produit. Les peptides dérivés des protéines sont présents dans de nombreux aliments soit par production *in situ* soit par incorporation lors de la formulation des produits sous forme d'hydrolysats. Des questions subsistent pour savoir i) si des règles générales d'assemblage existent ou non pour expliquer comment les peptides et les protéines interagissent entre eux et ii) et si une réorganisation se fait au sein des matrices ici laitières pour induire de nouvelles propriétés fonctionnelles ou les modifier. L'objectif de la thèse était de déterminer comment et jusqu'à quel niveau les peptides sont capables de modifier la texture, la rhéologie et la microstructure des matrices à haute teneur en caséines.

Nous avons ainsi pu montrer i) quels changements de structure et de texture avaient lieu à différentes échelles en variant le type de peptides produits, leur proportion et la concentration de la matrice dans laquelle ils étaient ajoutés et ii) les interactions entre peptides et caséines et la non-linéarité des phénomènes expliquant les évolutions parfois contradictoires des propriétés de texture des matrices caséiniques.

Sur la base de ces travaux, on peut considérer les peptides comme des briques élémentaires dont les propriétés physico-chimiques peuvent être choisies en vue de les réincorporer dans différentes matrices laitières de composition et de complexité croissante.

Mots clés: Peptides, caséines, propriétés fonctionnelles, texture, hydrolyse, fromage, systèmes concentrés, système dilués