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Biochemical and enzymological characterization of an isomaltase family in the yeast *Saccharomyces cerevisiae*

Xu Deng

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de Toulouse

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Xu DENG

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Titre :

Biochemical and enzymological characterization of an isomaltase family in the yeast *Saccharomyces cerevisiae*

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SUMMARY

Most enzymatic systems for sugar uptake and assimilation rely on multigene families in the yeast *Saccharomyces cerevisiae*. The *IMA* / *MAL* family has been used as a model system to study the molecular mechanisms that govern evolution of duplicated genes. The five *IMA* multigene family members encode four isomaltases sharing high sequence identity from 65% to 99%, of which *IMA3* and *IMA4* are 100% identical to encode the same isomaltase. In this work, the functional diversity of *IMA* family was further explored, with exhaustive in-vitro characterization of their biochemical and enzymological properties.

Ima1p and Ima2p were similar to biochemical properties; Ima3p showed some differences from the two proteins; amongst them, Ima5p was the most distant protein. The four isomaltases were highly sensitive to Tris and Fe^{3+} , but were unaffected by the addition or the removal of Ca^{2+} despite conservation of the calcium binding site. Besides, four isoenzymes exhibited a preference for the α -(1,6) disaccharides isomaltose and palatinose, with Michaelis-Menten kinetics and inhibition at high substrates concentration. They were also able to hydrolyse trisaccharides bearing an α -(1,6) linkage, but also α -(1,2), α -(1,3) and α -(1,5) disaccharides including sucrose, highlighting their substrate ambiguity.

While Ima1p and Ima2p presented almost identical characteristics, the results nevertheless showed many singularities within this protein family. In particular, Ima3p presented lower activities than Ima2p despite only 3 different amino acids between these two isoforms. The Ima3p_R279Q variant recovered activity levels of Ima2p, while the Leu-to-Pro substitution at position 240 significantly increased the stability of Ima3p and supported the role of prolines in thermostability. Ima5p presented the lower optimal temperature and was also extremely sensitive to temperature.

Isomaltose hydrolysis by Ima5p challenged previous conclusions about the requirement of specific amino acids for determining the specificity for α -(1,6) substrates. We finally found a mixed inhibition by maltose for Ima5p while, contrary to a previous work, Ima1p inhibition by maltose was competitive at very low isomaltose concentrations and uncompetitive as the substrate concentration increased.

The presented Ph.D's work provided preliminary insights into determining structural factors within this family, exemplifying for example the role of proline residues for thermostability. Moreover, it was illustrated that a gene family encoding proteins with strong sequence similarities can lead to enzyme with notable differences in biochemical and enzymological properties.

Key words: α -Glucosidase, Isomaltase, Isomaltose, Maltose, Substrate ambiguity, Substrate inhibition, Thermostability, Proline substitution

RESUME

La levure *Saccharomyces cerevisiae* est capable d'utiliser une grande variété de sucres comme source de carbone et d'énergie. La plupart des enzymes impliquées dans l'utilisation de ces sucres sont codées par des gènes issus de familles multigéniques. C'est le cas de la famille *IMA* identifiée comme impliquée dans l'utilisation de l'isomaltose. Cette famille comprend cinq gènes qui codent pour quatre isomaltases partageant une forte identité de séquence (de 65% à 100 %). Dans ce travail, la diversité fonctionnelle de la famille *IMA* a été étudiée, en caractérisant de façon exhaustive *in vitro* leurs propriétés biochimiques et enzymologiques.

Ima1p et Ima2p possèdent des propriétés biochimiques identiques (pH, température, et thermostabilité) mais Ima3p se distingue par rapport à ces deux protéines bien que n'ayant que trois acides de différence avec Ima2p (thermostabilité plus faible). Ima5p quant à elle, est la protéine la plus dissemblable (température optimale plus faible et demi-vie basse dès 37°C). Les quatre isomaltases sont cependant très sensibles au Tris et aux ions Fe^{3+} . Les quatre isoenzymes présentent une préférence pour les disaccharides liés en α -1,6 (isomaltose et palatinose), avec une cinétique de type Michaëlis-Menten et une inhibition par le substrat à une concentration élevée. Les isomaltases Imap sont cependant aussi capables d'hydrolyser les disaccharides α -1,2, α -1,3 et α -1,5 ainsi que les trisaccharides portant une liaison α -1,6, ce qui met en évidence leur ambiguïté de substrat.

Nos résultats ont toutefois montré de nombreuses singularités dans cette famille de protéines. Alors que Ima1p et Ima2p présentent des propriétés très semblables, l'activité catalytique de Ima3p est globalement très faible malgré sa forte ressemblance avec Ima2p. Le variant Ima3p_R279Q retrouve des niveaux d'activité proches de ceux d'Ima2p, tandis que la substitution d'une leucine par une proline à la position 240 a permis d'augmenter de manière significative la stabilité d'Ima3p confirmant le rôle

des prolines dans la thermostabilité des protéines. L'hydrolyse de l'isomaltose par Ima5p réfute les conclusions précédemment publiées sur l'exigence d'acides aminés spécifiques pour déterminer la spécificité de α -1,6 puisque le variant IMA5-MQH ne permet pas de restaurer une activité semblable à Ima1p malgré la présence des trois résidus MQH. Nous avons également trouvé qu'Ima5p est inhibé par le maltose suivant une inhibition mixte tandis qu'Ima1p est inhibée de façon compétitive à faible concentration et de manière incompétitive à forte concentration en isomaltose.

Le travail de doctorat présenté montre qu'une famille de gènes codant pour des protéines avec de fortes similarités de séquences peut conduire à des protéines avec des propriétés biochimiques et enzymologiques différentes. Il fournit également des indications préliminaires sur la détermination des facteurs structurels au sein de cette famille, illustrant par exemple le rôle des résidus proline dans la thermostabilité.

Mots clés: α -Glucosidase- Isomaltase- Isomaltose- Ambiguïté de substrat- Inhibition par le substrat- Thermostabilité- Substitution par la Proline

ABBREVIATIONS

α -MG: α -Methylglucopyranoside

CSR: Conserved Sequence Region

DSF: Differential Scanning Fluorimetry

GH: Glycoside Hydrolase Family

HPAEC: High Performance Anion Exchange Chromatography

IMA protein: isomaltase encoded by *IMA* gene

Ima1p: Isomaltase encoded by *IMA1*; Ima2p: Isomaltase encoded by *IMA2*

Ima3p: Isomaltase encoded by *IMA3*; Ima5p: Isomaltase encoded by *IMA5*

IMO: Isomaltooligosaccharide

K_{cat} : Catalytic constant; K_i : inhibition constant; K_m : Michaelis constant

ORF: Open Reading Frame

PAD: Pulsed Amperometric Detection

pNPG: 4-Nitrophenyl-D-glucopyranoside

qPCR: quantitative PCR, or real-time PCR

SGD: *Saccharomyces cerevisiae* Genome Database

SmDG: *Streptococcus mutans* dextran glucosidase

$T_{1/2}$: Half life; T_m : Melting temperature

WGD: *Saccharomyces cerevisiae* Whole-genome Duplication

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1 INTRODUCTION

Isomaltase (EC 3.2.1.10) whose systematic name is oligosaccharide α -1,6-glucohydrolase, is more generally accepted as oligo-1,6-glucosidase. They belong to the ' α -amylase' family of enzymes, which includes proteins with almost 30 different specificities, *e.g.* retaining glycoside hydrolases and transferases acting on α -glucosidic substrates such as starch, dextran, sucrose, etc (MacGregor E. A., 1988). Isomaltases have been classified in the subfamily 31 of the glycoside hydrolase family 13 [GH13_31, (Stam M.R. *et al.*, 2006)] according to the CAZy database that assigns carbohydrate-active enzymes into GH families sharing structural fold and stereochemical mechanism (Cantarel, B.L., *et al.*, 2008). α -1,6-glucosidases are divided in two specificities based on substrate size preference: (i) glucan α -1,6-glucosidases preferring isomaltooligosaccharides (IMO) longer than isomaltose (IG2) and active on dextran; (ii) oligo- α -1,6-glucosidases inactive on dextran and preferring shorter IMO with highest activity on IG2 (Saburi W. *et al.*, 2006). Contrary to α -1,6-glucosidases such as the dextran glucosidase from *Streptococcus mutans* (SmDG) that act on long-chain isomalto-oligosaccharides (IMOs), isomaltases preferentially cleave shorter IMOs (MacGregor E. A., 1988).

A gene encoding a yeast isomaltase was first isolated from a *S. cerevisiae* cDNA library and turned out to be *YGR287c* encoding a 589 amino acid protein, able to hydrolyse isomaltose and α -MG, but totally inactive on maltose (Yamamoto K. *et al.*, 2004). The crystal structure of this protein was solved, underlying the importance of some amino acids in the structure of the active site and explaining its specificity for isomaltose (Yamamoto K. *et al.*, 2010 and 2011). Three years ago, it has been found that *S. cerevisiae* S288c genome actually bears five related coding sequences located in the subtelomeres of different chromosomes, which compose the IMA multigene family (Teste M.A. *et al.*, 2010; Naumoff D.G. and G. I. Naumov, 2010). Two of them, namely *IMA3* and *IMA4* are strictly identical and encode the same protein (Ima3p, *id.* Ima4p). The Ima2p and Ima3p exhibit only 3

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different amino acids, while the most distant gene *IMA5* encodes a protein sharing 65% sequence identity with the remaining members of the family. All four IMA proteins are functional (Teste M.A. *et al.*, 2010). They possess the highly conserved regions of the GH13 family (consensus sequences I to IV), which include the catalytic triad (Asp215, nucleophile; Glu277, proton donor and Asp352, transition-state stabilizer) and the valine residue identified as a key signature of α -1,6 hydrolytic activity (Val216 from *Ima1p*) (Yamamoto K. *et al.*, 2004).

Previous works on purified α -glucosidases from baker's and brewer's yeasts showed that isomaltase hydrolyses isomaltose and α -methylglucoside (α -MG) but not maltose, whereas maltase hydrolyses maltose but not isomaltose and α -MG. However, strong discrepancies were noticed in published data, *i.e.* substrates specificities and kinetic parameters of the isomaltase from the yeast *S. cerevisiae* and from closely related yeast species, probably because enzymological properties have been determined from different isoenzymes, or from purified fractions that contained a mix of these isoenzymes.

Current facilities for gene expression and protein purification allowed us to carry out a detailed biochemical and enzymological characterization of the four isomaltases encoded by the yeast *S. cerevisiae*, including a revision of the biochemical properties of the protein encoded by *IMA1*. Targeted mutagenesis further provided key results to understand the role of important amino acid residues in substrate specificities and thermostability of these IMA proteins. From these results, we could expect opening the road to better understanding of the reasons that explain the presence of four isomaltases in *S. cerevisiae*. Especially, is this gene redundancy a simple consequence of the dynamic evolution of genomes in these specific subtelomeric regions, or, is there a real physiological relevance for yeast to withstand evolutionary diversification of both the expression and the properties of these enzymes?

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The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

2 BIBLIOGRAPHY

2.1 The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

2.1.1 Generality

Yeasts are single-cell organisms that belong to the group of organisms called "fungi", which exist almost everywhere in nature. Yeasts are very helpful, especially with respect to baking, wine making, and brewing. Yeasts reproduce rapidly and grow especially well in substances containing sugar. Yeast can grow vegetatively as either haploid or diploid cells. There are two forms in which yeast cells can survive and grow, haploid and diploid. The haploid cells undergo a simple lifecycle of mitosis and growth, and under conditions of high stress will generally simply die. The diploid cells (the preferential "form" of yeast in "nature") similarly undergo a simple lifecycle of mitosis and growth, but under conditions of stress can undergo sporulation, entering meiosis and producing a variety of haploid spores, which can go on to mate (conjugate), reforming the diploid.

Yeast has graduated from a position as the premier model for eukaryotic cell biology to become the pioneer organism that facilitated the establishment of entirely new fields of study called "functional genomics" and "systems biology". There are many recent technological advances: the study of gene expression and regulatory networks, for instance, the genome-wide assessment of translation rates (Ingolia N. *et al.*, 2009); the research of gene interaction networks as well as protein interaction networks, for example, the two hybrid method for identifying protein–protein interactions (Uetz P. *et al.*, 2000); the development of integrating co-expression and protein-gene interaction networks, including the analyses of gene and protein interactions which have been successfully applied to data on plant (Lee J.H. *et al.*, 2011). These technological advances enable yeast biologists to determine the

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The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

properties of living cells and organisms and to gain a deep understanding of the organization and regulation of eukaryotic cells (Botstein D. and G. R. Fink, 2011).

2.1.2 General aspects of genome and evolution in *Saccharomyces cerevisiae*

2.1.2.1 The sequence

The genome of the yeast *Saccharomyces cerevisiae* has been completely sequenced and was published on April 1996 through international efforts. It was the first complete sequence of a eukaryote genome (Goffeau A. *et al.*, 1996). The *S. cerevisiae* genome contains approximately 5800 genes on 16 chromosomes ranging from 0.2 – 1.5 Mb in length. Since 1996, the fraction of the 5800 genes encoding protein with a defined function has risen from 30% to 85% (Botstein D. and G. R. Fink, 2011). Nearly 1000 yeast genes (*i.e.*, ~17%) are members of orthologous gene families associated with human disease (Heinicke S. *et al.*, 2007).

Giaever *et al.* published that a deletion of 6000 genes in the *Saccharomyces cerevisiae*, 1000, which has proved to be useful for basic functional medium, and at that time only half of the genes (57%) were previously known to be essential (Giaever G. *et al.*, 2002). Fleischer *et al.* have reported a study on the systematic identification and functional screens of uncharacterized proteins in yeast, which provided more information on the biochemical and physiological context of protein function (Fleischer T.C. *et al.*, 2006). In January 2014, the total ORFs are up to 6607 according to the *Saccharomyces* Genome Database (SGD; <http://www.yeastgenome.org/>). Figure 2.1 displays that 76.78% (5073 ORFs) of the yeast genes which are currently described as “functionally characterized”, being members of well-defined protein families, or exhibiting strong homology to proteins with known biochemical functions. This means that there is still 11.91% (787 ORFs) classified as Uncharacterized and 11.31%

BIBLIOGRAPHY

The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

(747 ORFs) as Dubious, respectively. A future challenge therefore resides on finding a fine physiological function of these remaining uncharacterized genes.

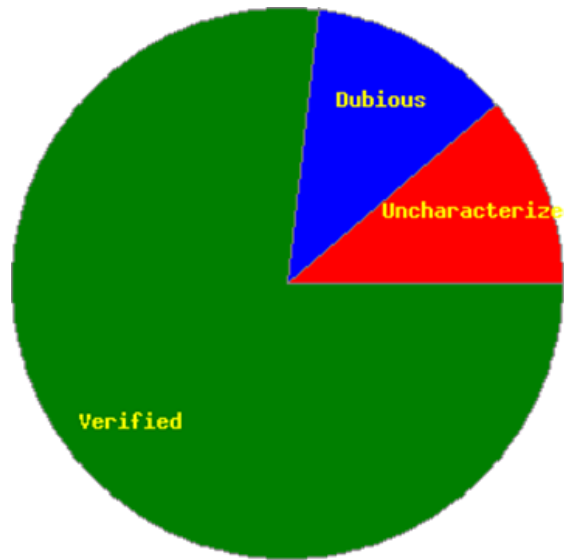


Figure 2. 1 Graphical View of Protein Coding Genes on January, 2014

2.1.2.2 Genome evolution

Saccharomyces cerevisiae still provides a unique opportunity to explain molecular mechanisms that underlie yeast evolutionary genomics, for instance, gene duplications and mutations (Lynch M. *et al.*, 2001), as well as acquisition of novel genetic material (reviewed by Dujon B., 2010). The evolutionary period, which starts with the ancestor, corresponds to a time during which the *Saccharomyces cerevisiae* lineage became increasingly adapted to rapid fermentative growth (Thomson J. M. *et al.*, 2005). This period faced the extensive rearrangement of the genome, including the deletion of thousands of redundant copies of duplicated genes (Scannell D. R. *et al.*, 2007) that mainly appeared at the time of the WGD event (see below).

It was nevertheless published that in experimental cultures of *Saccharomyces cerevisiae*, duplications of large chromosomal segments (tens to hundreds of kilobases) containing many genes appeared spontaneously at high frequency in both haploid and diploid (Koszul R. *et al.*, 2004;

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The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

Schacherer J. *et al.*, 2007). Four types of chromosomal structures are formed with different degrees of stability during subsequent generations (Koszul R. *et al.*, 2006). This process of spontaneous segmental duplications in *Saccharomyces cerevisiae* was recently reviewed (shown in Figure 2. 2) (Dujon B., 2010). Whatever their origin, the existence of sets of two or more genes encoding proteins with identical or very similar sequences (redundancy) provided the raw material for the evolution of novel functions (Ohno S., 1970). Duplication is indeed in charge of the formation of paralogous gene families, and functional diversification may occur within this process (Lynch M. *et al.*, 2001) through subtle alteration of structures and catalytic motifs to achieve new functions. Studies of proteins have demonstrated that aspects of catalysis are often conserved between evolutionarily related proteins that catalyze different reactions. Understanding the evolution of enzymes has implications for many areas of biology, including genome annotation, function prediction and protein engineering (Glasner M.E *et al.*, 2007).

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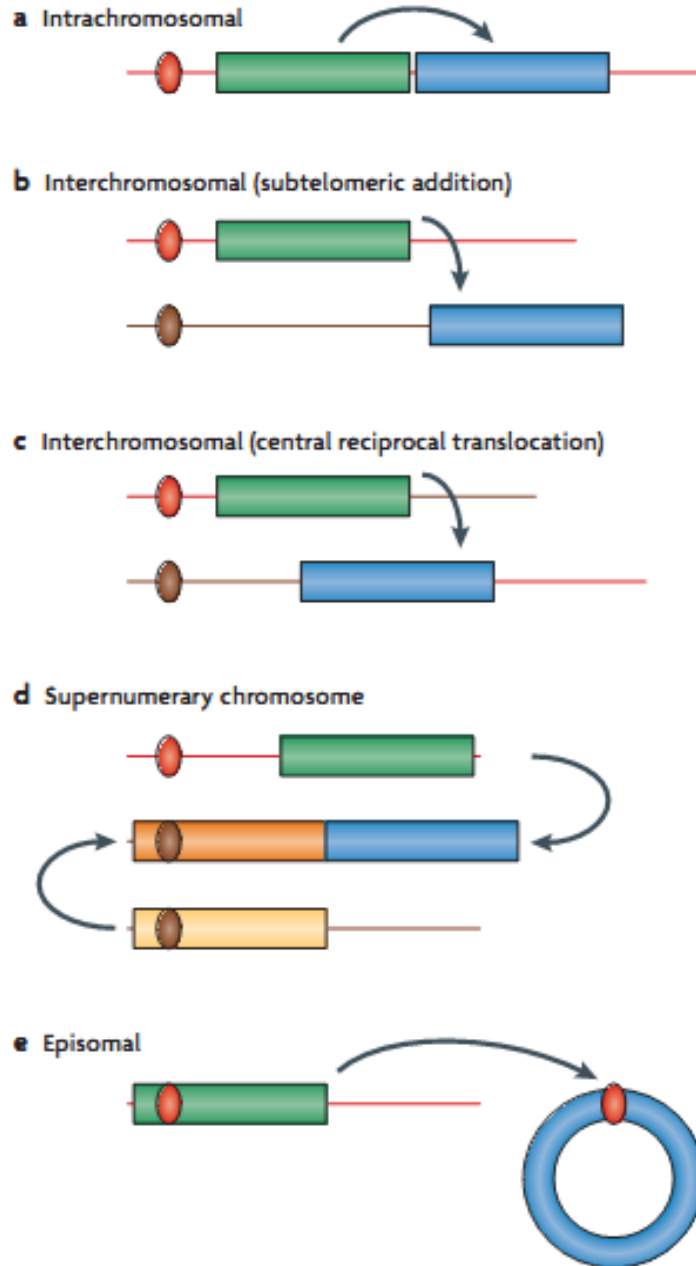


Figure 2. 2 Types of spontaneous segmental duplications in *S. cerevisiae*

(Dujon B., 2010).

[Blue sequences symbolize new copies of green sequences. Orange sequences symbolize new copies of yellow sequences. Tandem intrachromosomal duplications (a) are the most frequent structures observed and are unstable at meiosis. Interchromosomal duplications forming a novel chromosomal end (b) are also frequently observed. Interchromosomal duplications accompanied by reciprocal translocations of distal chromosome segments (c) are stable. Duplications encompassing centromeres (coloured ovals) can form supernumerary chromosomes (d) or circular episomes (e)]

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The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

2.1.2.2.1 The whole genome duplication (WGD)

Gene duplication is recognized as a crucial mechanism in evolution. Gene duplication allows asymmetric evolution of preexisting promiscuous function in a protein, such that these prior functions can be further optimized (subfunctionalization) (Ohno S., 1970). Mewes *et al.* have systematically observed the genome for clusters of genes that have been produced by local duplication events (Mewes H.W. *et al.*, 1997). The large number of fungal genome sequences has provided firm support for the idea that the *S. cerevisiae* genome resulted from a whole-genome duplication (Wolfe K.H and D. C. Shields, 1997; reviewed in Scannell *et al.*, 2007). After the genome duplication, one gene copy is apt to preserve the initial function while the other copy is free to diverge. Most studies indeed assume that enzymes change function from one specialized catalytic role to another specialized role, after being freed to change function, usually through an extra copy of the gene for the enzyme being created through gene duplication (Zhang J.Z., 2003). Duplicated genes can supply genetic raw material for the emergence of new functions through the forces of mutation and natural selection (Kellis B. *et al.*, 2004). In principle, coordinate duplication of an entire genome may allow for large-scale adaptation to new environments. Kellis and coworkers published the model of WGD followed by massive gene loss of *Kluyveromyces* and *Saccharomyces*, see Figure 2. 3 (Kellis B. *et al.*, 2004). To counterbalance the uncontrolled genome expansion, massive gene loss and gene inactivation was researched following the WGD (Liti G. and E. J. Louis, 2005).

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The yeast *Saccharomyces cerevisiae*, a model eukaryotic organisms

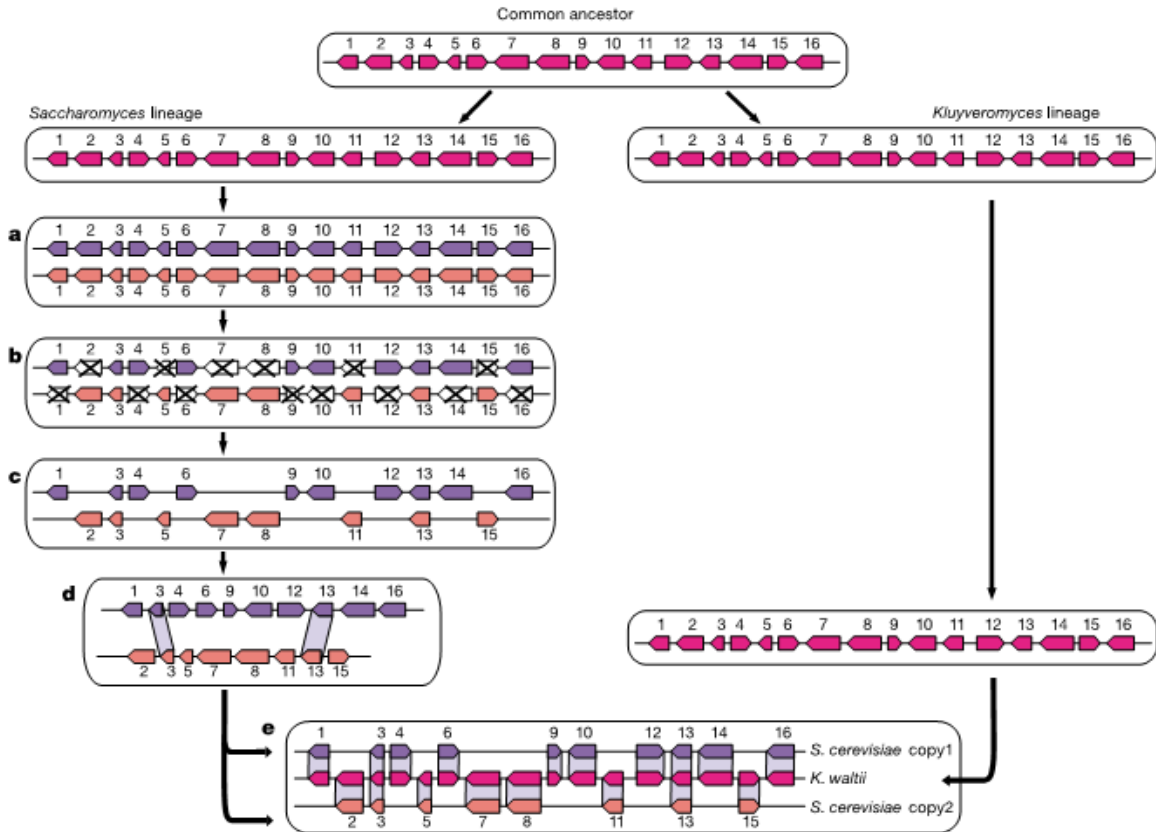


Figure 2. 3 Model of WGD followed by massive gene loss

(Kellis B. *et al.*, 2004).

[a, After divergence from *Kluyveromyces waltii*, the *Saccharomyces* lineage underwent a genome duplication event, creating two copies of every gene and chromosome. b, The vast majority of duplicated genes underwent mutation and gene loss. c, Sister segments retained different subsets of the original gene set, keeping two copies for only a small minority of duplicated genes, which were retained for functional purposes. d, Within *Saccharomyces cerevisiae*, the only evidence comes from the conserved order of duplicated genes (numbered 3 and 13) across different chromosomal segments; the intervening genes are unrelated. e, Comparison with *Kluyveromyces waltii* reveals the duplicated nature of the *Saccharomyces cerevisiae* genome, interleaving genes from sister segments on the basis of the ancestral gene order.]

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2.1.2.2.2 Multigene families

Multigene families are a group of genes that have descended from a common ancestral gene and therefore have comparable functions and similar DNA sequences (Li W.H, 1997). Most of these multigene families in *S. cerevisiae* are found either in subtelomeric regions (Viswanathan M. *et al.*, 1994) or organized as tandem repeats (Leh-Louis V. *et al.*, 2004). The concentration of multigene families in the telomere-adjacent regions has been proposed to reflect a recombination-mediated dispersal mechanism (Zakian, V. A. 1996). As compared to non-subtelomeric genes, their subtelomeric location, which is characterized by an extraordinary instability, mainly explains their fast expansion through gene recombination, and evolution through functional divergence of the alleles (Brown C.A. *et al.*, 2010; Sickmann A. *et al.*, 2003)). Besides, a recent study by Liao *et al.* showed that duplication is an important contributor to phenotypic evolution and a large number of morphological and physiological traits are controlled by multigene families (Liao B.Y. *et al.*, 2010), promoting adaptation to novel niches.

2.2 Yeast Sugar metabolism

Sugars are preferential carbon sources for yeasts, and vast amount of information is available on the components of the pathways for sugar utilization in the yeast *Saccharomyces cerevisiae*. The glycolysis is the general pathway for conversion of glucose to pyruvate and energy, while an alternative mode of glucose oxidation is the hexose phosphate pathway also known as the pentose phosphate cycle. Two principal modes of pyruvate utilization for energy production can be distinguished: respiration and fermentation (Figure 2. 4). Under aerobic condition, the yeast is capable of degrading glucose (or similar monosaccharides such as fructose and mannose) by an oxidative metabolism, leading to the respiration of glucose into CO₂ and H₂O. This is an energetic metabolism and allows an increase in the cellular biomass with a high yield. While under anaerobic or aerobic condition with high glucose concentration, it is converts the sugar into ethanol and CO₂ by the process of fermentation. These sugars are called fermentable sugars and the transformation is energetically favorable, although less energy than oxidative metabolism. The relationship between respiration and the utilization of sugar is reviewed by Fukuhara (Fukuhara Hiroshi, 2003).The use of other sugars such as sucrose, galactose, maltose or trehalose in *S. cerevisiae* depends on both the genetic variability of strains and the regulatory mechanisms.

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Yeast Sugar metabolism

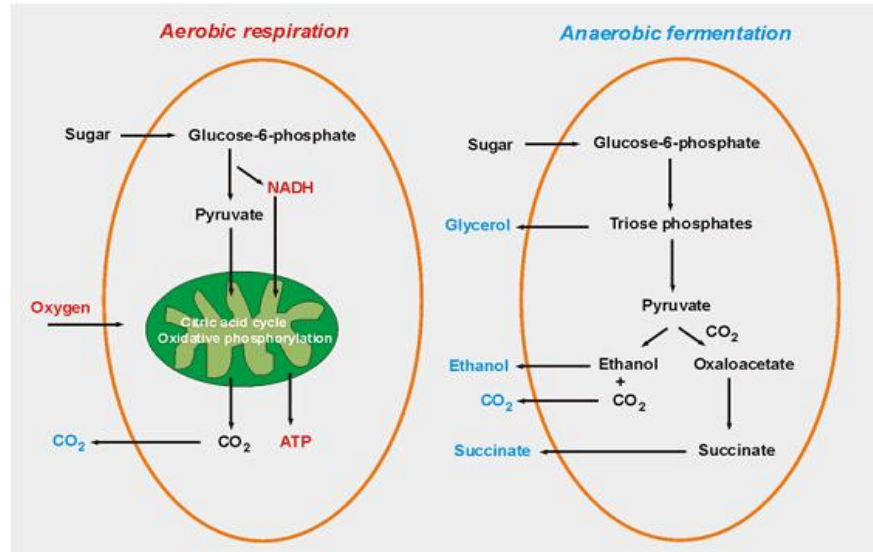


Figure 2. 4 Metabolism in yeast under aerobic and anaerobic conditions.

2.2.1 The monosaccharides

2.2.1.1 Glucose and fructose

Glucose and related sugars repress the transcription of genes encoding enzymes required for the utilization of alternative carbon sources (Gancedo J.M, 1998), such as mono-(galactose), di-(maltose, sucrose) or oligosaccharides, and those involved in gluconeogenesis, in the respiration in the TCA cycle or the glyoxylate cycle). The mechanisms involved in this process, called catabolite repression are not yet all clear. Indeed, catabolite repression involves many intermediaries through cascades of complex regulations. Entian was firstly reported the role of *HXK2* in catabolite repression, which was an important step in understanding the mechanism of regulation (Entian K.D., 1980). The study reported by Rodriguez revealed the mechanism of gene regulation whereby the product of a glycolytic gene, interacted directly with nuclear proteins to regulate the transcription of the *HXK1* and *GLK1* genes and to autoregulate its own transcription (Rodriguez A. *et al.*, 2001).

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Yeast Sugar metabolism

2.2.1.2 Galactose

Galactose is used by the Leloir pathway (Caputto R. *et al.*, 1949) (Figure 2. 5). This pathway allows the conversion of galactose to glucose-6-phosphate, which enters through the glycolysis. Timson and Pannala researched and published the galactose metabolism (Timson D. J., 2007; Pannala V. R. *et al.*, 2010). These *GAL* genes encode proteins necessary for the hydrolysis and the use of galactose (Sellick *et al.*, 2008), and they are involved in controlling structural and regulatory gene expression (Rubio-Teixeira M., 2005). Members of this pathway are the target of catabolite repression in the presence of glucose, but galactose also triggers catabolite repression of other genes like *MLS1* of the glyoxylate shunt, *FBP1* of gluconeogenesis, and so on (Gancedo J.M., 1998).

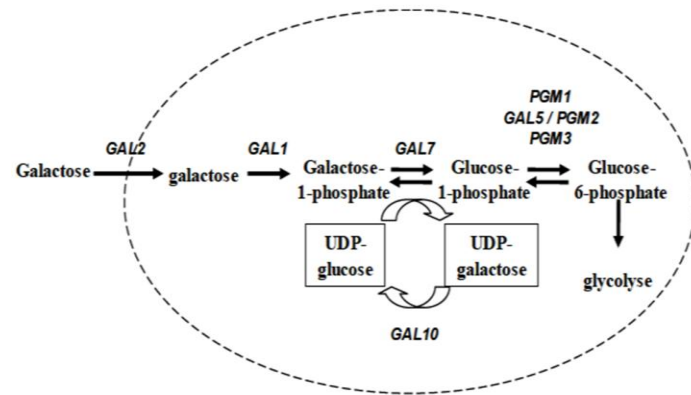


Figure 2. 5 Leloir pathway: metabolism of galactose

2.2.2 The di- and tri-saccharides

There are two modes to assimilate disaccharides. The first step is their passage through the plasma membrane followed by hydrolysis in the cytoplasm (*e.g.* maltose). For other sugars, they are hydrolyzed in the periplasm by specific enzymes such as invertases for raffinose or sucrose, and then their products are used as mentioned for monosaccharides (*i.e.* melibiose, which is cleaved into glucose and galactose) (Jules M. *et al.*, 2008). These two patterns may coexist for some other sugars (*i.e.* trehalose).

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2.2.2.1 Disaccharides (Glucose → Glucose)

Trehalose [*O*- α -D-glucosyl-(1→1)- α -D-glucose] is a nonreducing sugar formed from two glucose units. Trehalose can be found in animals, plants, and microorganisms, but not in mammals. However, mammals can assimilate trehalose from their diets because of acid trehalase in their intestine (Ruf J. *et al.*, 1990). Trehalose can protect cells against various environmental stresses and prevent the deactivation of proteins (Jiang T. *et al.*, 2013). Trehalose is synthesized by the trehalose synthase complex (*Tps1p*, *Tps2p*, *Tsl1p*, *Tps3p*) and hydrolysed by neutral (*Nth1p*, *Nth2p*) or acid, extracellular (*Ath1p*) trehalases (Francois J. and J. L Parrou, 2000; Jules M. *et al.*, 2008).

Kojibiose (*O*- α -D-glucosyl-(1→2)- α -D-glucose) occurs in koji extract, honey, starch hydrolysate, and beer. This disaccharide has been regarded as a product from dextrin by a transfer reaction of α -glucosidase (Takahashi M. *et al.*, 1969). Kojibiose exerts a prebiotic effect that can increase beneficial bacterial populations in the human gut (Sanz M.L. *et al.*, 2005). Very recently, Jung *et al.* reported that a novel kojibiose catabolic gene cluster responsible for kojibiose utilization was identified in the genome of *Pyrococcus sp. ST04* (Jung J. H. *et al.*, 2014). The encoded proteins converted kojibiose into G6P, a substrate of the glycolytic pathway. Their research could help to understand the kojibiose utilization pathway via phosphorolysis. However, kojibiose assimilation by yeast is still unknown.

Nigerose [*O*- α -D-glucosyl-(1→3)- α -D-glucose] is an unfermentable sugar obtained by partial hydrolysis of nigeran. Nigerose is also readily extracted from the dextrans found in rice molds and many other fermenting microorganisms (Matsuda K. *et al.*, 1961). Nigerose can served as acceptors for a glucosyltransferase GTF-I from cariogenic *Streptococcus sobrinus* to give a series of homologous acceptor products (Mukasa H. *et al.*, 2000).

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Yeast Sugar metabolism

Maltose [*O*- α -D-glucosyl-(1 \rightarrow 4)- α -D-glucose] is an important carbon source for *Saccharomyces cerevisiae* during beer fermentation and leavening of dough. The metabolism of maltose is initiated by the disaccharide transport across the plasma membrane, after hydrolysis by maltase (α -glucosidase), the productions of glucose enter into glycolysis. The structural genes for the use of maltose are grouped in a multigene locus: *MAL1*, *MAL2*, *MAL3*, and *MAL4 MAL6*, which are generally called *MALx*. Each locus consists of three essential genes for maltose utilization (Charron M. J. *et al.*, 1986; Needleman R., 1991).

Isomaltose [*O*- α -D-glucosyl-(1 \rightarrow 6)- α -D-glucose] is an isomeric form of maltose. The structure of isomaltose was firstly reported in 1958 (Wolfrom M.L. *et al.*, 1958). Its caloric value is less than 50% that of sucrose and its viscosity is lower than that of maltose (Harrison M.D. *et al.*, 2008). Isomaltose is a member of the isomalto-oligosaccharide group, whose members contain at least one α -1,6-glycosidic linkage, such as pannose, isomaltotriose, isomaltotetraose, nigerose, and isopanose (Lee H.C. *et al.*, 2008). Isomaltose has potential applications, not only in the food industry, such as in confections, processed fruits and vegetables, and canned and bottled food, but also as an ingredient in animal feed, cosmetics, and medicines. It is generated as a by-product during chemical and enzymatic reactions that use liquefied starch or a glucose-containing solution as a reactant (Harrison M.D. *et al.*, 2008).

2.2.2.2 Disaccharides (Glucose \rightarrow Fructose)

Sucrose [*O*- α -D-glucosyl-(1 \rightarrow 2)-D-fructose] is a non-reducing sugar because its glycosidic bond is formed between the reducing ends of both glucose and fructose. The hydrolysis of sucrose is performed by the invertase (β -fructofuranosidase, EC 3.2.1.26) encoded by the multigene family *SUCx*. This family includes seven genes (*SUC1-SUC5*; *SUC7* and *SUC8*) (Carlson M. and D. Botstein, 1983; Naumoff D.G. and G. I. Naumov, 2010). All these genes are located in the telomeric regions of different chromosomes except *SUC2* which is not very close to a telomere (Carlson M. *et al.*, 1985).

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Turanose [*O*- α -D-glucosyl-(1 \rightarrow 3)-D-fructose] is an analog of sucrose not metabolized by higher plants, but rather acquired through the action of sucrose transporters for intracellular carbohydrate signaling. It can also be used as a carbon source by many organisms including numerous species of bacteria and fungi (Loreti E. *et al.*, 2000). Turanose is actively transported by Agt1p (Han E.K. *et al.* 1995) and can be utilized by ester-producing yeasts *Pichia sp.*, *Hyphopichia sp.*, and *Zygosaccharomyces cidri*. Specific disaccharide signaling, independent of metabolism, is also demonstrated by using non-metabolizable sucrose analogs, such as palatinose and turanose (Ramon M. *et al.*, 2008).

Maltulose [*O*- α -D-glucosyl-(1 \rightarrow 4)-D-fructose] is an isomer of maltose which can be obtained from the hydrolysis of corn starch by α -amylase. Maltulose is formed by the chemical isomerization of the reducing end glucose units during enzyme liquefaction and autoclaving of starch in the enzyme-enzyme process of glucose manufacture (Dias F. *et al.*, 1987). There are few references to the utilization of the isomeric glucosyl-fructoses (trivially designated trehalulose, turanose, maltulose, leucrose, and palatinose) by microorganisms (Robrish S.A. *et al.*, 2001; Thompson J. *et al.*, 2001).

Leucrose [*O*- α -D-glucosyl-(1 \rightarrow 5)-D-fructose] is a bond isomer of sucrose that was first detected as a byproduct of microbial dextran production. Only some of the yeasts, for example, *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and *Saccharomyces cerevisiae* can utilize leucrose (Peltroche-Llacsahuanga H. *et al.*, 2001). The substrate properties of α -glucosidase for leucrose in yeast were determined by Ziesenitz and coworkers, and these data were compared with those of maltose and sucrose (Ziesenitz S.C. *et al.*, 1989). In addition, leucrose is a noncariogenic disaccharide and thus represents a highly promising sugar substitute for caries prevention.

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Palatinose [*O*- α -D-glucosyl-(1 \rightarrow 6)-D-fructose] has the same function as sucrose acting as an energy source, and it is a natural constituent of honey and sugar cane. Lemaire *et al.* reported that palatinose was unable to trigger a G Protein-Coupled Receptor1-dependent cAMP response in the Yeast *Saccharomyces cerevisiae* (Lemaire K. *et al.*, 2004) Peltroche-Llacsahuanga and coworkers tested 30 yeast strains by an auxanographic sugar assimilation assay. They found that only *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and *S. cerevisiae* utilized leucrose and/or palatinose well (Peltroche-Llacsahuanga H. *et al.*, 2001).

2.2.2.3 Trisaccharides

Maltotriose [*O*- α -D-glucosyl-(1 \rightarrow 4)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose] is most commonly produced by the digestive enzyme alpha-amylase on amylose in starch. Londesborough reported that most yeast strains used maltotriose only after maltose was exhausted (Londesborough John, 2001). The specific features of maltotriose metabolism leading to incomplete or delayed utilization of this sugar by the yeast remain elusive (Salema-Oom M. *et al.*, 2005). There have been conflicting reports on whether α -glucoside transporters in *Saccharomyces cerevisiae* (*MALx1*, *AGT1*, and *MPH2* and *MPH3* transporters) allow efficient maltotriose utilization by yeast cells (Alves S. L. *et al.*, 2008; Duval E. H. *et al.*, 2010).

Isomaltotriose [*O*- α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 6)-D-glucose] The hydrolysis of isomaltotriose by oligo-1,6-glucosidase was firstly reported in 1950s (Larner Joseph, 1955). Yoon *et al.* studied the specificity of the yeast *Saccharomyces cerevisiae* on the removal of carbohydrates by fermentation. Isomaltotriose and melezitose were not hydrolyzed: They suggested that the addition of a second α -(1 \rightarrow 6)-linked d-glucose unit in isomaltotriose completely stops the hydrolysis of this substrate by the yeast α -glucosidase, preventing its removal from the broth medium (Yoon S.H. *et al.*, 2003).

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Melezitose [*O*- α -D-glucosyl-(1 \rightarrow 3)- β -D-fructosyl-(2 \rightarrow 1)-D-glucose] is a nonreducing sugar that is produced by many plant sap eating insects. In 1988, Needleman and Perkins identified two essential genes by genetic complementation: *MAL64^c*, *trans* regulatory genes *MAL6x*, and a new gene called *MPT1* located near the *MAL1* locus on chromosome VII (Perkins E. L. and R. B. Needleman, 1988). The active transport of melezitose is responsible by *AGT1* permease, which is an α -glucoside-H⁺ symporter in *Saccharomyces cerevisiae* (Stambuk B.U. *et al.*, 1999). It was reported that in *Saccharomyces cerevisiae*, invertase is not able to hydrolyze the D-fructose linkage to give d-glucose when a D-glucose unit is attached α -(1 \rightarrow 3) to the D-fructose moiety of melezitose, and further the α -glucosidase is not able to hydrolyze the D-glucose attached to the D-fructose unit of sucrose in melezitose, making it completely resistant to fermentation (Yoon S.H. *et al.*, 2003).

Panose [*O*- α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose] is constituted by a maltose molecule bonded to a glucose molecule by an alpha-1,6-glycosidic bond. Panose can also be considered prebiotic for stimulating the growth of benefic microorganisms (Fernandes F. A. and S. Rodrigues, 2007). Makelainen *et al.* studied the prebiotic effects of panose and the fermentation of panose by the endogenous microbiota (Makelainen H. *et al.*, 2009). To our knowledge there is no study reporting panose utilization by yeast. However, researches on bacteria could help us to understand trisaccharides utilization by yeast. For example, Andersen and his team performed the transcriptional analysis of oligosaccharide utilization by *Bifidobacterium lactis* Bl-04, such as maltotriose, isomaltose, raffinose and panose. Their study identified the differential gene expression for utilization of potential prebiotics highlighting the extensive capabilities to utilize oligosaccharides (Andersen J.M. *et al.*, 2013).

Raffinose [*O*- α -D-galactopyranosyl-(1 \rightarrow 6)- α -D-glucopyranosyl-(1 \rightarrow 2)- β -D-fructofuranose] is composed of galactose, glucose, and fructose. Raffinose cannot be transported as such in the interior of

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the cell. It must be hydrolyzed by the extracellular invertase. Both types of invertase, cytoplasmic and periplasmic have identical affinity for raffinose (K_m : 150mM) (Gascon S. *et al.*, 1968). Yeast cells grown on raffinose, as a sole carbon source, differ from glucose. The results obtained by Guaragnella and his colleagues, together with the role of raffinose-grown pathway in extending replicative life span make yeast an attractive model organism of molecular biology (Guaragnella N. *et al.*, 2013).

2.2.3 The polysaccharides

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic bonds. They range in structure from linear to highly branched. Examples include storage polysaccharides such as starch and glycogen, and structural polysaccharides such as cellulose and chitin. Hydrolysis of polysaccharides is facilitated by the presence of extracellular enzymes. Very often, heterologous enzymes such as amylases, cellulases, pectinases and xylanases, are expressed in *Saccharomyces cerevisiae* so that it can ferment a number of larger carbohydrates.

In this paragraph, I just concerned the IMO (Isomaltooligosaccharide) which is related to α -1,6-glucosidases in present work. IMOs, specifically, are glucose oligomers with α -D-(1,6)-linkages. They are generated as by-products during chemical and enzymatic reactions that use liquefied starch or a glucose-containing solution as reactants (Harrison M.D. *et al.*, 2011). Recently, Moller *et al.* reported the α -1, 6 glucosidases of the family GH13_31 which were bacterial enzymes but belong to the same subfamily as isomaltases (Moller M. S. *et al.*, 2012). They found these enzymes were able to confer degradation of IMO. Moreover, Majzlova *et al.* reviewed the GH13 specificities, and they presented only two enzymes, oligo-1,6-glucosidase and dextran glucosidase can hydrolyze isomaltooligosaccharides (Majzlova K. *et al.*, 2013)

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Enzymes in carbohydrate metabolism

2.3 Enzymes in carbohydrate metabolism

2.3.1 Carbohydrate metabolic process genes

Carbohydrate metabolism contains a wide variety of biochemical processes responsible for the formation, breakdown and interconversion of carbohydrates in living organisms. Carbohydrate metabolic process in yeast consist of seven main processes: polysaccharide metabolic process, carbohydrate catabolic process, negative regulation of carbohydrate metabolic process, positive regulation of carbohydrate metabolic process, regulation of carbohydrate metabolic process, single-organism carbohydrate metabolic process and cellular carbohydrate metabolic process. Nowadays, there are 98 genes known to be involved in these processes in the yeast *S. cerevisiae*.

Gene Name	Description
ALG13	Catalytic component of UDP-GlcNAc transferase; required for the second step of dolichyl-linked oligosaccharide synthesis; anchored to the ER membrane via interaction with Alg14p; similar to bacterial and human glycosyltransferases; protein abundance increases in response to DNA replication stress
AMS1	Vacuolar alpha mannosidase; involved in free oligosaccharide (fOS) degradation; delivered to the vacuole in a novel pathway separate from the secretory pathway
ATG26	UDP-glucose:sterol glucosyltransferase; conserved enzyme involved in synthesis of sterol glucoside membrane lipids; in contrast to ATG26 from <i>P. pastoris</i> , <i>S. cerevisiae</i> ATG26 is not involved in autophagy
ATH1	Acid trehalase required for utilization of extracellular trehalose; involved in intracellular trehalose degradation during growth recovery after saline stress
BGL2	Endo-beta-1,3-glucanase; major protein of the cell wall, involved in cell wall maintenance
CAT5	Protein required for ubiquinone (Coenzyme Q) biosynthesis; localizes to the matrix face of the mitochondrial inner membrane in a large complex with ubiquinone biosynthetic enzymes; required for gluconeogenic gene activation
CAT8	Zinc cluster transcriptional activator; necessary for derepression of a variety of genes under non-fermentative growth conditions, active after diauxic shift, binds carbon source responsive elements; relative distribution to the nucleus increases upon DNA replication stress
CDA1	Chitin deacetylase; together with Cda2p involved in the biosynthesis ascospore wall component, chitosan; required for proper rigidity of the ascospore wall
CDA2	Chitin deacetylase; together with Cda1p involved in the biosynthesis ascospore wall component, chitosan; required for proper rigidity of the ascospore wall
CRH1	Chitin transglycosylase; functions in the transfer of chitin to beta(1-6) and beta(1-3) glucans in the cell wall; similar and functionally redundant to Utr2; localizes to sites of polarized growth; expression induced by cell wall stress
CRR1	Putative glycoside hydrolase of the spore wall envelope; required for normal spore wall assembly, possibly for cross-linking between the glucan and chitosan layers; expressed during sporulation
CTS1	Endochitinase; required for cell separation after mitosis; transcriptional activation during the G1 phase of the cell cycle is mediated by transcription factor Ace2p
CTS2	Putative chitinase; functionally complements <i>A. gossypii</i> cts2 mutant sporulation defect

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EMI2	Non-essential protein of unknown function; required for transcriptional induction of the early meiotic-specific transcription factor IME1; required for sporulation; expression regulated by glucose-repression transcription factors Mig1/2p; EMI2 has a paralog, GLK1, that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress
EXG1	Major exo-1,3-beta-glucanase of the cell wall; involved in cell wall beta-glucan assembly; exists as three differentially glycosylated isoenzymes; EXG1 has a paralog, SPR1, that arose from the whole genome duplication
EXG2	Exo-1,3-beta-glucanase; involved in cell wall beta-glucan assembly; may be anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor
FBA1	Fructose 1,6-bisphosphate aldolase; required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; localizes to mitochondrial outer surface upon oxidative stress
FBP1	Fructose-1,6-bisphosphatase; key regulatory enzyme in the gluconeogenesis pathway, required for glucose metabolism; undergoes either proteasome-mediated or autophagy-mediated degradation depending on growth conditions; glucose starvation results in redistribution to the periplasm; interacts with Vid30p
GAL1	Galactokinase; phosphorylates alpha-D-galactose to alpha-D-galactose-1-phosphate in the first step of galactose catabolism; expression regulated by Gal4p; GAL1 has a paralog, GAL3, that arose from the whole genome duplication
GAL10	UDP-glucose-4-epimerase; catalyzes the interconversion of UDP-galactose and UDP-D-glucose in galactose metabolism; also catalyzes the conversion of alpha-D-glucose or alpha-D-galactose to their beta-anomers
GAL3	Transcriptional regulator; involved in activation of the GAL genes in response to galactose; forms a complex with Gal80p to relieve Gal80p inhibition of Gal4p; binds galactose and ATP but does not have galactokinase activity; GAL3 has a paralog, GAL1, that arose from the whole genome duplication
GAL4	DNA-binding transcription factor required for activating GAL genes; responds to galactose; repressed by Gal80p and activated by Gal3p
GAL7	Galactose-1-phosphate uridyl transferase; synthesizes glucose-1-phosphate and UDP-galactose from UDP-D-glucose and alpha-D-galactose-1-phosphate in the second step of galactose catabolism
GAL80	Transcriptional regulator involved in the repression of GAL genes; involved in the repression of GAL genes in the absence of galactose; inhibits transcriptional activation by Gal4p; inhibition relieved by Gal3p or Gal1p binding
GAS1	Beta-1,3-glucanosyltransferase; required for cell wall assembly and also has a role in transcriptional silencing; localizes to the cell surface via a glycosylphosphatidylinositol (GPI) anchor; also found at the nuclear periphery
GAS2	1,3-beta-glucanosyltransferase; involved with Gas4p in spore wall assembly; has similarity to Gas1p
GAS3	Putative 1,3-beta-glucanosyltransferase; has similarity to other GAS family members; low abundance, possibly inactive member of the GAS family of GPI-containing proteins; localizes to the cell wall; mRNA induced during sporulation
GAS4	1,3-beta-glucanosyltransferase; involved with Gas2p in spore wall assembly; has similarity to Gas1p; localizes to the cell wall
GAS5	1,3-beta-glucanosyltransferase; has similarity to Gas1p; localizes to the cell wall
GDB1	Glycogen debranching enzyme; contains glucanotransferase and alpha-1,6-amyloglucosidase activities; required for glycogen degradation; phosphorylated in mitochondria; activity is inhibited by Igd1p; protein abundance increases in response to DNA replication stress
GFA1	Glutamine-fructose-6-phosphate amidotransferase; catalyzes the formation of glucosamine-6-P and glutamate from fructose-6-P and glutamine in the first step of chitin biosynthesis; GFA1 has a paralogous region, comprising ORFs YMR084W-YMR085W, that arose from the whole genome duplication
GLC3	Glycogen branching enzyme, involved in glycogen accumulation; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern; relocalizes from nucleus to cytoplasmic foci upon DNA replication stress
GLC7	Type 1 serine/threonine protein phosphatase catalytic subunit; cleavage and polyadenylation factor (CPF) component; involved in various processes including glycogen metabolism, sporulation, mitosis; accumulates at mating projections by interaction with Afr1p; interacts with many regulatory subunits; involved in regulation of the nucleocytoplasmic shuttling of Hxk2p; import into nucleus is inhibited during spindle assembly checkpoint arrest
GLC8	Regulatory subunit of protein phosphatase 1 (Glc7p); involved in glycogen metabolism and chromosome segregation; proposed to regulate Glc7p activity via conformational alteration; ortholog of the mammalian protein phosphatase inhibitor 2; protein abundance increases in response to DNA replication stress
GLK1	Glucokinase; catalyzes the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism; one of three glucose phosphorylating enzymes; expression regulated by non-fermentable carbon sources; GLK1 has a paralog, EMI2, that arose from the whole genome duplication
GPD1	NAD-dependent glycerol-3-phosphate dehydrogenase; key enzyme of glycerol synthesis, essential for growth under osmotic stress; expression regulated by high-osmolarity glycerol response pathway; protein abundance increases in response to DNA replication stress; constitutively inactivated via phosphorylation by the protein kinases YPK1 and YPK2, dephosphorylation increases catalytic activity; GPD1 has a paralog, GPD2, that arose from the whole genome duplication

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GPD2	NAD-dependent glycerol 3-phosphate dehydrogenase; expression is controlled by an oxygen-independent signaling pathway required to regulate metabolism under anoxic conditions; located in cytosol and mitochondria; constitutively active but is inactivated via phosphorylation by energy-stress responsive kinase SNF1; GPD2 has a paralog, GPD1, that arose from the whole genome duplication
GPH1	Glycogen phosphorylase required for the mobilization of glycogen; non-essential; regulated by cyclic AMP-mediated phosphorylation; expression is regulated by stress-response elements and by the HOG MAP kinase pathway
GRR1	F-box protein component of an SCF ubiquitin-ligase complex; modular substrate specificity factor which associates with core SCF (Cdc53p, Skp1p and Hrt1p/Rbx1p) to form the SCF(Grr1) complex; SCF(Grr1) acts as a ubiquitin-protein ligase directing ubiquitination of substrates such as: Gic2p, Mks1p, Mth1p, Cln1p, Cln2p and Cln3p; involved in carbon catabolite repression, glucose-dependent divalent cation transport, glucose transport, morphogenesis, and sulfite detoxification
GUT1	Glycerol kinase; converts glycerol to glycerol-3-phosphate; glucose repression of expression is mediated by Adr1p and Ino2p-Ino4p; derepression of expression on non-fermentable carbon sources is mediated by Opi1p and Rsf1p
HXK1	Hexokinase isoenzyme 1; a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves hexokinase Hxk2p; HXK1 has a paralog, HXK2, that arose from the whole genome duplication
HXK2	Hexokinase isoenzyme 2; catalyzes phosphorylation of glucose in the cytosol; predominant hexokinase during growth on glucose; functions in the nucleus to repress expression of HXK1 and GLK1 and to induce expression of its own gene; phosphorylation/dephosphorylation at serine-14 by protein kinase Snf1p and protein phosphatase Glc7p-Reg1p regulates nucleocytoplasmic shuttling of Hxk2p; HXK2 has a paralog, HXK1, that arose from the whole genome duplication
IMA1	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase); required for isomaltose utilization; specificity for isomaltose, alpha-methylglucoside, and palatinose; member of the IMA isomaltase family
IMA2	Isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase); specificity for isomaltose, alpha-methylglucoside, and palatinose; not required for isomaltose utilization, but Ima2p overexpression allows the ima1 null mutant to grow on isomaltose
IMA3	Alpha-glucosidase; weak, but broad substrate specificity for alpha-1,4- and alpha-1,6-glucosides; member of IMA isomaltase family; not required for isomaltose utilization, but Ima3p overexpression allows the ima1 null mutant to grow on isomaltose; identical to IMA4
IMA4	Alpha-glucosidase; weak, but broad substrate specificity for alpha-1,4- and alpha-1,6-glucosides; member of IMA isomaltase family; not required for isomaltose utilization, but Ima4p overexpression allows the ima1 null mutant to grow on isomaltose; identical to IMA3
IMA5	Alpha-glucosidase; specificity for isomaltose, maltose, and palatinose, but not alpha-methylglucoside; member of the IMA isomaltase family; not required for isomaltose utilization, but Ima5p overexpression allows the ima1 null mutant to grow on isomaltose
MAL12	Maltase (alpha-D-glucosidase); inducible protein involved in maltose catabolism; encoded in the MAL1 complex locus; hydrolyzes the disaccharides maltose, turanose, maltotriose, and sucrose
MAL32	Maltase (alpha-D-glucosidase); inducible protein involved in maltose catabolism; encoded in the MAL3 complex locus; functional in genomic reference strain S288C; hydrolyzes the disaccharides maltose, turanose, maltotriose, and sucrose
MDH1	Mitochondrial malate dehydrogenase; catalyzes interconversion of malate and oxaloacetate; involved in the tricarboxylic acid (TCA) cycle; phosphorylated
MDH2	Cytoplasmic malate dehydrogenase; one of three isozymes that catalyze interconversion of malate and oxaloacetate; involved in the glyoxylate cycle and gluconeogenesis during growth on two-carbon compounds; interacts with Pck1p and Fbp1
MDH3	Peroxisomal malate dehydrogenase; catalyzes interconversion of malate and oxaloacetate; involved in the glyoxylate cycle
MIG1	Transcription factor involved in glucose repression; sequence specific DNA binding protein containing two Cys2His2 zinc finger motifs; regulated by the SNF1 kinase and the GLC7 phosphatase; regulates filamentous growth along with Mig2p in response to glucose depletion; shuttles between cytosol and nucleus depending on external glucose levels and its phosphorylation state
MIG2	Zinc finger transcriptional repressor; cooperates with Mig1p in glucose-induced repression of many genes; under low glucose conditions Mig2p relocalizes to mitochondrion, where it interacts with Ups1p and antagonizes mitochondrial fission factor, Dnm1p, indicative of a role in mitochondrial fusion or regulating morphology; regulates filamentous growth along with Mig2p in response to glucose depletion; MIG2 has a paralog, MIG3, that arose from the whole genome duplication
MPA43	Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
NQM1	Transaldolase of unknown function; transcription is repressed by Mot1p and induced by alpha-factor and during diauxic shift; NQM1 has a paralog, TAL1, that arose from the whole genome duplication
PCL10	Pho85p cyclin; recruits, activates, and targets Pho85p cyclin-dependent protein kinase to its substrate; PCL10 has a paralog, PCL8, that arose from the whole genome duplication
PCL6	Pho85p cyclin of the Pho80p subfamily; forms the major Glc8p kinase together with Pcl7p and Pho85p; involved in the control of glycogen storage by Pho85p; stabilized by Elongin C binding; PCL6 has a paralog, PCL7, that arose from the whole genome duplication
PCL7	Pho85p cyclin of the Pho80p subfamily; forms a functional kinase complex with Pho85p which phosphorylates Mmr1p and is regulated by Pho81p; involved in glycogen metabolism, expression is cell-cycle regulated; PCL7 has a paralog, PCL6, that arose from the whole genome duplication
PCL8	Cyclin; interacts with Pho85p cyclin-dependent kinase (Cdk) to phosphorylate and regulate glycogen synthase, also activates Pho85p for Glc8p phosphorylation; PCL8 has a paralog, PCL10, that arose from the whole genome duplication

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PGM1	Phosphoglucomutase, minor isoform; catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; PGM1 has a paralog, PGM2, that arose from the whole genome duplication
PGM2	Phosphoglucomutase; catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase; protein abundance increases in response to DNA replication stress; PGM2 has a paralog, PGM1, that arose from the whole genome duplication
PGU1	Endo-polygalacturonase; pectolytic enzyme that hydrolyzes the alpha-1,4-glycosidic bonds in the rhamnogalacturonan chains in pectins
PKP1	Mitochondrial protein kinase; involved in negative regulation of pyruvate dehydrogenase complex activity by phosphorylating the ser-133 residue of the Pda1p subunit; acts in concert with kinase Pkp2p and phosphatases Ptc5p and Ptc6p
PKP2	Mitochondrial protein kinase; negatively regulates activity of the pyruvate dehydrogenase complex by phosphorylating the ser-133 residue of the Pda1p subunit; acts in concert with kinase Pkp1p and phosphatases Ptc5p and Ptc6p; relocates from mitochondrion to cytoplasm upon DNA replication stress
PMI40	Mannose-6-phosphate isomerase; catalyzes the interconversion of fructose-6-P and mannose-6-P; required for early steps in protein mannosylation
PRM15	Phosphoribomutase; catalyzes interconversion of ribose-1-phosphate and ribose-5-phosphate; has some phosphoglucomutase activity but primary activity in vivo is phosphoribomutase; contributes to ribose recycling in the pentose phosphate pathway; transcription induced in response to stress; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus; non-essential
RBK1	Putative ribokinase
ROT2	Glucosidase II catalytic subunit; required for normal cell wall synthesis; mutations in rot2 suppress tor2 mutations, and are synthetically lethal with rot1 mutations
RPE1	D-ribose-5-phosphate 3-epimerase; catalyzes a reaction in the non-oxidative part of the pentose-phosphate pathway; mutants are sensitive to oxidative stress
SCW10	Cell wall protein with similarity to glucanases; may play a role in conjugation during mating based on mutant phenotype and its regulation by Ste12p; SCW10 has a paralog, SCW4, that arose from the whole genome duplication
SCW11	Cell wall protein with similarity to glucanases; may play a role in conjugation during mating based on its regulation by Ste12p
SCW4	Cell wall protein with similarity to glucanases; scw4 scw10 double mutants exhibit defects in mating; SCW4 has a paralog, SCW10, that arose from the whole genome duplication
SGA1	Intracellular sporulation-specific glucoamylase; involved in glycogen degradation; induced during starvation of a/a diploids late in sporulation, but dispensable for sporulation
SHB17	Sedoheptulose bisphosphatase involved in riboneogenesis; dephosphorylates sedoheptulose 1,7-bisphosphate, which is converted via the nonoxidative pentose phosphate pathway to ribose-5-phosphate; facilitates the conversion of glycolytic intermediates to pentose phosphate units; also has fructose 1,6-bisphosphatase activity but this is probably not biologically relevant, since deletion does not affect FBP levels; GFP-fusion protein localizes to the cytoplasm and nucleus
SNF1	AMP-activated serine/threonine protein kinase; found in a complex containing Snf4p and members of the Sip1p/Sip2p/Gal83p family; required for transcription of glucose-repressed genes, thermotolerance, sporulation, and peroxisome biogenesis; involved in regulation of the nucleocytoplasmic shuttling of Hxk2p; regulates filamentous growth in response to starvation; SUMOylation by Mms21p inhibits its function and targets Snf1p for destruction via the Slx5-Slx8 Ubiquitin ligase
SNF4	Activating gamma subunit of the AMP-activated Snf1p kinase complex; additional subunits of the complex are Snf1p and a Sip1p/Sip2p/Gal83p family member; activates glucose-repressed genes, represses glucose-induced genes; role in sporulation, and peroxisome biogenesis; protein abundance increases in response to DNA replication stress
SOL1	Protein with a possible role in tRNA export; shows similarity to 6-phosphogluconolactonase non-catalytic domains but does not exhibit this enzymatic activity; homologous to Sol3p and Sol4p; SOL1 has a paralog, SOL2, that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress
SOL2	Protein with a possible role in tRNA export; shows similarity to 6-phosphogluconolactonase non-catalytic domains but does not exhibit this enzymatic activity; homologous to Sol3p and Sol4p; SOL2 has a paralog, SOL1, that arose from the whole genome duplication
SOL3	6-phosphogluconolactonase; catalyzes the second step of the pentose phosphate pathway; weak multicopy suppressor of los1-1 mutation; homologous to Sol2p and Sol1p; SOL3 has a paralog, SOL4, that arose from the whole genome duplication
SOL4	6-phosphogluconolactonase; protein abundance increases in response to DNA replication stress; SOL4 has a paralog, SOL3, that arose from the whole genome duplication
SPR1	Sporulation-specific exo-1,3-beta-glucanase; contributes to ascospore thermoresistance; SPR1 has a paralog, EXG1, that arose from the whole genome duplication
SUC2	Invertase; sucrose hydrolyzing enzyme; a secreted, glycosylated form is regulated by glucose repression, and an intracellular, nonglycosylated enzyme is produced constitutively
TAL1	Transaldolase, enzyme in the non-oxidative pentose phosphate pathway; converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate; TAL1 has a paralog, NQM1, that arose from the whole genome duplication
UTR2	Chitin transglycosylase; functions in the transfer of chitin to beta(1-6) and beta(1-3) glucans in the cell wall; similar to and functionally redundant with Crh1; glycosylphosphatidylinositol (GPI)-anchored protein localized to bud neck

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XKS1	Xylulokinase; converts D-xylulose and ATP to xylulose 5-phosphate and ADP; rate limiting step in fermentation of xylulose; required for xylose fermentation by recombinant <i>S. cerevisiae</i> strains
XYL2	Xylitol dehydrogenase; converts xylitol to D-xylulose; expression induced by xylose, even though this pentose sugar is not well utilized by <i>S. cerevisiae</i> ; null mutant has cell wall defect
YBR056W	Putative glycoside hydrolase of the mitochondrial intermembrane space
YDR109C	Putative kinase
YDR248C	Putative gluconokinase; sequence similarity to bacterial and human gluconokinase; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; upregulated by deletion of the RNAP-II associated factor, PAF1
YHR210C	Putative aldose 1-epimerase superfamily protein; non-essential gene; highly expressed under anaerobic conditions
YIR007W	Putative glycosidase; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YIR007W is a non-essential gene
YLR446W	Putative hexokinase; transcript is upregulated during sporulation and the unfolded protein response; YLR446W is not an essential gene
YMR085W	Putative protein of unknown function; YMR085W and adjacent ORF YMR084W are merged in related strains, and together are paralogous to glutamine-fructose-6-phosphate amidotransferase GFA1
YMR099C	Glucose-6-phosphate 1-epimerase (hexose-6-phosphate mutarotase); likely involved in carbohydrate metabolism; GFP-fusion protein localizes to both the nucleus and cytoplasm and is induced in response to the DNA-damaging agent MMS
YNR071C	Putative aldose 1-epimerase
ZWF1	Glucose-6-phosphate dehydrogenase (G6PD); catalyzes the first step of the pentose phosphate pathway; involved in adapting to oxidative stress; homolog of the human G6PD which is deficient in patients with hemolytic anemia; protein abundance increases in response to DNA replication stress

Table 2. 1 Carbohydrate Metabolic Gene Ontology Annotations in *S. cerevisiae*

2.3.2 Classification of Glycoside hydrolases

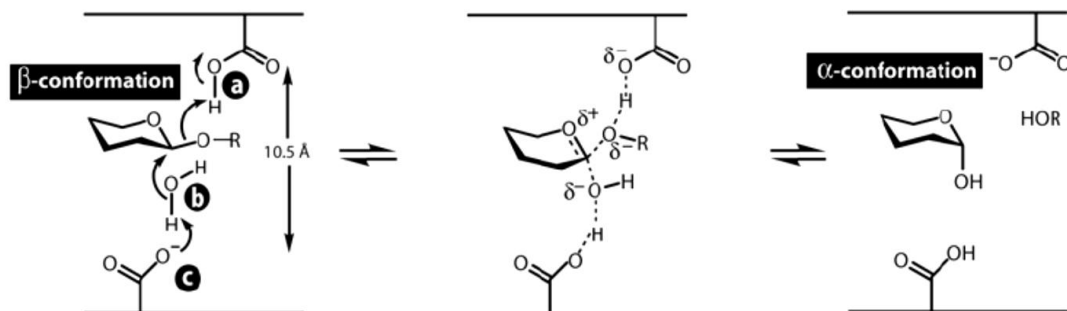
Glycoside hydrolases (glycosidases) are one of the major classes of carbohydrate-active enzymes, which catalyze hydrolysis of glycosidic linkages. In all glycoside hydrolases, enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis that requires generally two invariant carboxylic acids found in the active cleft that act as proton donor or acid, and a nucleophile or base. This hydrolysis occurs via two major mechanisms that result in either inversion ($\alpha \rightarrow \beta$; $\beta \rightarrow \alpha$) or retention ($\alpha \rightarrow \alpha$; $\beta \rightarrow \beta$) of the anomeric configuration of the glycosidic bond (See Figure 2. 6) (Rye C.S. and G. S. Withers, 2000; Heightman T. D. and A.T Vasella, 1999). Glycoside hydrolases are classified in 114 families (Cantarel, B.L., *et al.*, 2008), and each GH family contains enzymes with different specificities, accompanying similar tertiary structures and mechanisms. Typically about 1% of the genome of any organism encodes for glycoside hydrolases (Vocadlo D. J. and J. D. Gideon, 2008). Moreover, the primary sequences of thousands of glycoside hydrolases, as well as their evolutionary relationships were concerned recently (Cantarel, B.L. *et al.*, 2009). The evolutionary relationships were

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described for the entire GH13 family (Janecek, S, 1994) and some closely related specificities, *i.e.* subfamilies (Oslancova A. and S. Janecek, 2002), as well as the close evolutionary relatedness.

A. Inversion of the anomeric conformation



B. Retention of the anomeric conformation

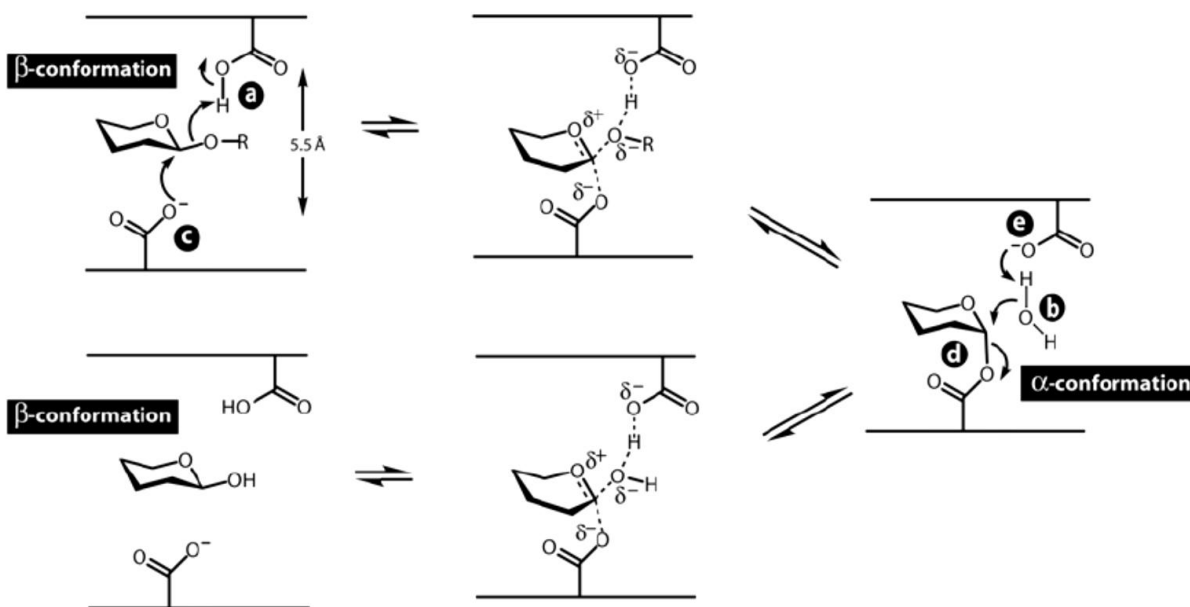


Figure 2. 6 Hydrolytic mechanisms for (A) an inverting glycosidase and (B) a retaining glycosidase.

(Adapted from Rye C.S. and G. S. Withers, 2000)

[Legend: (a) general acid; (b) water-based nucleophilic attack; (c) carboxylate base; (d) intermediate with the glycosyl residue covalently linked to the enzyme; (e) base form of the catalyst acid]

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2.3.3 GH13 family

The GH13 family, also known as the α -amylase family, has been identified very early (MacGregor E. A., 1988). It is the largest sequence-based family of glycoside hydrolases. There are more than 13,500 sequences classified currently in the family GH13. These GH13 genes encode proteins with different enzyme activities and substrate specificities acting on α -glycosidic bonds (Stam M. R. *et al.*, 2006). All the members share 4–7 CSRs (Conserved Sequence Regions), the α -amylase-type of $(\beta/\alpha)_8$ -barrel catalytic domain, catalytic machinery, and retaining reaction mechanism (Janecek S. *et al.*, 2013).

Among thousands of sequences and 30 different enzymes specificities (Cantarel B. L. *et al.*, 2008), many GHs are closely related to each other and the GH13 family has officially been subdivided into 35 subfamilies (Stam M.R. *et al.*, 2006; Table 2.2). Two novel GH13 subfamilies were identified since August 2006, i.e. the oligo-1,6-glucosidase and the neopullulanase subfamilies (Oslancova A. and S. Janecek, 2002; Machovic, M. and S. Janecek, 2008). The largest subfamilies of GH13 are subfamilies GH13_15, GH13_9 and GH13_11 which count 303, 132 and 119 members, respectively. Only five subfamilies (GH13_19, GH13_31 (which includes isomlatases), GH13_20, GH13_2 and GH13_4) contain more than one reported activity. All these proteins are now classified within the CAZY database (<http://www.cazy.org/GH13.html>) (REFERENCES on CAZY) according to the CAZY classification system of glycoside hydrolases based on the sequence homology

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Subfamily	EC number ^a	Reported enzyme activities	Taxonomical range ^b
GH13_1	3.2.1.1	α -Amylase	Fungi (48)
GH13_2	3.2.1.1	α -Amylase	Bacteria (38), Archaea (4)
	2.4.1.19	Cyclodextrin glucanotransferase	
	3.2.1.133	Maltogenic α -amylase	
GH13_3	ND	Unknown activity	Bacteria (33), Archaea (1)
GH13_4	2.4.1.4	Amylosucrase	Bacteria (11)
	3.2.1.-	Sucrose hydrolase	
GH13_5	3.2.1.1	α -Amylase	Bacteria (52), Eukaryota (1)
GH13_6	3.2.1.1	α -Amylase	Viridiplantae (44)
GH13_7	3.2.1.1	α -Amylase	Euryarchaeota (9)
GH13_8	2.4.1.8	1,4- α -Glucan branching enzyme	Eukaryota (58), Bacteria (1)
GH13_9	2.4.1.8	1,4- α -Glucan branching enzyme	Bacteria (130), Eukaryota (2)
GH13_10	3.2.1.141	4- α -(1,4- α -Glucano)trehalose -trehalohydrolase	Bacteria (38) Archaea (5)
GH13_11	3.2.1.68	Isoamylase	Bacteria (100), Archaea (6) and Eukaryota (13)
GH13_12	3.2.1.41	Pullulanase	Firmicutes (12)
GH13_13	3.2.1.41	Pullulanase	Bacteria (16), Eukaryota (7)
GH13_14	3.2.1.41	Pullulanase	Bacteria (40)
GH13_15	3.2.1.1	α -Amylase	Metazoa (300), Bacteria (3)
GH13_16	5.4.99.16	Maltose α -glucosyltransferase	Bacteria (38), Archaea (1)
GH13_17	3.2.1.20	α -Glucosidase	Metazoa (18)
GH13_18	2.4.1.7	Sucrose phosphorylase	Bacteria (31)
GH13_19	3.2.1.1	α -Amylase	Bacteria (27)
	3.2.1.98	Maltohexaose-forming α -amylase	
	3.2.1.-	Maltopentaose-forming α -amylase	
GH13_20	3.2.1.54	Cyclomaltodextrinase	Bacteria (56)
	3.2.1.133	Maltogenic α -amylase	
	3.2.1.135	Neopullulanase	
GH13_21	3.2.1.20	α -Glucosidase	Proteobacteria (22) Deinococcus-Thermus (1)
GH13_22	2.4.1.183	α -1,3-Glucan synthase	Fungi (14)
GH13_23	ND	Unknown activity	Proteobacteria (15)
GH13_24	3.2.1.1	α -Amylase	Metazoa (24)
GH13_25	3.2.1.33	Amylo- α -1,6-glucosidase	Eukaryota (15)
GH13_26	5.4.99.15	(1,4)- α -Glucan 1- α -glucosylmutase	Bacteria (43), Archaea (6)
GH13_27	3.2.1.1	α -Amylase	Proteobacteria (19)
GH13_28	3.2.1.1	α -Amylase	Firmicutes (16), Actinobacteria (1)
GH13_29	3.2.1.93	α -Phosphotrehalase	Bacteria (69)
GH13_30	3.2.1.20	α -Glucosidase	Actinobacteria (18)
GH13_31	3.2.1.70	Glucan 1,6- α -glucosidase	Bacteria (23)
	3.2.1.10	Oligo-1,6-glucosidase	
GH13_32	3.2.1.1	α -Amylase	Bacteria (13)
GH13_33	5.4.99.16	Trehalose synthase	Bacteria (6)
GH13_34	NA	Amino acid transporter	Eukaryota (23)
GH13_35	NA	Amino acid transporter	Eukaryota (7)

Table 2. 2 Composition of the 35 subfamilies within glycosidase family GH13

(Stam M.R. *et al.*, 2006)

[ND, not determined; NA, not applicable; a, Experimentally determined; b, The number of sequences for each taxon (data from 26 July 2005) was given in brackets].

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2.3.4 Alpha-glucosidases

Within the GH13 family, alpha-glucosidases (α -D-glucoside glucohydrolase) are carbohydrate hydrolases produced by many microbes in response to growth on starch-containing media and are involved in the degradation of oligosaccharides produced by the action of amylolytic enzymes on starch. α -glucosidases hydrolyze terminal non-reducing 1-4 linked alpha- glucose residues to release α -glucose molecules. The α -glucosidases characterized to date belong to two major families, GH13 and GH31 (Henrissat Bernard, 1991; Henrissat B. and A. Bairoch, 1993), and two minor families, GH4 and GH97 (Roach P.J., 2002). GH13 α -glucosidase is observed in bacteria, brewer's yeast and insects, which hydrolyzes the heterogeneous substrates such as sucrose and *p*-nitrophenyl (PNP) α -glucoside of synthetic substrate, more rapidly than the homogeneous substrates of maltooligosaccharide and shows low or no activity towards the polymer substrate (soluble starch, raw starch, and glycogen) (Nakai H. *et al.*, 2005).

Kelly and Fogarty summarized the properties of microbial α -glucosidases to show that the substrate specificity of α -glucosidase differs greatly with the source of the enzyme. The majority of bacterial and fungal α -glucosidases have the highest activity towards maltose but not towards sucrose except *B. amyloliquefaciens* and yeast α -glucosidases (Kelly C.T. and W.M. Fogarty, 1983). Nashiru *et al.* reported that in mesophilic (Suzuki Y. *et al.*, 1982) and thermophilic bacteria, α -glucosidases exhibited preferential specificity towards maltose and maltooligosaccharides, but little activity towards aryl glucosides (Nashiru O., *et al.*, 2001). α -glucosidases was also found to catalyse transglycosylation reactions when high concentrations of glucosyl acceptor were present in the reaction system (Plant A.R. *et al.*, 1988). Besides, important amino acid residues of α -glucosidases have been identified by mutational analyses. McCarter and Withers suggested the identity of Asp-214 as the catalytic

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nucleophile in yeast α -glucosidase was consistent with the available mutagenesis and structural data (McCarter J.D. and S.G. Withers, 1996). Later, Pons and coworkers carried out a detailed analysis of the site-directed mutations which indicated a direct implication of the conserved residues Asp/Glu (region I), Asp (region II), and Glu (region III) in substrate binding and hydrolysis (Pons T. *et al.*, 2003).

On the other hand, substances that reduce an enzyme's activity are very important in controlling many biological activities. The α -glucosidase inhibitors delay the degradation of oligosaccharides, decreasing the absorption of glucose, which would inhibit the increase in postprandial hyperglycemia (Loizzo M.R. *et al.*, 2008). Most α -glucosidase inhibitors have been reported to be competitive (Breitmeier D. *et al.*, 1997). These "starch blockers" are effective at lowering postprandial glucose levels without causing hypoglycemia, because carbohydrates can be absorbed more distally in the small intestine or be available for colonic metabolism (Topping D.L. and P.M. Clifton, 2001). Some α -glucosidase inhibitors such as acarbose, miglitol and voglibose have been developed and have received considerable attention (Scheen A. J. 2003; Ismail S. *et al.*, 2012).

2.3.5 Isomaltases

Isomaltases belong to glycoside hydrolase family 13 subfamily 31 [GH13_31] (Stam, M.R. *et al.*, 2006) according to the CAZy database classification. Isomaltase, whose systematic name is oligosaccharide α -1,6-glucohydrolase, are more generally accepted as oligo-1,6-glucosidase. In 1965, Huston and Manners were the first to measure an isomaltase activity (EC 3.2.1.10) in a commercial preparation of maltase (Hutson D. H. and D. J. Manners, 1965). Two years later (1967), the purification of α -methylglucosidase demonstrated that this enzyme was able to hydrolyze isomaltose (Eaton & Khan, 1967). Then, this was associated that the *MGLx* genes encoding proteins can hydrolyze maltose

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as well as isomaltose. In 2004, Yamamoto and coworkers proved that the protein Ygr287cp had isomaltase function, which was obtained from an induced culture by maltose after cloning the gene from total RNA (Yamamoto K. *et al.*, 2004). Alike to maltase, isomaltase is an intracellular enzyme and, therefore, a transporter is needed to take up isomaltose inside the cell. For this purpose, the *AGT1* transporter which has a broad substrate specificity, becomes to be responsible for their uptake, this gene belongs to *MAL1* locus (Han E.K. *et al.* 1995).

Isomaltases hydrolyse (1→6)- α -D-glucosidic linkages at the nonreducing end of substrates with retention of anomeric configuration, as for instance in isomaltose and in some oligosaccharides produced from starch and glycogen by EC3.2.1.1 (α -amylase). They also hydrolyse isomaltulose (palatinose), isomaltotriose and panose (see §2.2.2 for the description of these sugars), but they have no action on glycogen or phosphorylase limit dextrin (Sjostrom, H. *et al.*, 1980). The enzyme from intestinal mucosa is a single polypeptide chain that also catalyses the reaction of sucrose α -glucosidase (EC3.2.1.48). Isomaltases differ from amylo- α -1,6-glucosidase (EC3.2.1.33) in their preference for short-chain substrates and from the fact that they do not require the 6-glucosylated residue to be at a branch point, *i.e.* linked at both C-1 and C-4.

α -1,6-glucosidases are actually divided in two specificities based on substrate size preference:

(i) glucan α -1,6-glucosidases preferring IMOs longer than isomaltose (IG2) and active on dextran (Saburi W. *et al.*, 2006); (ii) oligo- α -1,6-glucosidases inactive on dextran and preferring shorter IMO with highest activity on IG2. Contrary to α -1,6-glucosidases such as the dextran glucosidase from *Streptococcus mutans* (SmDG) that prefer long-chain IMOs (Saburi W. *et al.*, 2006), isomaltases preferentially cleave shorter IMOs (MacGregor E.A, 2001). Oligo-1,6- α -glucosidase has been reported from the thermophilic bacterium *Bacillus thermoglucosidius* KP1006 and was apparently unique in

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demonstrating high specificity towards isomaltose (Plant A.R. *et al.*, 1988). Oligo- α -1,6-glucosidases of *Bifidobacterium* species have been also studied, including the AlgAp from *Bifidobacterium adolescentis* that showed high activity towards the natural α -1,6 substrates isomaltose and isomaltotriose (Van den Broek L. *et al.*, 2003).

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Isomaltases from the yeast *Saccharomyces cerevisiae*

2.4 Isomaltases from the yeast *Saccharomyces cerevisiae*

2.4.1 Characterization of the *IMA* multigene family

In 2010, while looking for uncharacterized genes encoding α -glucosidases in the *Saccharomyces cerevisiae* genome, Teste *et al.* identified five genes encoding putative α -1, 6-glucosidases (Teste M.A. *et al.*, 2010). They blasted the whole yeast genome with the *MAL12/YGR292w* sequence, and besides *MAL32/YBR299w*, five additional genes were retrieved: *YGR287c*, *YIL172c*, *YJL216c*, *YJL221c*, and *YOL157c*. The ORF *YGR287c* was first isolated from a *Saccharomyces cerevisiae* cDNA library (Yamamoto K. *et al.*, 2004) and characterised as a gene encoding for an isomaltase. *YJL221c* was previously known as *FSP2*, which was denoted as “Flocculent Specific Protein”, but no function has yet been reported for this gene (Backhus L.E. *et al.*, 2001). These five genes actually encoded proteins with α -1, 6-glucosidase activities, a function that was biochemically validated *in vitro* and *in vivo* (Teste M.A. *et al.*, 2010). At the same time, another group used bioinformatical tools to describe the existence of this gene family (Naumoff D.G. and G. I. Naumov, 2010), which was taken as a model gene family to study expansion and functional divergence of subtelomeric multigene families in yeast (Brown et al 2010). Alike to other yeast multigene families which contain a large number of members located in subtelomeres (Pryde, F. E., and E. J. Louis), such as *MAL* (23 members), *SUC* (7 members), or *MEL* (10 members), the *IMA* family (5 members) is involved in sugar fermentation.

From common dialogue between these three groups and the SGD staff, these genes were therefore renamed *IMA* for IsoMAItase as *IMA1*, *IMA2*, *IMA3*, *IMA4*, and *IMA5*. These five related coding sequences form the *IMA* multigene family (list in Table 2.3), and are all located in the subtelomeric region of the corresponding chromosomes (Figure 2. 7 A~D).

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Systematic Name	Gene Name	Genome Localization
YGR287c	<i>IMA1</i>	ChrVII: 1068991 to 1067222
YOL157c	<i>IMA2</i>	ChrXV: 24294 to 22525
YIL172c	<i>IMA3</i>	ChrIX: 18553 to 16784
YJL221c	<i>IMA4</i>	ChrX: 18536 to 16767
YJL216c	<i>IMA5</i>	ChrX: 26086 to 24341

Table 2. 3 Genome localization and homology of the five *IMA* genes.

[The genome localization is annotated by SGD.]

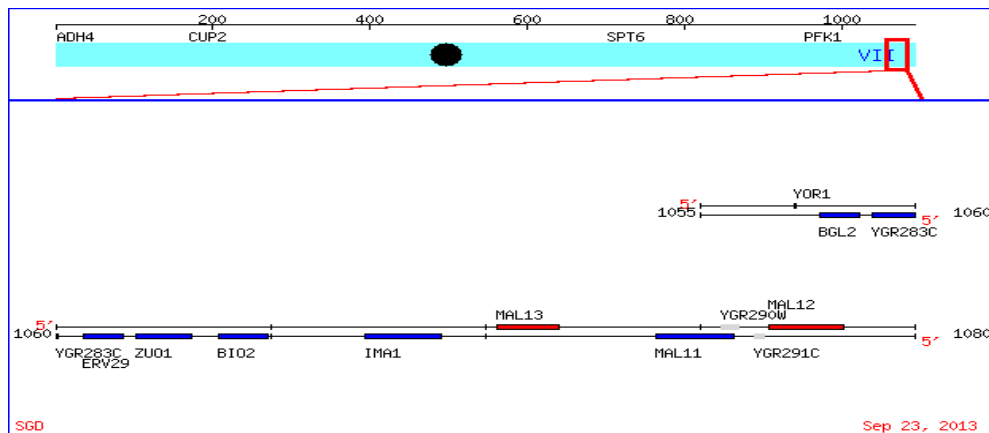


Figure 2. 7-A Chromosome VII features that span coordinates 1057222 - 1078991 bp.

[It shows the ORF location of the *IMA1* in the subtelomeric region.]

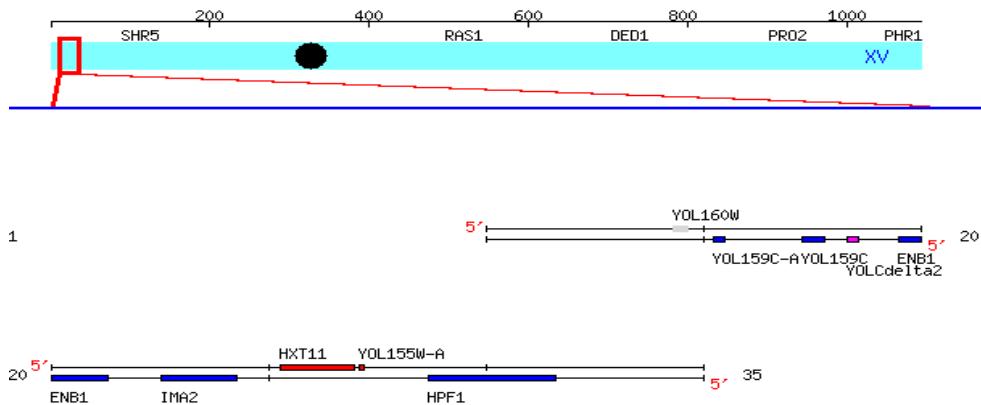


Figure 2. 7-B Chromosome XV features that span coordinates 12525 - 34294 bp.

[It shows the ORF location of the *IMA2* in the subtelomeric region.]

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Isomaltases from the yeast *Saccharomyces cerevisiae*

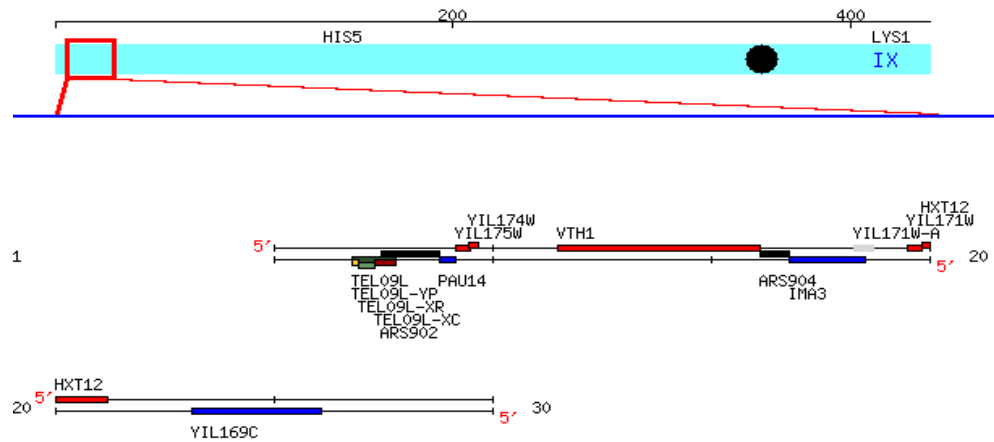


Figure 2. 7-C Chromosome IX features that span coordinates 6784 - 28553 bp.

[It shows the ORF location of the IMA3 in the subtelomeric region.]

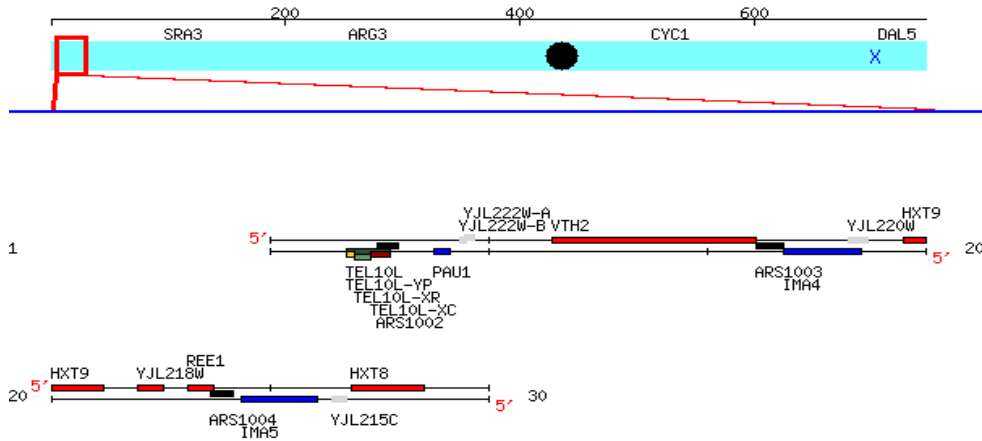


Figure 2. 7-D Chromosome X features that span coordinates 6767 - 28536 bp.

[It shows the ORF location of both *IMA4* and *IMA5* in the subtelomeric region.]

When comparing these genes two by two, *IMA3* and *IMA4*, which are located on different chromosomes, are 100% identical (Table 2.4). *IMA2* is 98% identical to *IMA3* (id. to *IMA4*), and *IMA1* possesses 89 and 88% identities with *IMA2* and *IMA3* or *IMA4*, respectively. *IMA5* is the most distant within the family, because it only exhibits an overall 67% sequence identity with the other four genes. Similarity varies between the flanking sequences (promoters): from no significant (~46%) to 99.8% identity between *IMA3* and *IMA4* upstream regions.

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Isomaltases from the yeast *Saccharomyces cerevisiae*

Gene Name	Sequence identity				
	<i>IMA1</i>	<i>IMA2</i>	<i>IMA3</i>	<i>IMA4</i>	<i>IMA5</i>
<i>IMA1</i>	100				
<i>IMA2</i>	89	100			
<i>IMA3</i>	88	98	100		
<i>IMA4</i>	88	98	100	100	
<i>IMA5</i>	66	67	67	67	100

Table 2. 4 Pairwise analysis of the five *IMA* genes.

[Sequence identities at the nucleotide sequence level.]

2.4.2 Biochemical evidence for isomaltase activity

The *YGR287c/IMA1* encodes a 589 amino acids protein. This protein was characterized and our recent analysis corroborated previous results obtained by these data (Yamamoto K. *et al.*, 2004). For example, this isomaltase hydrolyzed isomaltose and α -MG but not maltose, whereas maltase was previously shown to hydrolyze maltose but not isomaltose and α -MG (Matsusaka K. *et al.*, 1977). During their preliminary biochemical characterization of the entire protein family, Teste *et al* performed an assay with *IMAx* gene overexpression in yeast cells and measure of α -glucosidase activity from cells crude extracts (Teste M.A. *et al.*, 2010).

As shown in Figure 2. 8, the determination with the hydrolysis of α -p-nitrophenyl α -D-glucopyranoside (*p*NPG) showed that they bear α -glucosidase activity. The α -1,4-glucosidase activity was measured using maltose or maltotriose as substrates, and the α -1,6-glucosidase activity was determined on isomaltose or on α -MG alternatively. Remarkable α -glucosidase activity was obtained

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on α -MG and isomaltose in crude extracts prepared from cells overexpressing *IMA1* and *IMA2*, suggesting that these two genes encode proteins cleaving preferentially α -1,6-linkages. Extracts from *IMA5*-overexpressing cells was able to act on isomaltose, but was totally unable to cleave α -MG. A slight activity could be observed on maltose, even if it was hardly higher than the activity observed in the background assay. *IMA3* and *IMA4* were weakly active on the different sugars tested, but authors suspected a broad substrate specificity with both α -1,4- and α -1,6-glucosidic activity. Nevertheless, their results showed biochemically that these five unlinked genes encode proteins bearing isomaltase activity.

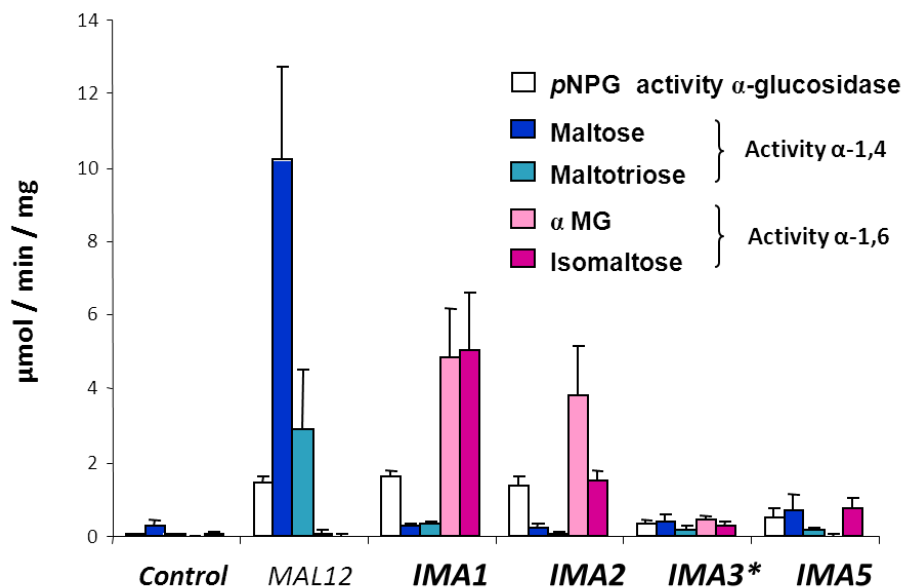


Figure 2. 8 Biochemical activities on α -1,6- and α -1,4-glucosidic substrates.

(Teste M.A. *et al.*, 2010)

[The α -glucosidase activity on different α -glucosidic substrates was assayed on crude extract from JF1811 yeast strain overexpressing *MAL12* or ORFs of this gene family. * Because *IMA3* and *IMA4* are 100% identical, they could not be differentiated. The Control JF1811 strain was transformed with the empty YEplac181-PGK/CYC1 plasmid.]

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Isomaltases from the yeast *Saccharomyces cerevisiae*

To have an insight of *MAL* gene families (see also below, §2.4.4), Brown and coworkers analyzed the capability of yeast strains expressing these genes, to grow on a different set of substrates. Combinatorial knockouts of *MALS* alleles in *Saccharomyces cerevisiae* S288c, overexpression of *MALS* alleles in S288c, and purified enzyme assays of encoded proteins, were used therefore to determine *MALS* allele specificity (see Figure 2. 9). They suggested that clades (*YOL157C*, *YJL216C*, and *YGR287C*) have evolved specificity for the carbohydrates, such as palatinose, isomaltose, and methyl- α -glucoside.

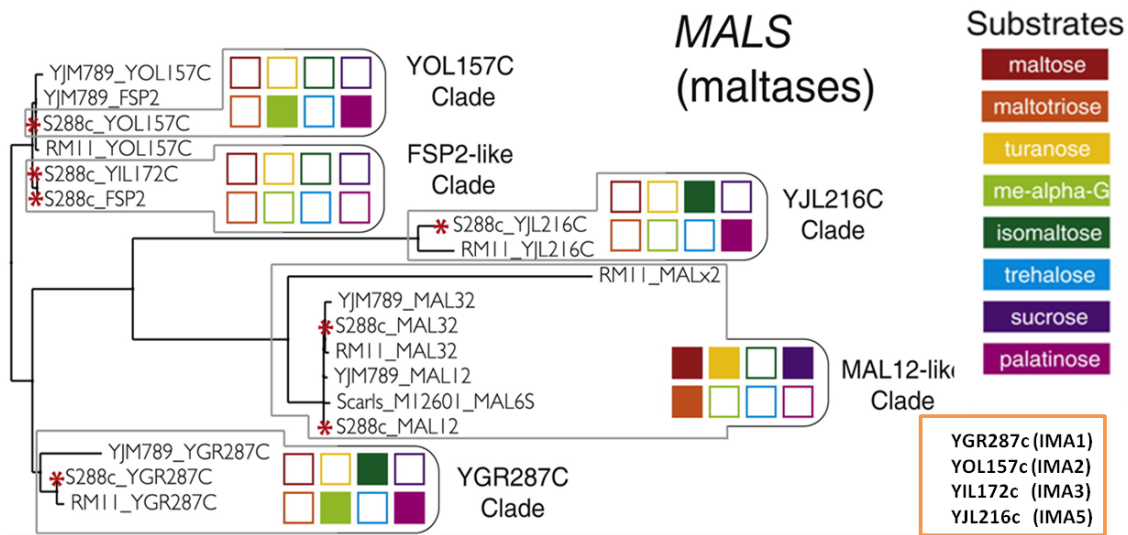


Figure 2. 9 Gene families correlated with specificity towards specific substrates

(Adapted from Brown C.A. *et al.*, 2010)

[The phylogeny of the MAL maltases, determined from multiple protein sequence alignments, is shown for alleles from *Saccharomyces cerevisiae* strains S288c, YJM789, and RM11, as well as alleles deposited in GenBank from *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. The MAL maltases subfamily is outlined with grey boxes, in which specificity is denoted by a colored barcode. A red asterisk to the left of the allele name denotes that the function of this allele was experimentally investigated. The function of alleles that are not marked by an asterisk was not experimentally verified (in these cases, the function was only inferred from the sequence similarity with other alleles in the same clade). Activity of a subfamily is summarized for maltose (red), maltotriose (orange), turanose (yellow), methyl- α -glucoside (lime green), isomaltose (green), trehalose (light blue), sucrose (purple), and palatinose(magenta). Activity towards a specific substrate is indicated by a solid colored square, while lack of activity for a specific substrate is depicted by white boxes with colored outlines.]

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2.4.3 Amino acid sequence and structural elements of isomaltase

Isomaltase consists of 589 amino acid residues (Yamamoto K. *et al.*, 2004). Alike to other GH13 family members, isomaltase possesses the four highly conserved sequence regions (consensus I to IV). Although regions I, II, and IV were rigorously identical, the consensus III sequence showed some variability between these five proteins, as illustrated in Figure 2. 10.

	I	II	III	IV
		↓		
		★	★	★
<i>MAL12</i>	106-DLVINH-111	209-DGFRIDTAG-217	275-GEVAH-279	344-YIENHD-349
<i>MAL32</i>	106-DLVINH-111	209-DGFRIDTAG-217	275-GEVAH-279	344-YIENHD-349
<i>YGR287c/IMA1</i>	107-DLVINH-112	210-DGFRIDVGS-218	276-GEMQH-280	347-YIENHD-352
<i>YOL157c/IMA2</i>	107-DLVINH-112	210-DGFRIDVGS-218	276-GEMQH-280	347-YIENHD-352
<i>YIL172c/IMA3</i>	107-DLVINH-112	210-DGFRIDVGS-218	276-GEMRH-280	347-YIENHD-352
<i>YJL221c/IMA4</i>	107-DLVINH-112	210-DGFRIDVGS-218	276-GEMRH-280	347-YIENHD-352
<i>YJL216c/IMA5</i>	102-DLVINH-107	205-DGFRIDVGS-213	271-GEVGI-275	342-YIENHD-347
		↑		

Figure 2. 10 Four conserved regions of isomaltase

(Teste M.A. *et al.*, 2010)

[ClustalW amino acid sequence alignment of *MAL12*, *MAL32* as well as five *IMA* genes encoding maltase and isomaltase respectively. The three most important catalytic residues were written in boldface and marked with a star. The arrows indicated the position of the amino acid residue that discriminates α -1,6- (Val) from α -1,4- (Thr) linkages of glucosidic substrates.]

Particularly, *Ima1p* and *Ima2p* possessed the same GEMQH consensus; the *Ima3p* and *Ima4p* consensus was GEMRH, whereas the *Ima5p* consensus diverged from the others with the GEVGI sequence. Despite these differences, the Glu277 E residues is nevertheless conserved and probably involved in acid/base catalysis (McCarter J.D. and S.G. Withers, 1996). All members proteins indeed hold the catalytic triad formed in *Ima1p* by the Asp215 (nucleophile), Glu277 (proton donor) and Asp352 (transition-state stabilizer).

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The three-dimensional structure of numerous GH13 family members has been determined. The first high-resolution three-dimensional structure of GH13 family was determined for Taka-amylase A in 1984 (Matsuura K. *et al.*, 1984). In 1997, the crystal structure of oligo-1,6-glucosidase from *Bacillus cereus* was solved (Watanabe K. *et al.*, 1997). Then the structures of dextran glucosidase in an uncomplexed form, and of its mutant in complex with isomaltotriose, were determined (Hondoh H. *et al.*, 2008). A latest 3D structure of GH13_31 was reported by Caner *et al.* They not only presented a 3D structure but also revealed an oligosaccharide binding site (Caner S. *et al.*, 2013).

Almost simultaneously, the crystal structure of Ima1p from yeast *Saccharomyces cerevisiae* was solved at resolution of 1.30 Å, underlying the importance of some amino acids in the structure of active site (Yamamoto K. *et al.*, 2010) The overall structural features of isomaltase are similar to the other GH 13 family members (Yamamoto K. *et al.*, 2010). Isomaltase consists of three domains: A, B, and C, as shown in Figure 2. 11. Domain A (residues 1–113 and 190–512) consists of eight alternating parallel β -strands and α -helices, which make up the $(\beta/\alpha)_8$ -barrel common to GH family 13 enzymes. Domain A also includes the catalytic residues Asp215, Glu277 and Asp352 at the C-terminal side of the barrel. Domain B (residues 114–189) possesses a loop-rich structure containing one short helix and an antiparallel β -sheet. It is notable that the fold of domain C differs from those of other GH 13 family enzymes. Domain C (residues 513–589) of *Saccharomyces cerevisiae* isomaltase comprises an antiparallel five-stranded β -sheet; thus, only half of the eight-stranded β -barrel motif close to domain A is present in Ima1p. One calcium ion is bound at the loop located just before helix 1 of domain A (Figure 2. 11). This calcium ion has an octahedral geometry coordinated by the side chains.

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Isomaltases from the yeast *Saccharomyces cerevisiae*

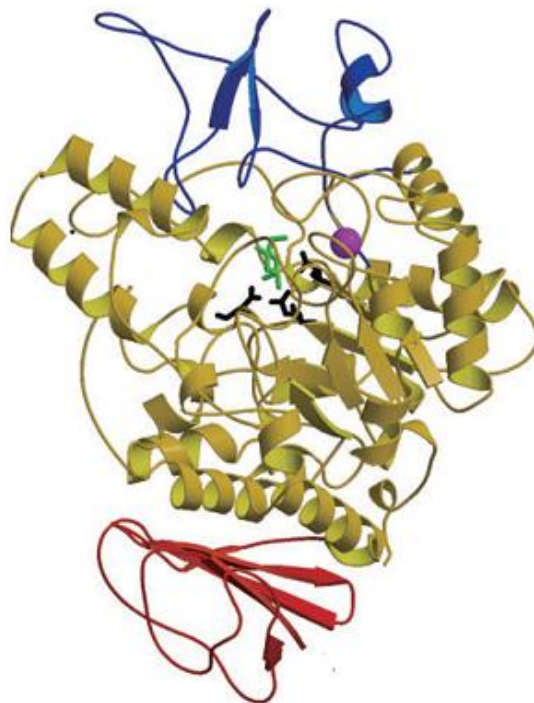


Figure 2. 11 Stereoview of the overall structure of isomaltase in complex with maltose

(Yamamoto K. *et al.*, 2010)

[Domain A is shown in yellow, domain B in blue, and domain C in red. A calcium ion is shown as a magenta sphere. The reducing end of the glucose residue is displayed in green. The three catalytic acidic residues are shown as a stick model.]

For the α -1,6-glucosidic linkage hydrolyzing enzymes, mutational analyses indicated that Val216, located just after the catalytic nucleophile in the consensus region II, must be involved in the recognition of α -glucosidic linkages and was clearly identified as a key signature of α -1,6 hydrolytic activity, contrary to the Thr residue in maltases (Yamamoto K. *et al.*, 2004; Hondoh H. *et al.*, 2008;). The detailed structural analyses of ligand binding was performed by Yamamoto with an activity-deficient isomaltase mutant, E277A (Glu277Ala) and obtention of crystal structures in complex with isomaltose at resolutions of 1.80 Å (Yamamoto K. *et al.*, 2011). As shown in Figure 2. 12 at subsite -1, the non-reducing end glucose residue of isomaltose is bound at the bottom of the active site pocket by 8 hydrogen bonds, and it stacks against Tyr72. The binding manner is the same as that observed in the structure of the isomaltase-maltose complex (Yamamoto K. *et al.*, 2010). Six of the 8

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Isomaltases from the yeast *Saccharomyces cerevisiae*

hydrogen bonds are conserved among α -amylase and other GH family 13 enzymes (MacGregor E.A, 2001). Yamamoto *et al.* also showed that two isomaltase mutant enzymes – V216T and Q279A – hydrolyzed maltose. Thus, the amino acid residues at these positions may be largely responsible for determining the substrate specificity of α -glucosidases (Yamamoto K. *et al.*, 2011).

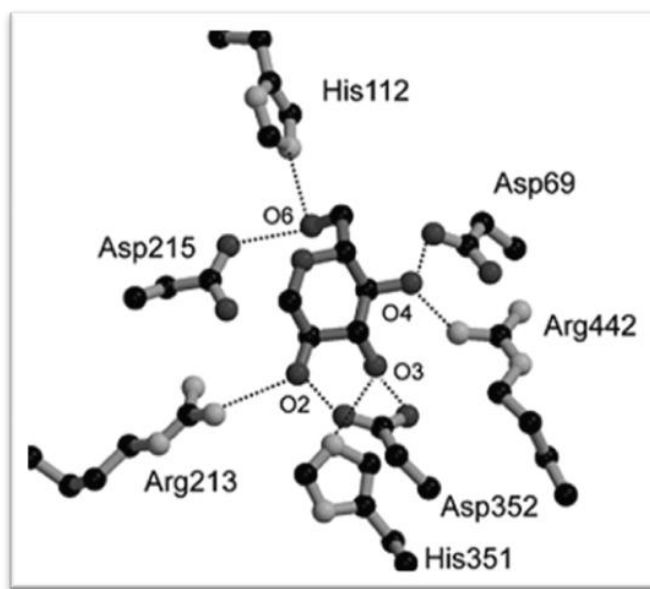


Figure 2. 12 The active site structure of E277A-isomaltose

(Yamamoto K. *et al.*, 2011)

[Non-reducing end of glucose residue is bound to subsite -1. Hydrogen bonds are depicted by broken lines.]

2.4.4 Enzyme evolution

2.4.4.1 Isomaltase in phylogenetic tree

To construct the phylogenetic tree of α -glucosidases, Naumoff and Naumov performed pairwise and multiple comparison of ten catalytic domains of proteins from the GH13 and GH31 families of *Saccharomyces cerevisiae* S288C (Naumoff D.G. and G. I. Naumov, 2010). The phylogenetic analysis (shown in Figure 2. 13) demonstrated the existence of two distinctly isolated α -glucosidase clusters (with a more than 99% bootstrap support). One cluster combines three maltases; the other, isomaltase

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and its three close paralogs. In the diagnostic site, all the three maltases comprise the Thr–Ala–Gly tripeptide whereas four proteins from the isomaltase cluster bear the Val–Gly–Ser tripeptide.

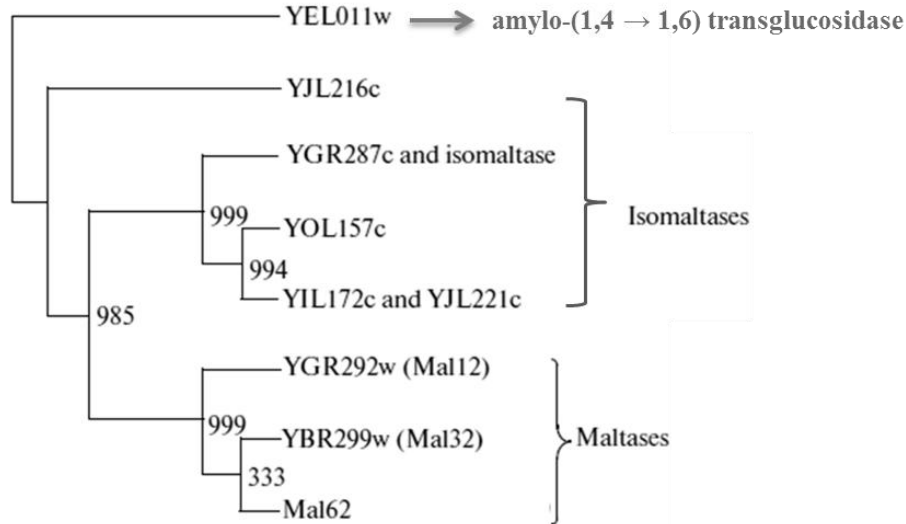


Figure 2. 13 Phylogenetic tree of GH13 family α -glucosidases

(Adapted from Naumoff D.G. and G. I. Naumov, 2010)

[The phylogenetic tree was constructed by the maximum-parsimony method using the PHYLIP program package. The maltase and isomaltase were from other *S. cerevisiae* strains rather than S288C. The YEL011w was used as an outgroup. The statistical significant of phylogenetic tree nodes was estimated using bootstrap analysis; The number of confirming pseudoreplicas (out of 1000) is indicated near each node. The curly brackets mark the cluster corresponding to isomaltases and maltases.]

In order to establish robust groups that show an improved correlation between sequence and enzymatic specificity, Stam has performed a large-scale analysis of 1691 family GH13 sequences by combining clustering, similarity search and phylogenetic methods (Stam M.R. *et al.*, 2006). In their annotated phylogenetic tree (Figure 2. 14), all the subfamilies correspond to a subtree of the phylogenetic tree. Isomaltase encoding genes cluster in the GH13_31 subfamily, while maltases(α -glucosidases) distribute in GH13_17 and GH13_21.

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Isomaltases from the yeast *Saccharomyces cerevisiae*

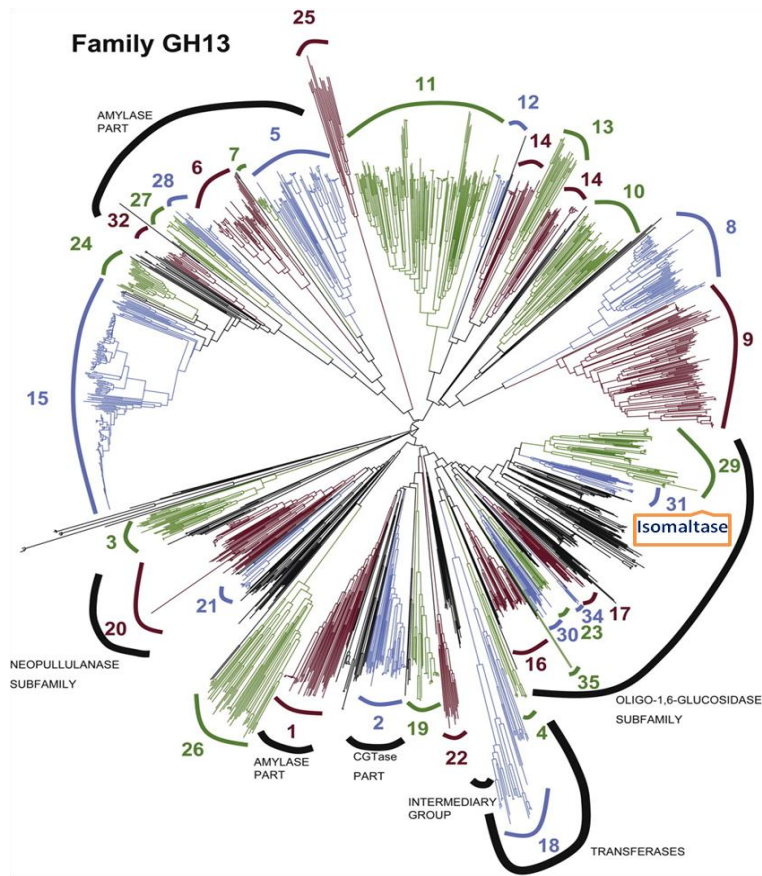


Figure 2. 14 Phylogenetic tree of family GH 13

(Stam M.R. *et al.*, 2006)

[Sequences classified into subfamilies 1–35 are shown in color. The sequences that were not included into subfamilies appear in black. The external black arcs cover subfamilies that were made previously]

2.4.4.2 Duplication events and functional divergence

Brown *et al.* studied the evolution and functional divergence of subtelomeric gene families in the yeast lineage (Brown C.A. *et al.*, 2010). They focused on three related subtelomeric *MAL* gene families that show typical patterns of rapid expansion and evolution. Recently, the same authors still used as a model system these *IMA* and *MAL* gene families (Voordeckers K. *et al.*, 2012) to further study the molecular mechanisms that govern evolution of duplicated genes (Conant G.C and K.H. Wolfe, 2008; Innan H. and F. Kondrashov, 2010). In order to determine the activity of all resurrected ancestral enzymes for different substrates, Voordeckers and coworkers have done the reconstruction of

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Isomaltases from the yeast *Saccharomyces cerevisiae*

a series of ancestral metabolic enzymes (Voordeckers K. *et al.*, 2012). The activity data presented in Figure 2.15 showed how this promiscuous ancestral protein with relatively poor activity for several substrates evolved to the seven present-day enzymes that show high activity for a subset of substrates, and little or no activity for others. In two separate instances, a major shift in specificity was observed, from maltose-like sugars to isomaltose-like sugars (transition from *ancIMA5* to *IMA5*, and from *ancMAL-IMA* to *ancIMAI-4*). The shift in activity from *ancMAL-IMA* to *ancIMAI-4* was particularly pronounced. The *ancMAL-IMA* enzyme hydrolyzed maltose, sucrose, turanose, maltotriose, and maltulose but had hardly any measurable activity for isomaltose and palatinose, whereas *ancIMAI-4* can only hydrolyze isomaltose and palatinose (and also sucrose). According to these authors, duplication enables an organism to circumvent adaptive constraints on a multifunctional gene by optimizing the subfunctions separately in different paralogs.

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Isomaltases from the yeast *Saccharomyces cerevisiae*

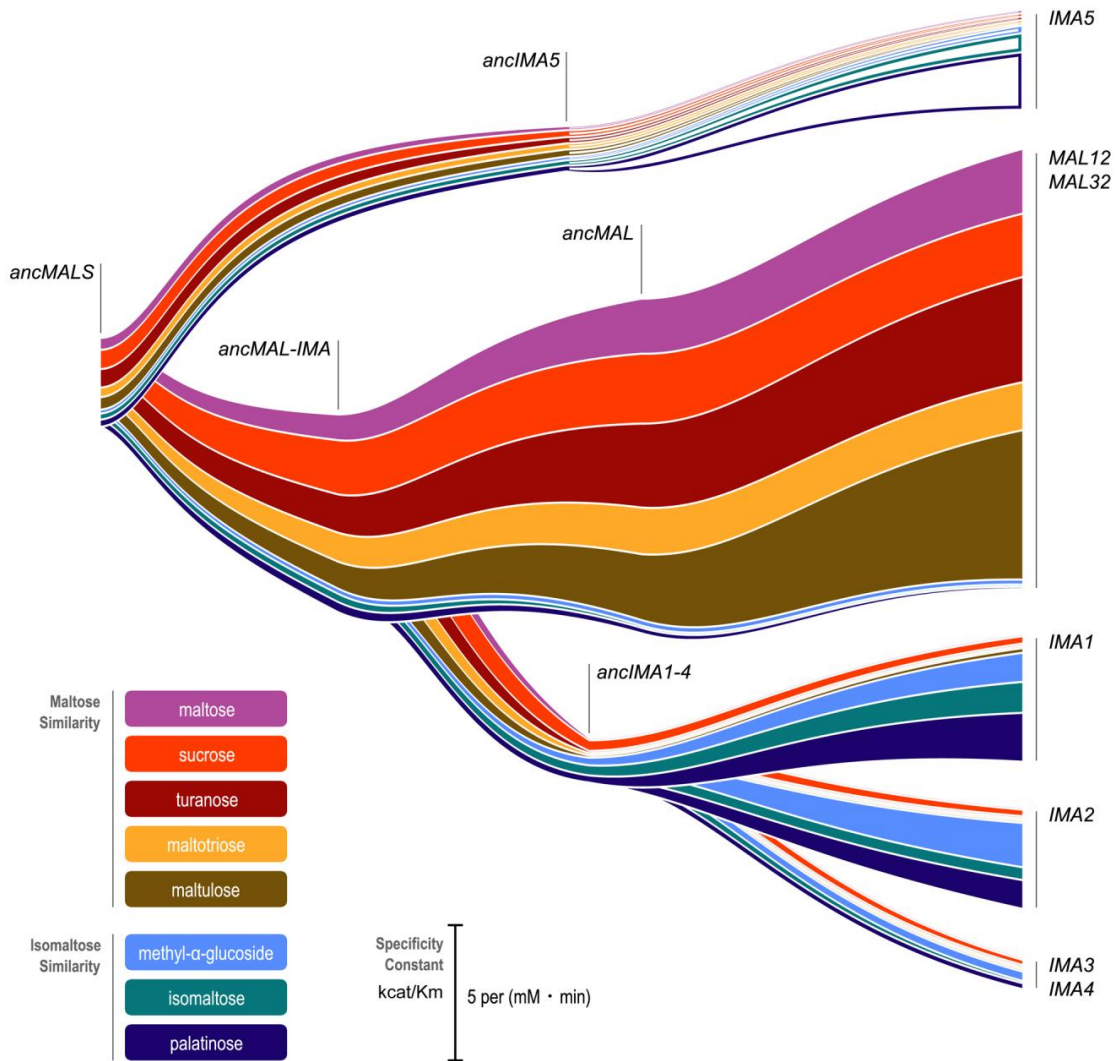


Figure 2. 15 Duplication events and changes in specificity and activity in evolution of *Saccharomyces cerevisiae* MalS enzymes.

(Voordeckers K. *et al.*, 2012)

[The hydrolytic activity of all seven present-day alleles of Mal and Ima enzymes as well as key ancestral (anc) versions of these enzymes was measured for different α -glucosides. The width of the colored bands corresponds to k_{cat}/K_m of the enzyme for a specific substrate. Note that in the case of present-day Ima5, no purified protein was obtained active. Here, the width of the colored (open) bands represents relative enzyme activity in crude extracts derived from a yeast strain overexpressing *IMA5* compared to an *ima5* deletion mutant. While these values are a proxy for the relative activity of Ima5 towards each substrate, they can therefore not be directly compared to the other parts of the figure.]

3 RESULTS AND DISCUSSIONS

Before undertaking the comprehensive and systematic characterization of the biochemical and enzymological properties of *S. cerevisiae* IMA proteins, the construction of plasmids containing *IMAx* genes, along with their expression in the host *Saccharomyces cerevisiae* and their purification were fundamental. In addition, conditions for measurement of optimal enzymatic activity had to be established. The major part of the present PhD study was then the biochemical and enzymological characterization of the four *S. cerevisiae* IMA proteins and their mutagenized variants.

RESULTS AND DISCUSSIONS

Purity and stability of IMA proteins

3.1 Purity and stability of IMA proteins

3.1.1 Expression and purification of IMA proteins

For the characterization of the isomaltase family, we expressed and purified the recombinant IMA proteins from *Saccharomyces cerevisiae*. Firstly, *IMAx* genes were amplified by PCR and cloned into the yeast expression pYES2.1/V5-His-TOPO[®] vector carrying GAL1 promoter and 6xHis Tag. When cells were cultivated, the expression from the GAL1 promoter was induced with galactose for 6 hours. With the His tag, IMA proteins were purified with affinity cobalt resin.

A key step of purification is to identify the purity of proteins. Therefore, we loaded these purified proteins along with crude extract on a gel and analysed their purity by SDS-PAGE. We could observe after Coomassie-blue staining of the gel that the recombinant protein was the major band in the cell lysate (Figure 3.1, line 1) and the only visible band after purification (lanes 2 & 4-6), with apparent MW of approximately 70 kDa. Therefore, we could consider high purity of these IMA proteins, suitable for further enzymological characterization.

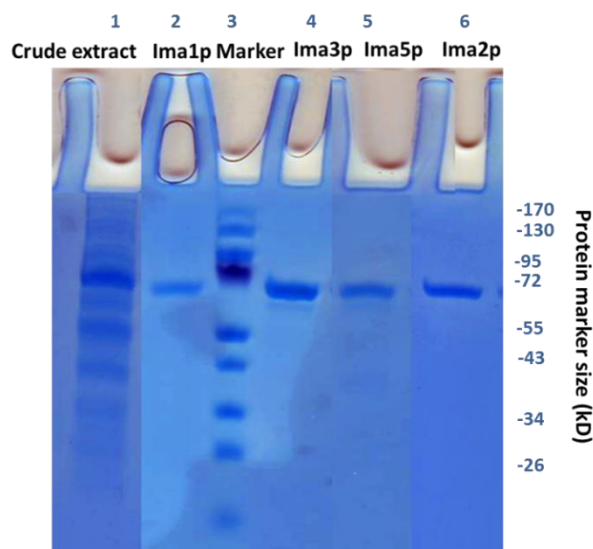


Figure 3. 1 SDS-PAGE of of recombinant IMA proteins from *Saccharomyces cerevisiae*.

[Line 1, IMA protein crude extract; Line 3, protein marker; Line 2/4/5/6, purified IMA proteins following the cobalt affinity chromatography purification. Gels were revealed by Coomassie Blue]

RESULTS AND DISCUSSIONS

Purity and stability of IMA proteins

3.1.2 Evaluation of conditions for stabilization and perservation

Following purification, it is often necessary to store proteins for extended periods of time before performing detailed biophysical, enzymatic, and structural studies. Therefore, it is essential that the pure target protein maintains its original biological (or functional) behavior over an extended period of storage. Generally, enzymes can be stored under appropriate temperature and pH range, in the presence of glycerol, BSA or similar substances to retain activity and prevent aggregation. Various conditions were therefore tested for optimizing long term storage and preserve activity of our enzymes (*i.e.* 24h storage or more).

Dignum and coworkers reported that the addition of certain amount of glycerol could be a good method for the storage of β -glucosidase (Dignum M.J.W. *et al.*, 2001). Therefore, the influence of glycerol was determined firstly. Two concentrations of glycerol (25%, 40%) were tested at 4°C and -20°C respectively (Table 3.1). The results showed that after one night, without addition of glycerol, the four IMA proteins kept approximately 60% of their activities when conserved at 4°C, while they were almost totally inactivated after freezing at -20°C. In presence of 25% glycerol, all these IMA proteins kept 60-80% of activity, except for Ima3p with only 25% remaining activity after one night at -20°C. Notably for Ima1p and Ima2p, the residual activity ratio exceeded 80% overnight at both 4°C and -20°C. However, in spite of an increase of glycerol concentration to 40%, no positive effect was observed, especially for Ima3p and Ima5p that were totally inactivated. We therefore confirmed that glycerol at moderate concentration (25%) helped to preserve activity to a certain extent, but not enough to significantly improve the storage.

RESULTS AND DISCUSSIONS

Purity and stability of IMA proteins

Conditions	Residual activity (%)							
	Ima1p		Ima2p		Ima3p		Ima5p	
		Remove imidazole		Remove imidazole		Remove imidazole		Remove imidazole
Fresh proteins (T ₀)	100	100	100	100	100	100	100	100
4 °C	67,9	42,8	77,3	52,9	57,8	88,4	70,6	80,4
-20 °C	10,7	41,5	10,5	61,2	0	85,4	0,9	89,3
Glycerol 25% 4°C	84,3	52,2	85,7	59,4	59,4	89,0	70,8	86,9
Glycerol 25% -20 °C	82,9	51,6	-	59,0	25,0	86,6	56,9	89,8
Glycerol 40% 4°C	54,3	46,1	84,5	60,2	0	57,3	0	7,6
Glycerol 40% -20 °C	80,0	55,7	72,2	70,7	0	72,0	1,4	13,5

Table 3. 1 Influence of freezing and glycerol for overnight conservation

[In all cases, *p*NPG 5mM was used as the substrate, and determined in the Citrate-Potassium Phosphate buffer pH7, at 30°C. These data were obtained from an average of 3 independent assays. “-” denoted to value needed further conformation]

Previous results indicated that the presence of imidazole did not interfere with proteins, and the stabilizing effect of imidazole could be of use for His-tagged proteins (Hamilton S. *et al.*, 2003). Molina *et al.* published the influence of imidazole on measuring the specific activities of enzymes, with the aim of characterizing its interference (Molina F. *et al.*, 1996). In this study, we removed imidazole that was added during the last step of the purification (elution), to verify whether this compound did not interfere with the storage of isomaltases. As can be seen in the table, Ima3p and Ima5p were more stable in the absence of imidazole under all the six conditions. Significantly, preserved at -20°C, Ima3p kept more than 85% of activity after removal of imidazole, while it was nearly inactivated in its presence. Contrary to our expectation, the activity of Ima1p and Ima2p dropped more after removal of

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imidazole, regardless of the conditions. We therefore had no clear evidence to show the stabilizing effect of imidazole for all the four isomaltases.

Ammonium sulphate is commonly used to precipitate and store proteins for long term, as it is usually completely innocuous and preserves the native state of proteins. In this assay, ammonium sulfate was mixed to the protein solutions. Khan and Eaton published that the addition of $(\text{NH}_4)_2\text{SO}_4$ could stabilize the yeast alpha-methyl glucosidase during purification and long-term storage (Khan N. A. and N. R. Eaton, 1967). Following their report, the effects of 5% and 10% $(\text{NH}_4)_2\text{SO}_4$ were tested in the present work (shown in Table 3.2). It was confirmed that the addition of 10% $(\text{NH}_4)_2\text{SO}_4$ on purified isomaltases was able to maintain almost 100% of their specific activity after freezing for 24 hours. Subsequently four days' storage, the residual activity ratio for Ima1p in presence of 5% and 10% $(\text{NH}_4)_2\text{SO}_4$ were 25.9%, 79% respectively. Ten days later, this activity nevertheless dropped by 20% with 10% $(\text{NH}_4)_2\text{SO}_4$ and even worse to only 9.3% activity remaining with 5% $(\text{NH}_4)_2\text{SO}_4$.

	Fresh protein		OVERNIGHT		4 DAYS		10 DAYS		
Residual Activity Ratio (%)			Concentration of $(\text{NH}_4)_2\text{SO}_4$						
		Control	2,50%	5%	10%	5%	10%	5%	10%
		100	6,1	2,4	87,8	98,3	25,9	79	9,3

Table 3. 2 Storage of Ima1p in presence of $(\text{NH}_4)_2\text{SO}_4$ at -20°C

[pNPG 5mM was used as the substrate, and determined in the Citrate-Potassium Phosphate buffer pH7, at 30°C . The result came from one experiment with an average of 3 technical replicates]

Further, more conditions were performed. We studied the influence of pH value with the addition of Tris at three concentrations. Meanwhile, we also determined the long term storage for one week in the same assay, including in presence of 25% glycerol. Our results were shown in Table 3.3. Either at pH 6 or pH 7, we didn't observe any improvement for long term storage. Tris and glycerol, no

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matter the concentration used, provided slightly longer residual activity of the four IMA proteins. There was nevertheless a remarkable decrease of activity after one week. Moreover, Tris apparently affected the stability of four IMA proteins. The higher concentrations of Tris, the more unstable were these proteins. Particularly for Ima1p and Ima2p following one week storage, when the concentration of Tris was up to 20mM, there was only approximately 20% activity resting. Table 3.3 also indicated that Ima5p was the most difficult protein to preserve, because after one week of storage, this enzyme lost over half of its activity.

Conditions	Residual activity ratio %										
	Fresh protein	Control	pH6				pH7				
				Tris 5mM	Tris 10mM	Tris 20mM	Gly 25%	Tris 5mM	Tris 10mM	Tris 20mM	Gly 25%
Ima1p overnight	100	69,3	72,0	69,3	32,0	16,0	96,0	80,0	58,7	45,3	84,0
Ima1p 1 week	100	65,3	60,0	56,0	45,3	19,3	72,0	45,3	34,7	26,7	76,0
Ima2p overnight	100,0	64,6	71,5	43,1	24,6	18,5	78,5	53,8	41,5	29,2	76,2
Ima2p 1 week	100,0	63,1	60,0	36,9	24,6	16,9	36,9	41,5	30,8	21,5	76,2
Ima3p overnight	100,0	45,5	60,6	60,6	60,6	54,5	63,6	66,7	60,6	54,5	63,6
Ima3p 1 week	100,0	42,4	36,4	48,5	42,4	36,4	27,3	54,5	42,4	30,3	45,5
Ima5p overnight	100,0	61,9	47,1	51,1	49,2	43,8	58,6	61,3	56,2	53,7	57,6
Ima5p 1 week	100,0	50,6	43,3	50,2	47,3	43,2	23,3	54,9	51,1	46,3	40,5

Table 3. 3 Influence of pH in presence of Tris and glycerol for conservation at 4°C

[In all cases, *p*NPG 5mM was used as the substrate, and determined in the Citrate-Potassium Phosphate buffer pH7, at 30°C. These values were calculated from an average of 3 technical replicates]

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Indeed, various conditions were attempted during the present work, such as the addition of glycerol, Tris or BSA (no data shown), the removal of the imidazole after protein purification, the influence of freezing (at 4°C, -20°C and -80°C, not shown), as well as under pH 6 and pH 7, and so on. However, none of these conditions could improve the stabilization and long term preservation of protein activity, to consider storage of these proteins before their use. Besides, taking into account pH and thermal inactivation processes (see below), we verified that the 4 isomaltases nevertheless kept their activity at 30°C in the Citrate-Potassium phosphate buffer pH 7.0, for at least 2 hours, which was enough for kinetic characterization. Therefore, Citrate-Potassium phosphate buffer pH 7.0, 30°C, was taken as the standard condition for all the measurements presented below in this manuscript, and all the enzymatic analyses were performed with freshly prepared and cation-free purified proteins.

3.1.3 Effects of chemical reagents and metal ions

The influences of various compounds and metal ions were also tested. The activity was measured with 5 mM *p*NPG as substrate in the Citrate-Potassium Phosphate buffer, pH 7 at 30°C in the absence (Control) or in the presence of different effectors (Figure 3.4). Glycine, urea as well as EDTA had no effect on IMA proteins with the exception of Ima3p, which was slightly inhibited by these compounds. All isomaltases were nevertheless highly sensitive to 5 mM Tris in the reaction mix with approximately 90% drop of activity. About metals, iron under its ferric Fe³⁺ form significantly inhibited the four enzymes, especially Ima1p with only 8% residual activity in the presence of 5mM Fe³⁺. Other cations, including Ca²⁺, had no effect on isomaltase activity, with the exception of Mg²⁺ and Mn²⁺ that slightly enhanced Ima1p activity.

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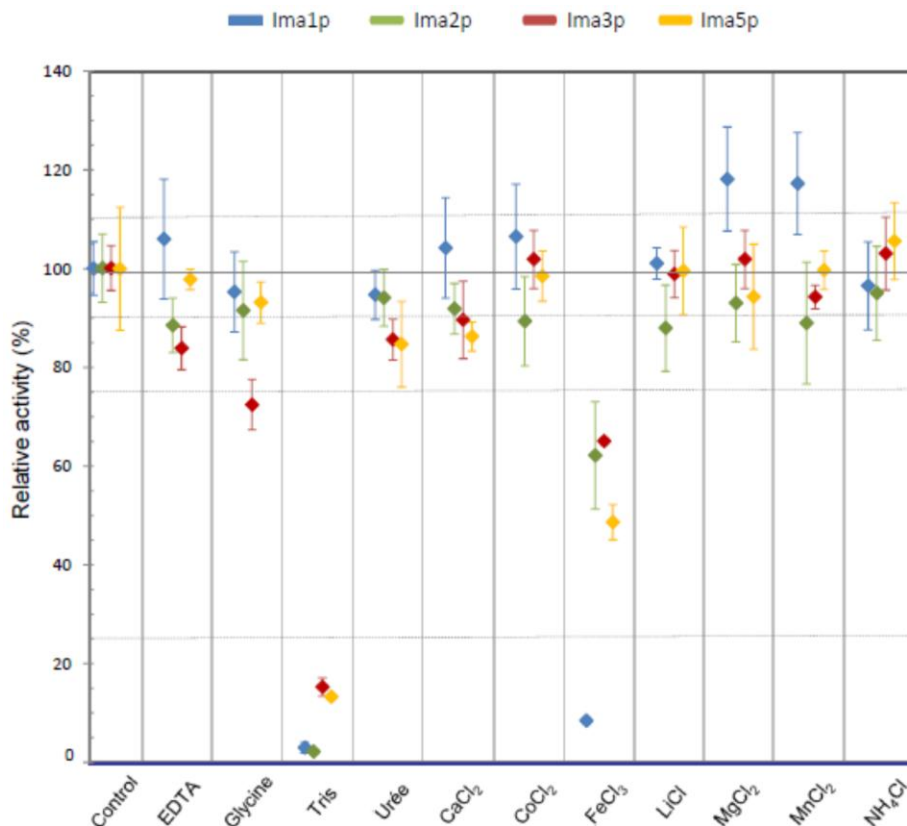


Figure 3. 2 Effects of chemical reagents and metal ions on purified isomaltases

[All these compounds were performed at 5 mM final concentration with the exception of 1 mM MnCl₂]

Glycine can be found in a series of buffers which provide an interesting set of solutions with limited side effects and in the range for most enzyme activities (Good N. E., *et al.*, 1966). Even though the results showed that there was no effect of glycine on most IMA proteins, the slight inhibition of Ima3p by glycine prevented a general use of buffers with glycine for studying this protein family. Our results also definitely excluded any possibility to use Tris-based buffers since all IMA proteins totally lost activity in the presence of this compound. For all these reasons, amine (Tris- and glycine-based) buffers which are widely used in protein purification (Stoll V.S. and J.S. Blanchard, 2009), have to be excluded in the study of IMA proteins.

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Contrary to a previous report (Herning T. *et al.*, 1992), the addition of $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixture did not increase their activities. The result (Table 3.4) showed that the ammonium sulfate significantly reduced their relative activities, with a drop to 75 % to nearly 15% residual activity when it was added at 1.25 to 10 % in the reaction mixture, respectively.

Relative activity ratio %	Control	Concentration of $(\text{NH}_4)_2\text{SO}_4$				
		1,25%	2,50%	3,50%	5%	10%
	100,00	75,6	67,2	51,2	34,4	15,3

Table 3. 4 Effects of $(\text{NH}_4)_2\text{SO}_4$ on purified Ima1p

[*p*NPG 5mM was used as the substrate, and determined in the Citrate-Potassium Phosphate buffer pH7, at 30°C. The result came from two assays with an average of 6 technical replicates]

As already briefly introduced, the metal ions such as Li^{1+} , Ca^{2+} , and Co^{2+} , did not change with statistical significance the activity of Ima isoforms. A striking result was the positive effect of Mg^{2+} and Mn^{2+} , only observed for Ima1p, which is contrary to inhibitory effect of these cations on *B. cereus* and *B. coagulans* enzymes (Suzuki Y. *et al.*, 1982; Suzuki Y. and Y. Tomura1986). Besides, with a perfect conservation of all 5 residues involved in the coordination of calcium in all IMA proteins shown in Figure 2.11 (Yamamoto K. *et al.*, 2010), it was suspected that Ca^{2+} probably played a role in optimizing activity of yeast isomaltases. Such a role was actually demonstrated for alpha-amylases (Vallee B. L. *et al.*, 1959) and more interestingly for the SmDG (*Streptococcus mutans* dextran glucosidase), a GH13 enzyme that identically to yeast isomaltase, bears a calcium ion tightly bound to the first beta-alpha loop of the core barrel (Kobayashi M. *et al.*, 2011). However, the addition of Ca did not change the activity of any of the IMA proteins.

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An inhibition by cobalt might have been a serious technical drawback, since it is used during protein purification of His-tagged IMA proteins. Although a normal use of the resin according to manufacturer instructions does not lead normally to metal leaching, cobalt concentrations in the range of μM in the elution fractions have been reported (Block H. *et al.*, 2009). This concentration is however much lower (1000-fold) than the concentration of cobalt that has been tested in the Ph.D's work. Therefore, any suspicion of inhibition of IMA protein activity by cobalt through leaching of the metal from the affinity resin had been deliberately excluded from the experiments.

In addition, our results clearly showed that EDTA, which is a chelating agent, had no effect on isomaltases activity with the exception of Ima3p (Figure 3. 4). In deep contrast with the importance of metals for the activity that was particularly emphasized, we can therefore argue that IMA proteins do not probably rely on the presence of a bound metal ion for their activity. We cannot however exclude that EDTA failed to chelate calcium due to tight binding of this ion as it was previously proposed (Yamamoto K. *et al.*, 2010). Alternatively, if chelation of calcium occurred during long-term incubation of the enzyme in the presence of EDTA, this result confirmed that fixation of calcium to its binding site is totally useless for protein activity.

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3.2 Biochemical characterization of IMA proteins

3.2.1 Optimal pH

This assay was determined with 5mM *p*NPG at 30°C. Optimal pH of the four isomaltases was obtained from the curve, activity as a function of pH value, by varying the pH from 2.6-9 in the appropriate buffer, *i.e.* the citrate-potassium phosphate broad-range buffer, allowing to test the activity at pH values in the range 2,6-8,0, and in the sodium phosphate buffer (range 7,0-9,0) to extend the analysis to higher pH values. Indeed, we tested the influence of buffer previously These IMA proteins exhibited the same stability and activity with the two buffers, both tried at pH 7 and pH 8 (no data shown), which did not prevent to perform this assay in a broader range of pH with different buffers. The four proteins presented similar pH optima between 7 and 8 (Figure 3.3). They were all inactive below pH 5,0 and significantly drop of activity above pH 8,0. The optimal pH of Ima1p, Ima2p, Ima3p and Ima5p were 7, 7.6, 8, and 7 respectively.

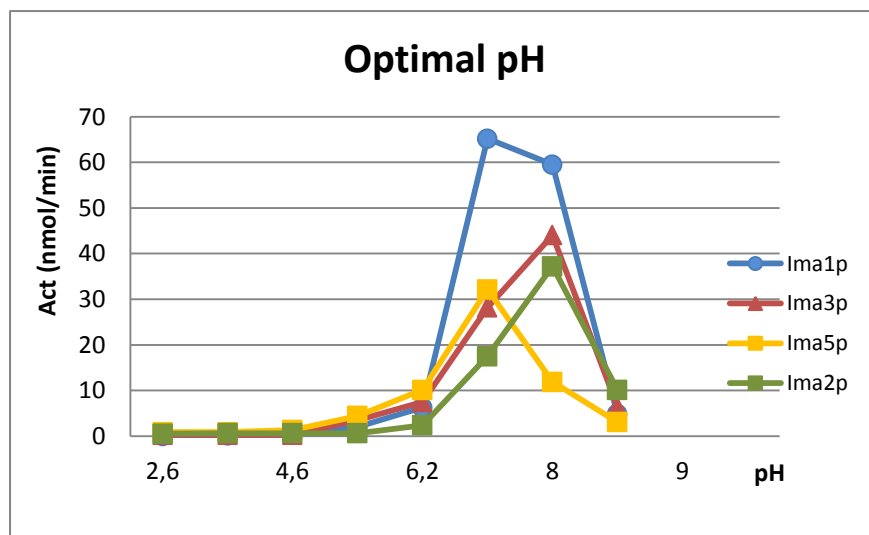


Figure 3. 3 pH dependence of isomaltases activity (Substrate: *p*NPG)

[Optimal pH was determined with 5mM *p*NPG at 30°C, from the curve activity as a function of pH by varying the pH from 2.6-10 in the appropriate buffer. (* Ima3p and Ima5p: $\times 10^{-1}$)]

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In general, the optimal pH reported for fungal α -glucosidases have been in the range of 3.5-5.0, whereas pH optima of yeast α -glucosidases tend to be in the range 6.5-7.0 (Kelly C. T and W.M. Fogarty, 1983). These points in Figure 3.3 were higher than the optimum pH of 5.5 reported for the α -glucosidase from yeast *Xanthophyllomyces dendrorhous* (Marin D. *et al.*, 2006), but agreed with other published data for yeast alpha-methyl glucosidase (Herning T. *et al.*, 1992). Furthermore, the optimal pH exhibited by these IMA proteins was in the range of the intracellular pH of yeast *S. cerevisiae* (OrijR. *et al.*, 2011).

However, the very narrow pH window for *in vitro* activity measurement did not allow deviating from this pH range, at risk of significant fall of activity but also rapid time-dependent deactivation of the protein as soon as the pH increases. We indeed observed that all IMA proteins faced denaturation with rapid loss of activity when assayed above their optimum pH, even at 30°C. Interestingly, Ima2p was the most sensitive protein to pH. It lost for example 50% of its activity after 6 minutes in solution at pH 8.0.

3.2.2 Optimal Temperature

The temperature optimum was determined with 5 mM *p*NPG as substrate in the Citrate-Potassium Phosphate buffer pH 7, by varying the temperature between 20°C and 60°C. These four isomaltases showed rather variable temperature optima in the range 36-46°C (see Figure 3.4). The optimum temperature for activity was slightly more discriminative than optimum pH. The Ima5p was the most singular with a maximal activity at 36°C, whereas the optimum temperature was 43°C for Ima2p and 46°C for both Ima1p and Ima3p. At temperatures higher than the optimum, the activity abruptly dropped with a complete loss of activity above 45°C for Ima5p.

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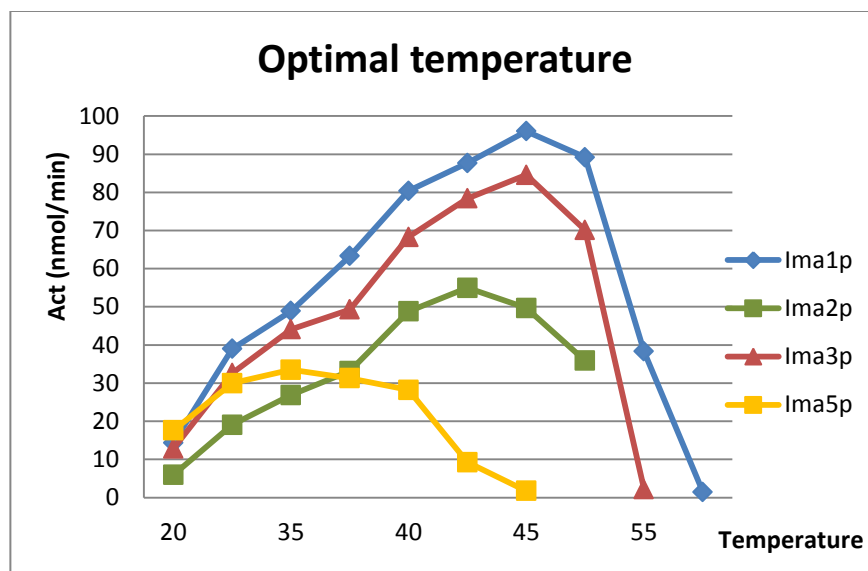


Figure 3. 4 Temperature dependence of isomaltases activity (Substrate: *p*NPG)

[The assay was determined with 5mM *p*NPG in the Citrate-Potassium Phosphate buffer pH7 by varying the temperature between 20°C and 60°C (* Ima3p and Ima5p: $\times 10^{-1}$)]

Indeed, the four proteins lost their activity above 55°C, which was similar to the α -glucosidases from *Bifidobacterium breve* and various *Bacillus species* (Suzuki Y. *et al.*, 1982; Yamamoto M. and K. Horikoshi, 1990; Van den Broek L. *et al.*, 2003; Pokusaeva K. *et al.*, 2009). However, the optimum temperature of IMA protein was totally different from that of α -glucosidase from a Hyperthermophilic Archaeobacterium, *Pyrococcus furiosus*, which exhibited a temperature optimum of 105 to 115°C (Costantino H.R. *et al.*, 1990). These values therefore consolidated the role of IMA proteins as intracellular catalysts in the yeast *S. cerevisiae*, a mesophile organism, and was for example in deep contrast with the high optimum activity at 60°C that has been reported for the yeast *Lipomyces starkeyi* extracellular α glucosidase (Kelly C. T. *et al.*, 1985).

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3.2.3 Half-Life

The half-life of IMA proteins was performed with 5 mM *p*NPG as substrate in the Citrate-Potassium Phosphate buffer pH 7. It was also observed that the kinetic curves declined rapidly with time (see Figure 3.5), even under standard conditions (either optimal pH or optimal temperature). This progressive decline demonstrated that isomaltase was subjected to inactivation or denaturation, leading to a progressive loss of activity. Thus the assay was carried out with each enzyme a time-dependent heat inactivation analysis at various defined temperatures.

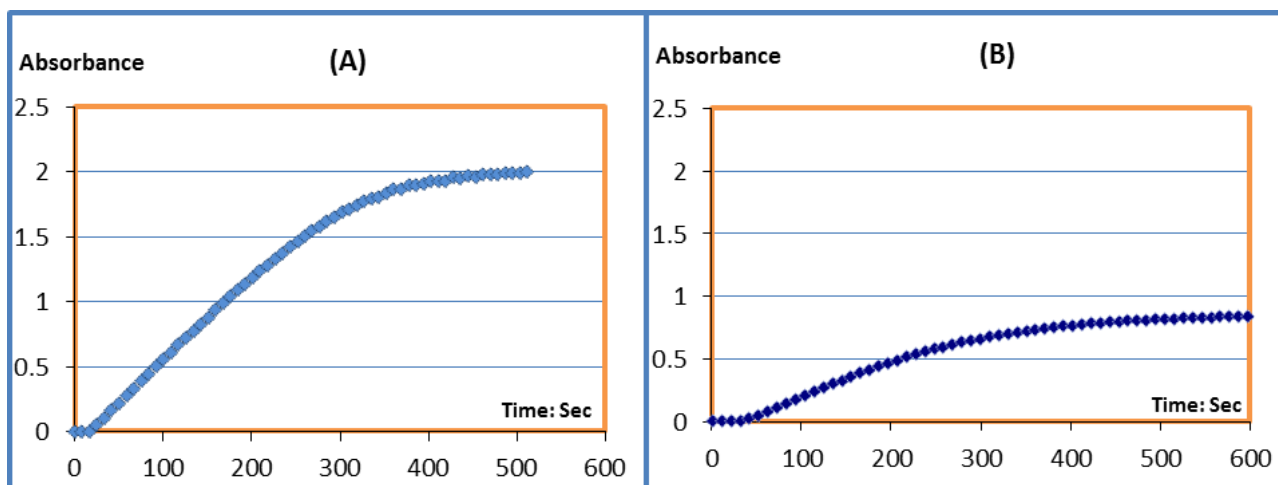


Figure 3. 5 the kinetic curves of isomaltase activity assay (Substrate: *p*NPG)

[This activity assay was performed under standard conditions (5 mM *p*NPG as substrate in the Citrate-Potassium Phosphate buffer pH 7 at 30°C). **Figure A** explicitly displayed that isomaltase activity followed the linear increase, until the technical limit (for this colorimetric reaction of *p*NPG, linearity is lost at the time when the absorbance reaches 1.5, with a plateau at 2. **Figure B** is an example of quick and continuous drop of activity, which completely stops around 500 seconds of reaction (plateau at 0.8 in this example, far below the technical limit of 1.5). In such a situation, estimation of initial velocity (100%) requires working during the first seconds of the reaction (Initial linear phase, or tangent to declining curve). At each time-point, tangent of the curve yields to the residual activity value.]

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Therefore, the half-life ($t_{1/2}$) was determined by incubating the enzyme at the specified temperature (37°C, 40°C or 42°C) (see Table 3.5). A temperature of 37°C already led to some drop of enzyme activity. While Ima5p activity fell to half the initial velocity after 3 minutes (half-life, $t_{1/2}$), the half-life of Ima3p was 23 min, whereas Ima2p / Ima1p were the most stable proteins with $t_{1/2}$ around 27 minutes. When the same experiment was performed at 40°C, the half-life was around 1 min for Ima5p, 8 min for Ima3p and 18 min for Ima1p / Ima2p, which illustrated the dramatic sensitivity of isomaltases to increasing temperature. As the temperature increased up to 42°C, the half-life was 11 min for Ima2p, whereas Ima3p decreased to 3 min.

Enzyme	Half life (min)		
	37°C	40°C	42°C
Ima1p	26.1 ± 1.7	17.6 ± 1.7	nd
Ima2p	27.4 ± 1.6	18.5 ± 1.6	11.2 ± 0.7
Ima3p	23.2 ± 1.4	7.6 ± 0.9	2.9 ± 0.5
Ima5p	2.9 ± 0.4	0.8 ± 0.1	nd

Table 3. 5 Half life of IMA protein at different temperatures

[The half-life was determined with 5mM *p*NPG in the Citrate-Potassium Phosphate buffer pH7 by incubating the enzyme at the specified temperature and by measuring the residual activity as a function of time. “nd”: not determined under the conditions of the assay]

The study essentially pointed out the low stability of the purified proteins towards heat, with rather short and heterogeneous half-life values within the family. A half-life in the minute range has been formerly reported by Khan and Eaton for purified yeast alpha-methyl glucosidase (*i.e.* isomaltase) exposed to 51°C (Khan N. A. and N. R. Eaton, 1967). Our data for Ima1p and Ima2p, which were the

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most stable proteins, were consistent with *Bacillus cereus* glucosidase (Watanabe K. and Y. Suzuki, 1998) when referring to their thermostability standard, for instance, the temperature required for 50% inactivation by 10 minutes incubation.

Thinking to the putative stabilization of the enzymes with cytosolic solutes, the transposition of the results to the *in vivo* stability of yeast isomaltases was especially difficult. The preliminary study of our team indeed indicated that the use of crude extract for assaying the thermal stability of the proteins led to significantly higher half-life values at 40°C, with $t_{1/2}$ higher than one hour for Ima1p and Ima2p and around 10 minutes for Ima5p. Such sugar-dependant proteins are commonly highly expressed while the inducer (substrate) is present in the culture medium. If IMA proteins are also highly unstable *in vivo*, the low stability of these cytosolic proteins may therefore be a mean to recover quickly the initial protein pool as soon as the substrate has been totally consumed. Western blots on the different members of the family, under different conditions of growth, could be a mean to check the correlation between *in vitro* data and *in vivo* stability.

In addition to initial reaction rates measurement, the stability of isomaltases through continuous reaction kinetics was evaluated (Figure 3. 6). These identical thermal denaturation curves for kinetics were determined up to 30 min, with and without EDTA. As shown in Figure 3. 6, all these four IMA proteins decreased their activity, whose residual activity ratios were lower than 60%. Apparently, these proteins faced almost the same loss of activity. Besides, we didn't observe obvious difference between absence and presence of EDTA. Our results suggested that EDTA was not responsible of any metal chelation and inactivation of the enzymes.

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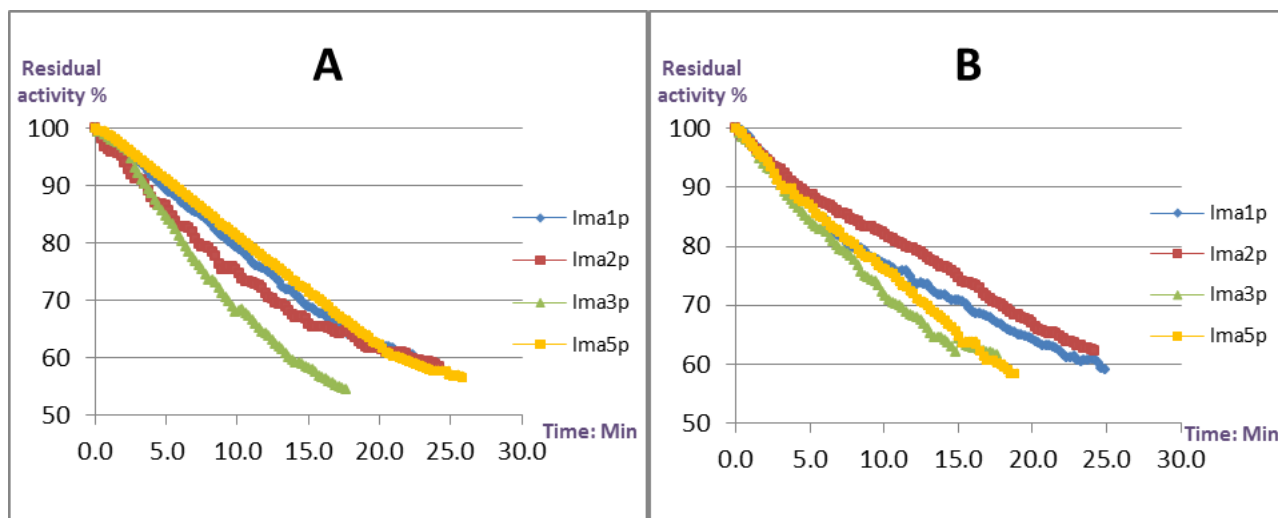


Figure 3. 6 Influence of EDTA on purified isomaltases at 30°C.

[The influence of EDTA was measured with 5 mM *p*NPG in the Citrate-Potassium Phosphate buffer pH 7.

Figure A, in the absence of EDTA; Figure B, in the presence of 10mM EDTA]

3.2.4 Melting Temperature

DSF experiments strengthened the variable sensitivity of the different proteins towards temperature. The melting temperatures (T_m) of the purified proteins were around 36°C, 39°C and 46°C for Ima5p, Ima3p and Ima1p or Ima2p, respectively (Table 3.6, pH 7). These values were remarkably influenced by the pH value since a decrease by only one pH unit (pH 6) increased the T_m by approximately 9°C, while an increase by one unit (pH 8) decreased the T_m by 5°C for Ima1p and by 13°C for Ima2p. In deep contrast, the T_m of Ima5p was almost insensitive to pH. A remarkable point within this IMA protein family was therefore the great heterogeneity of melting temperature values.

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Enzyme	Melting Temperature (°C)		
	pH 6	pH 7	pH 8
Ima1p	55.5 ± 0.6	46.6 ± 0.7	41.6 ± 0.2
Ima2p	55.1 ± 0.2	46.2 ± 0.8	33.2 ± 0.6
Ima3p	47.0 ± 0.3	38.8 ± 0.5	29.9 ± 0.3
Ima5p	36.6 ± 0.4	36.3 ± 0.4	35.7 ± 0.5

Table 3. 6 Melting temperature of IMA protein at different pH

[The melting temperature (T_m) was determined by Differential Scanning Fluorimetry (DSF), from five independent replicates for each IMA protein.]

The putative salt-bridge network is a relevant aspect for thermal stabilization, and may therefore differentiate these proteins. Especially, the presence of histidine-based salt bridges probably explains the huge impact of the pH on the melting temperature of Ima1p, Ima2p and Ima3p (Heyda J. *et al.*, 2010). Histidine is indeed the only amino acid whose pKa of the side chain may allow changes of electrical charge when increasing the pH value of the buffer from 6 to 8. The crystal structure of Ima1p revealed that five histidine-based salt-bridges could be identified (H7-E9; H98-D210; H252-E296; H280-D307; H505-E562)(Yamamoto K. *et al.*, 2010). Particularly, residue H280 is salt-bridged to D307, but this salt-bridge does not exist in Ima5p due to the substitution of H280 and D307 by I and S, respectively. However, in the absence of Ima5p crystal structure, it is difficult to verify whether these His-based salt bridges are really conserved and if alternative bridges may form. Though conversely to Ima1p, the independence of Ima5p T_m upon changing pH did not support the possibility of additional histidine-based bridges formation and/or their functional significance in this protein.

It is also known that the presence of ions and ligands into the binding site of an enzyme can strongly influence the melting temperature. For instance, many α -amylases, especially from GH13, are known to require Ca^{2+} to stabilize protein structures and increase melting temperature (Gupta R. *et al.*, 2003). Matulis *et al.* have observed a potential correlation between thermal stability and ligands affinity

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(Matulis D. *et al.*, 2005). A series of ligands were therefore analysed. The cations Ca^{2+} , Mg^{2+} and Mn^{2+} had positive influence on thermostability of the four proteins (See Table 3.7). Mg^{2+} enhanced the T_m about 2°C for Ima1p and Ima2p, whereas only 1°C for Ima3p and Ima5p. Fe^{3+} , EDTA, as well as glycine and had no effect on T_m of all proteins.

Ligands	Enzyme			
	Ima1p	Ima2p	Ima3p	Ima5p
Control	46.6 ± 0.7	46.2 ± 0.8	38.8 ± 0.5	36.3 ± 0.4
Tris	52.1 ± 0.4	52.0 ± 0.5	45.7 ± 0.3	42.8 ± 0.2
EDTA	47 ± 0.6	47 ± 0.2	38.7 ± 0.1	36.6 ± 0.1
Glycine	46.7 ± 0.2	45.7 ± 1.0	38.0 ± 0.7	36.2 ± 0.2
MgCl_2	48.9 ± 0.5	48.7 ± 0.6	39.8 ± 0.4	38.1 ± 0.1
CaCl_2	50.3 ± 0.2	50.2 ± 0.5	41.8 ± 0.7	41.0 ± 0.5
FeCl_3	47 ± 0.2	47.1 ± 0.1	38.5 ± 0.2	36.0 ± 0.1
MnCl_2	50.8 ± 0.2	50.7 ± 0.2	41.5 ± 0.8	40.2 ± 0.4

Table 3. 7 The influence of metal ions and ligands on IMA proteins

[The influence was determined from three independent assays with three parallel technical replicates for each metal ion and ligand on IMA proteins.]

Very interestingly however, it was observed that T_m values were significantly enhanced (about 5°C) in the presence of Tris, a substrate-mimicking molecule, which otherwise strongly inhibited these enzymes. This result could be consistent with the finding that a Tris molecule can bind the active site of GH13 enzymes such as amylosucrases (Skov L.K *et al.*, 2001; Guerin F. *et al.*, 2012; Champion E. *et al.*, 2012) and interfere with flexibility in this critical region (Skov L.K *et al.*, 2013). This significant effect probably leads to enhanced stability and favorable context for Ima5p crystallization perspectives. Strikingly and contrary to what might have been expected, the presence of ligands which were also found to bind the catalytic site (Yamamoto K. *et al.*, 2010) did not affect the melting temperature. Indeed, besides maltose, trehalose and melibiose were also tested as ligands to influence the melting temperature of the isomaltases. However, they played no apparent role on T_m of all the four proteins (results not shown).

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As already indicated in chapter 2.4.3, all IMA proteins have a perfect conservation of all 5 residues involved in the coordination of calcium (see Figure 2.11). We therefore investigated whether this cation may play a role in enhancing T_m of yeast isomaltases. Our results showed that both Ca^{2+} and Mn^{2+} increased approximately 3.5°C of the T_m for all the proteins. However, Calcium did increase T_m of all isomaltases which was in deep contrast to the previous results. They emphasized the importance of metals for SmDG along with alpha-amylases of various origins (Kobayashi M. *et al.*, 2011), whereas the calcium ion had no effect on specific activity nor half-life values of yeast isomaltases. The conservation of the residues involved in calcium coordination, which probably allows the tight binding of this ion, therefore raises the question of its functional relevance for yeast isomaltases.

3.2.5 Thermostabilization by single proline substitution

There are only three different amino acids of Ima2p *versus* Ima3p (A54/T54, P240/L240 and Q279/R279). The two proteins exhibited remarkable difference in their thermal stability, which prompted to investigate the importance of these substitutions in the enhanced thermotolerance of Ima2p relative to Ima3p. The proline residue at position 240 therefore appeared as a potential clue for the enhanced thermostability of Ima2p as compared to Ima3p, as supported by numerous articles emphasizing the role of proline residues in thermostabilization (Herning T. *et al.*, 1992; Li Y. *et al.*, 1997; Muslin E. H. *et al.*, 2002; Zhou C. *et al.*, 2010). To analyze this contribution, the two single mutations were constructed, L240P variant of Ima3p, R279Q variant of Ima3p (as a reference to study the proline residue). To understand the functions of proteins at a molecular level, it is necessary to know the protein primary structure which refers to amino acid linear sequence of the polypeptide chain. Primary sequences of isomaltases give more insight in the structure and function of the four proteins (Figure 3.7).

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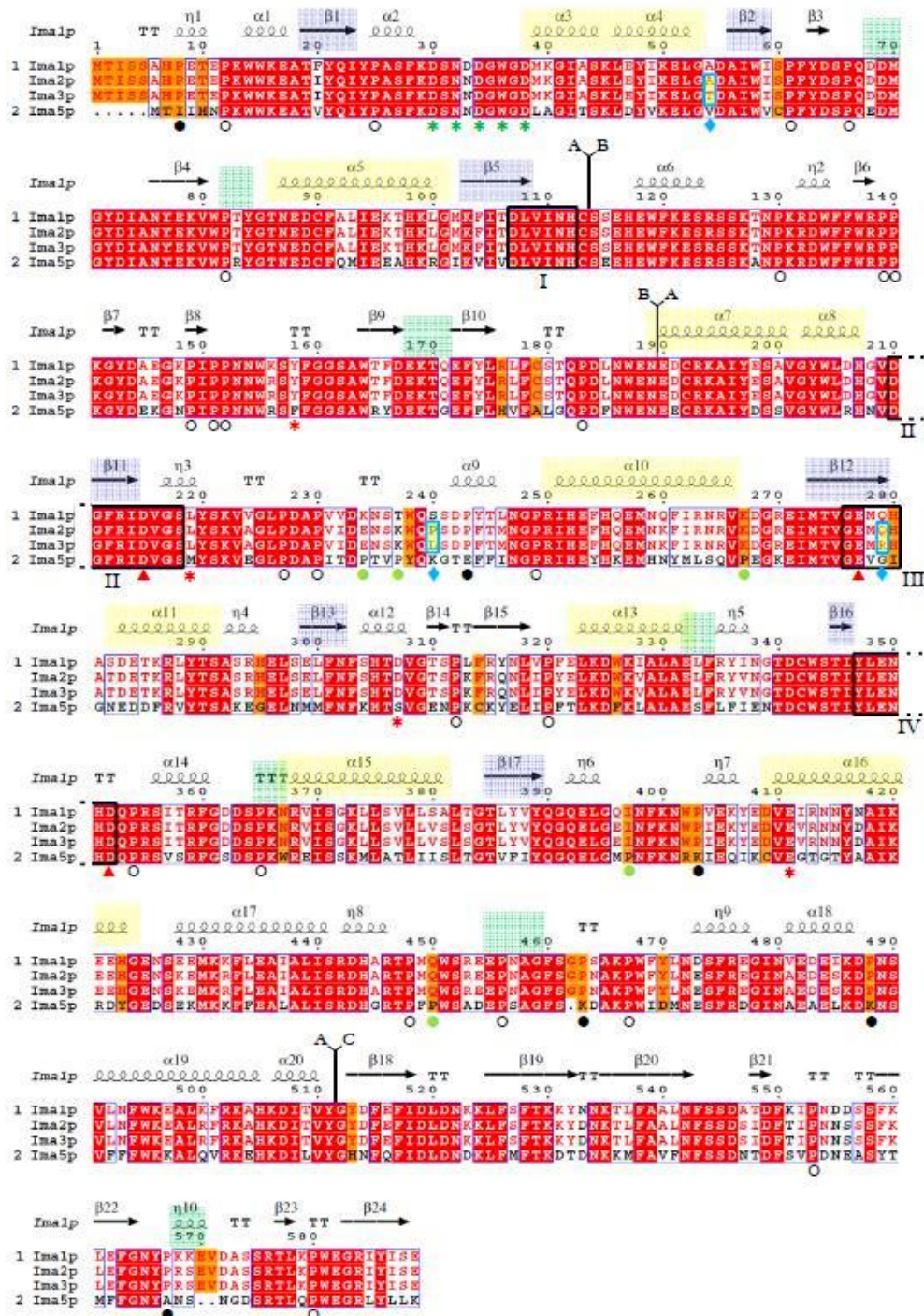


Figure 3. 7 Primary sequence alignment of the IMA proteins

This multiple sequence and structural alignment was generated with ESPrpt (Gouet P. *et al.*, 2003), from a Clustal W multiple sequence alignment of the four yeast isomaltases and secondary structure informations identified from the Ima1p PDB entry (ref 3AXH, *i.e.* cristal structure of Ima1p in complex with isomaltose). Numbering relative to Ima1p sequence. Conserved regions of the GH13 family (Black boxes); Three amino acids differentiating Ima2p and Ima3p (Blue diamonds); Catalytic triad as identified in Ima1p (Red triangles); Few amino acids amongst the variable residues mapped for substrate binding or located near the binding pocket (Red stars); Residues characterized in Ima1p as involved in the calcium binding (Green stars). Proline residues: Present in all four proteins (White dots); Only lacking in Ima5p (Black dots); Exclusively present in Ima5p (Green dots). Limits of the three independent folds (A|B, B|A, A|C). Eight α -helix and β -strands from the $(\alpha/\beta)_8$ barrel shaded in yellow and blue, respectively. Strict β -Turns as identified by EsPrpt (TT), and turns as depicted in the PDB entry (shaded in green)

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Half-life of Ima3p_R279 Q and Ima3p_L240P were also measured at the 37°C, 40°C and 42°C in triplicate. In addition, their melting temperatures were determined only under pH 7. The result shown in Table 3.8 illustrated that the mutation Ima3p_L240P led to a significant increase of the half-life, which positively shifted from 23.2 (Ima3p) to 26.5 min when the proteins were incubated at 37°C, or from 7.6 to 16.2 min at 40°C (Compared with Table 3.5). These indistinguishable half-life values of the Ima3p_L240P and Ima2p at both 37°C and 40°, demonstrated that the P240 residue is important for thermal stability of Ima2p. However, the half-life of Ima3_L240P variant was slightly lower than Ima2p (8.5 vs. 11.2 min) when the proteins were incubated at 42°C. To compare half-life of all these isomaltases family members, two figures of heat inactivation were drawn (Figure 3. 8).

Enzyme	Half life (min)			T_m (°C)
	37°C	40°C	42°C	pH 7
Ima2p	27.4 ± 1.6	18.5 ± 1.6	11.2 ± 0.7	46.2 ± 0.8
Ima3p	23.2 ± 1.4	7.6 ± 0.9	2.9 ± 0.5	38.8 ± 0.5
Ima3p_R279Q	9.5 ± 1.7	2.0 ± 0.3	nd	37.6 ± 0.8
Ima3p_L240P	26.5 ± 2.2	16.2 ± 1.8	8.5 ± 1.3	39.7 ± 0.4

Table 3. 8 Half life and melting temperature of single mutations

[The half-life was determined with 5mM *p*NPG in the Citrate-Potassium Phosphate buffer pH7; The melting temperature was determined by DSF; “nd”: not determined under the conditions of the assay]

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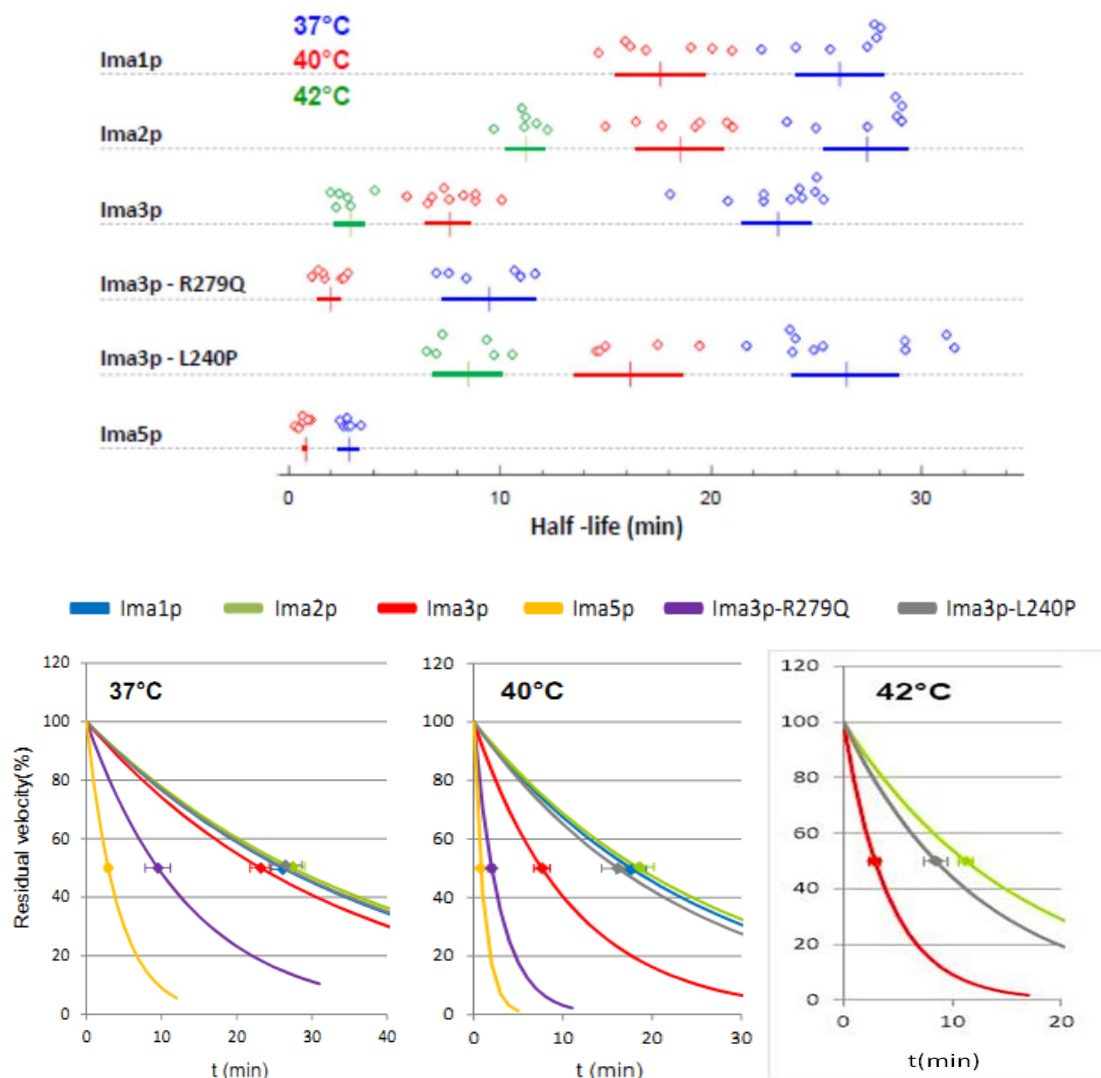


Figure 3. 8 Heat inactivation of isomaltases and variants

Upper panel: Half-life of isomaltases during heat inactivation [Enzymes were incubated at 37 °C (blue), 40°C (red) or 42°C (green) and activity decay was followed as a function of time. Half-life values ($t_{1/2}$, squares) were determined as described in the M&M section from an average of 7 independent curves for each temperature. Samples size and distribution allowed applying for some pairs of proteins a Student's t-test to ascertain that their respective mean, which are reported in table 1 as $t_{1/2} \pm 2$ SEM, are statistically different (confidence level of 95%, right column of the table).]

Lower panel: Models of time-dependent heat inactivation of isomaltases [Representative plots of the activity decay as a function of time, during incubation at 37 °C, 40 °C and 40 °C. Each curve of residual velocity ($v(t)$, in % of the initial reaction rate), was drawn from the equation: $v(t) = 100 e^{-(t \ln 2 / t_{1/2})}$, where $t_{1/2}$ is the mean half-life. Half-life was obtained from an average of 7 independent heat inactivation curves.]

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Moreover, DSF-based melting temperature results also showed that this proline substitution in Ima3p did not allow recovering the whole Ima2p thermal characteristics. Here, the L240P mutation did increase the T_m value from 38.8°C (Ima3p) to 39.7°C (L240P variant), but it remained much lower than the 46.2°C T_m of Ima2p. This result indicated that although the proline residue at position 240 in Ima2p was crucial to the difference in thermal stability between Ima2p and Ima3p, it was not the sole determinant. Considering that Ima3p_R279Q as a comparison, it slightly decreased the T_m to 37.6 °C, whereas it displayed a clear fall of the half-life from 23.2 to 9.5 min, 7.6 to 2 min when the protein was incubated at 37 °C or 40 °C, respectively.

3.2.6 Other possible issues for thermostabilization of Ima proteins

3.2.6.1 General aspects of thermostabilization

Thermostability is a beneficial character for enzyme applications. Some important factors have been revealed, which contribute to the remarkable stability of thermozymes (Fontana A, 1991; Ladenstein R. and G. Antranikian, 1998), although there is no single universal mechanism that promotes stability. Numerous studies on thermophilic properties of proteins have indicated that the stabilization mechanisms vary depending on the specific enzyme (Li W.F *et al.*, 2005). Some common features can be identified as contributing to thermostability. These aspects are as follows:

i) Amino Acid Composition

Protein amino acid composition is thought to be correlated to its thermostability. In relation to the idea that protein stability was determined by the stability and tight packing of its core, the propensity of the individual residues to participate in helical or strand structures was studied as a potential stability mechanism (Vieille C. and G. J. Zeikus, 2001). Ebrahimi *et al.* investigated the most important amino acid contributing to protein thermostability by prediction of any input protein sequence based on its amino acid composition (Ebrahimi M. *et al.*, 2011).

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ii) Prolines and Decreasing the Entropy of Unfolding

Among amino acids, proline can adopt only a few configurations and restricts the configurations allowed for the preceding residue (Sriprapundh D. *et al.*, 2000), along with its lowest conformational entropy. Suzuki reported a strong correlation between the increase in the number of proline residues and the rise in the thermostability of five oligo-1,6 glucosidases (Suzuki Y. *et al.*, 1987). Li and coworkers investigated the effect of introducing proline residues on the stability of *Aspergillus awamori* (Li Y. *et al.*, 1997). Zhou *et al.* published that thermostability was increased by introducing prolines at suitable sites in α -glucosidases (Zhou Z. *et al.*, 2010). When prolines were introduced in the corresponding locations in the *C. beijerinckii* enzyme, the residues Pro177 and Pro316 at the N termini of two helices and Pro24 in position 2 of a β -turn were shown to be stabilizing (Li C. *et al.*, 1999). Thermostability usually increased with the number of prolines introduced.

iii) Disulfide Bridges

Disulfide bridges are attributed to stabilize proteins mostly by decreasing the entropy of the protein's unfolded state. The entropic effect of the disulfide bridge increases in proportion to the logarithm of the number of residues separating the two cysteines bridged (Matsumura M. *et al.*, 1989). Genetically engineered additional disulphide bridges have been reported to increase protein thermostability by 5–30°C (Pace C.N, 1990). One successful example of thermostabilization by adding disulphide-bridges was reported for luciferase (Imani M. *et al.*, 2010).

iv) Electrostatic Interactions

It is known that thermostable proteins often have more electrostatic interactions than their less thermostable homologues (Szilogyi A. and P. Zovodszky, 2000). Typical charge-charge interactions between oppositely charged residues are known as salt bridges. Both salt-bridges and cation- π interactions are electrostatic interactions that stabilize protein structure, especially at high temperature

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(Robinson-Rechavi M. *et al.*, 2006). The increase of charged residues indicates that there are more salt bridges in proteins (Das R. and M. Gerstein, 2000).

v) **Hydrophobic Interactions**

Hydrophobic interactions are a stabilization mechanism in proteins. It was calculated that each additional methyl group buried in the enzyme gives an increase in stability of 1.3 (± 0.5) kcal mol⁻¹ (Pace C. N., 1992). Replacement of amino acids with more hydrophobic residues in a hydrophobic environment has been used as a site-directed mutagenesis strategy for example for chloramphenicol acetyltransferase (Chirakkal H. *et al.*, 2001), leading to improve of thermostability. A special case of hydrophobic interactions are the interactions between aromatic groups from the residues phenylalanine and tyrosine no further away from each other than 7 angstroms (Kumar S. *et al.*, 2000). There is one latest clear example in which extra aromatic pairs are responsible for the stability (Budyak I.L. 2013).

vi) **Hydrogen Bonds**

The number of hydrogen bonds can be correlated with the thermostability of proteins. Tanner *et al.* showed a strong correlation between GAPDH thermostability and the number of charged-neutral hydrogen bonds (Tanner J. J. *et al.*, 1996). Another example was showed by Vogt in a theoretical examination of several protein families with different thermal stabilities (Vogt G.S. *et al.*, 1997). Strong, low-barrier type of hydrogen bonds have been shown to contribute to improved stability of *T. reesei* cellobiohydrolase Cel6A at acidic pH (Wohlfahrt G. *et al.*, 2003).

vii) **Helix Dipole Stabilization**

Helix dipoles can be stabilized by negatively charged residues near their N-terminal end, as well as by positively charged residues near their C-terminal end (Vieille C. and G. J. Zeikus, 2001). Alanine residues in α -helices have been shown to stabilize the protein structure (Zhang X. *et al.*, 1995). Hennig *et al.* reported a dole-3-glycerol phosphate synthase in the *S. solfataricus* in, every helix dipole in the $(\alpha/\beta)_8$ barrel was stabilized versus six in the *E. coli* enzyme (Hennig M. *et al.*, 1995). In addition,

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Surzhik *et al.* studied the effect of point amino acid substitutions in an internal α -helix on thermostability of *Aspergillus awamori* X100 glucoamylase (Surzhik M. A. *et al.*, 2010).

viii) Metal Binding

Metals have long been known to stabilize and activate enzymes. The metal cofactor can stabilize the protein structure for example by coordinating, and thus stabilizing amino acids at the denaturation initiation site, as reported on *Bacillus licheniformis* xylose isomerase (Vieille C. and G. J. Zeikus, 2001). Indirect evidence for the role of metals in the stability of proteins is the difficulty encountered in removing the metals from the enzymes. The enhanced stability by Ca^{2+} was probably related to calcium binding in the $\beta \rightarrow \alpha$ loop 1 of the $(\beta/\alpha)_8$ barrel of SmDG (Kobayashi M. *et al.*, 2011). Most α -amylases contain a conserved calcium ion, which is essential for stability and activity (reviewed by (Prakash and Jaiswal, 2010). Since Ca^{2+} is preferentially binded, this metal is more likely than others to play a significant stabilizing role in proteins.

3.2.6.2 General comments on Ima proteins thermostabilization

With regard to *in vitro* thermostability of Ima proteins, a noticeable point within this family was the great heterogeneity of half-life and melting temperature values, except Ima1p and Ima2p that did not reveal any difference in thermal characteristics despite 39 differentiating amino acids in their primary sequence. The Ima2p/Ima3p comparison appeared as an excellent case-study since these two proteins showed evident differences in measured parameters while they differ in their primary sequence by only three amino acids. Suzuki published a strong correlation between the increase in the number of proline residues and the enhancement in the thermostability of bacillary oligo-1,6-glucosidases (Suzuki Y. *et al.*, 1987). Thereafter, it was reported that cumulative replacements with proline residues resulted in the additive enhancement in thermostability of an oligo-1,6-glucosidase from *Bacillus cereus* (Watanabe K. *et al.*, 1994; Watanabe K. *et al.*, 1997; Watanabe K. and Y. Suzuki, 1998). As a very similar

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approach, we could find that the replacement of L240 by a Proline in Ima3p strongly enhanced the thermostabilization of the protein. Since the Ima3_R279Q variant further accentuated the thermal sensitivity of the protein (Figure 3. 8), we could suggest that synergistically with this P240 residue, the short hydrophobic side chain of A54 residue found in Ima2p, which replaces the polar uncharged side chain of T54 residue, explains the residual increase of thermal stability observed for Ima2p.

Finally, as the most singular protein of the family relative to amino acid sequence, Ima5p displayed a complex situation which deeply contrasted with Ima1p and Ima2p. The sequence divergence of Ima5p virtually led to a highly unstable protein as compared to Ima1p and Ima2p. For different thermophilic enzymes, many factors contribute to stability towards temperature including disulfide bridges, compactness of the hydrophobic core, intensification of surface salt-bridge networks or alpha-helix dipole interactions, and substitution of X-to-Pro and Gly-to-Ala residues (Lehmann M. *et al.*, 2000). Some of these elements probably apply to our mesophilic proteins and may explain the clear differences among these IMA protein family members, especially for Ima5p. Ima5p contains seven cysteine residues and five of them are conserved in Ima1p, but 3D-analysis of their relative positioning in Ima1p 3-D structure indicated that none of them could initiate disulfide-bridges.

About prolines, Ima1p and Ima5p contain approximately the same number of residues (30 vs. 29, respectively, see Figure 3. 7), only few of them located differentially into these sequences and may explain the great temperature sensitivity of Ima5p isoenzyme. It is worth mentioning the location of the six proline residues specific to Ima1p. Four amongst them are located in the first N-terminus turn of alpha helices, which is regarded as a favourable position for helix stabilization, and one is found in a strict beta-turn, which also reinforces the rigidity of the loop. On the contrary, only one of the five residues specific to Ima5p is located in a beta turn, the four remaining residues being located outside assigned structural features. Altogether, these observations support a positive role of these specific

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prolines for improved thermostability of Ima1p relative to Ima5p, a hypothesis that might be tested through single or cumulative proline substitutions.

3.2.7 Conclusion

In the part, some biochemical properties of isomaltases were explored, such as optimal pH, temperature, half life as well as melting temperature. The effects of chemical reagents and metal ions, preservation conditions as well as thermostability of isomaltases were also investigated. A great effort has been done towards the biochemical characterization of highly purified enzymes. Moreover, we confirmed the central role of the proline residue at position 240 in thermal stability by inspecting thermostabilization of two proline substitutions: L240P variant of Ima3p and R279Q variant of Ima3p. From the present study. We have come to the conclusion that Ima1p and Ima2p were almost identical relative to analyzed biochemical parameters, all proteins could nevertheless be distinguished by few biochemical singularities.

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3.3 Enzymological characterization of IMA proteins

3.3.1 Substrates Specificities

Substrates specificities of purified yeast isomaltases were determined by applying a set of di- or tri-saccharides with different α -glycosidic linkages and with different type of sugars (Structures of sugars shown in Figure 3. 9). The specific enzyme activities were performed with 100mM of substrate, except for *p*NPG, which was assayed at 5mM, from an average of five independent experiments (Table 3.9). It was determined that the four isomaltases only reacted on the α -D-glucoopyranosides, since no pNP was released from β -*p*NPG as substrate. Specific activities clearly highlighted their preference for disaccharides bearing an α -1,6 bond, both between two glucose units (isomaltose, set to 100 %) and between glucose / fructose moieties (palatinose) for which relative activities with respect to isomaltose (taken as 100%) ranged from 60 (Ima5p) to 137% (Ima2p). The four proteins were ineffective on melibiose (O- α -D-galactosyl-(1 \rightarrow 6)- α -D-glucose), which indicated that a glucose moiety was required at the non-reducing end of the disaccharide for recognition and for binding the active site pocket.

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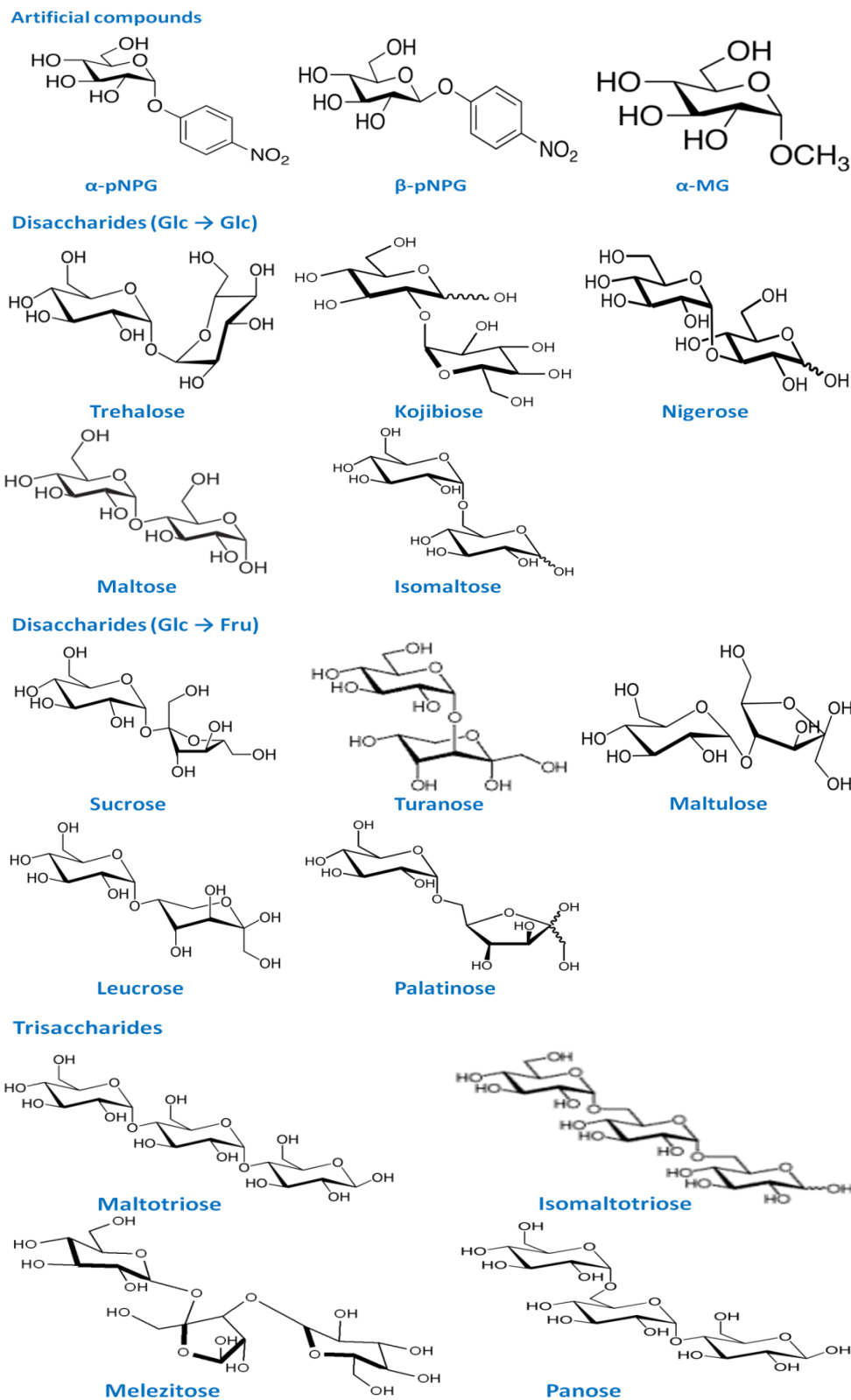


Figure 3. 9 Structures of sugars (Chair conformation)

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Substrate	Linkage	Specific activities($\mu\text{mol}/\text{min}/\text{mg}$)			
		Ima1 p	Ima2 p	Ima3 p	Ima5p
Artificial compounds					
α -pNPG	4-Nitrophenyl α -D-glucopyranoside	5.8 (10)	7.7 (14)	3.9 (54)	10 (15)
β -pNPG	4-Nitrophenyl β -D-glucopyranoside	0	0	0	0
α MG	α -Methylglucopyranoside	52 (87)	55 (102)	11 (153)	0 (0)
Disaccharides (Glc \rightarrow Glc)					
Trehalose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 1)- α -D-glucose	0	0	0	0
Kojibiose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 2)- α -D-glucose	7.7 (13)	6.9 (13)	0	2.5 (4)
Nigerose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)- α -D-glucose	6.5 (11)	6.8 (13)	2.5 (35)	4.2 (6)
Maltose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)- α -D-glucose	0	0	0	0
Isomaltose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucose	60 (100)	54 (100)	7.2 (100)	65 (100)
Disaccharides (Glc \rightarrow Fru)					
Sucrose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 2)- β -D-fructose	18.6 (31)	19.7 (36)	10.2 (142)	1.2 (2)
Turanose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)-D-fructose	4.3 (7)	3.5 (6)	1.8 (25)	1.2 (2)
Maltulose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)-D-fructose	0	0.53 (1)	0	0.36 (1)
Leucrose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 5)-D-fructose	8.6 (14)	8.6 (16)	0	1.1 (2)
Palatinose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)-D-fructose	52 (87)	74 (137)	5 (69)	39 (60)
Trisaccharides					
Maltotriose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose	3.5 (6)	3.4 (6)	0	3.4 (5)
Isomaltotriose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 6)-D-glucose	7.9 (13)	7.6 (14)	0	8.6 (13)
Melezitose (Glc \rightarrow Suc)	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)- β -D-fructosyl-(2 \rightarrow 1)-D-glucose	0	0	0	0
Panose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose	3.1 (5)	2.9 (5)	0	11 (17)

Table 3. 9 Activities of IMA proteins on different substrates

[This assay was performed in the Citrate-Potassium Phosphate buffer pH7, at 30°C. The substrate concentration was 100mM with an exception of 5mM pNPG. Between brackets: relative activity, taking as a reference for each enzyme the velocity on isomaltose (set to 100).

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Besides this activity on α -1,6 linkages, IMA proteins also exhibited α -1,2 glucosidase activity with the cleavage of substrates such as sucrose and kojibiose (Table 3.9), but with great disparity in the activity between the different isoenzymes. The activity on sucrose was approximately 35% relative to isomaltose for Ima1p and Ima2p, and only 2% for Ima5p. Interestingly, Ima3p was more active on sucrose than on isomaltose (142% relative activity), but this protein was totally inactive on kojibiose.

In an average range of 10-15% relative to the activity on isomaltose, it was found that the four IMA proteins could also hydrolyze the α -1,3 linkage of nigerose and turanose (except Ima3p), as well as the α -1,5 bond of leucrose. All these results further proved that most family members were available for hydrolyzing a much broader range of substrates than solely disaccharides bearing an α -1,6 bond. With such a substrate spectrum, IMA proteins were comparable to some broad substrate specificity α -glucosidases from honeybee (Ngiwsara L. *et al.*, 2011), *Bacillus breve* (Pokusaeva K. *et al.*, 2009), *Bacillus* sp. SAM1606 (Noguchi A. *et al.*, 2003) or *Geobacillus thermoglucosidasius* (Suzuki Y. *et al.*, 1979). We could therefore conclude that yeast isomaltase isoenzymes exhibit multispecificity, also called substrate ambiguity, meaning that they are able to act on structurally related substrates (Peric-Hassler L. *et al.*, 2010), which leads to the same class of activity and to EC numbers varying only by the fourth digit (Khersonsky O. and Dan S. Tawfik, 2010).

As it was previously observed for yeast isomaltase, all IMA proteins were totally inactive on maltose. However, it could be observed a very low, reproducible specific activity on maltulose for Ima2p and Ima5p, which was comparable to the *Bifidobacterium breve* Alg1 and alg2 glucosidases that were totally inactive on maltose while being able to cleave moderately maltulose (Pokusaeva K. *et al.*, 2009). In the same order of magnitude, the yeast *Torulaspota pretoriensis* α -glucosidase barely exhibited 1% relative activity on maltose as compared to isomaltose (Oda Y. *et al.*, 1993). Conversely

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and still under this clear-cut situation *i.e.* the possibility for an enzyme to cleave either maltose or isomaltose, not both, the *S. cerevisiae* maltase has been reported to be inactive on isomaltose (Khan N. A. and N. R. Eaton, 1967; Matsusaka K. *et al.*, 1977; Voordeckers K. *et al.*, 2012). The brewer's yeast maltase tested by Yoshikawa *et al.* showed some residual activity on isomaltose (30-fold reduction of the catalytic efficiency (Yoshikawa K. *et al.*, 1994), and the *A. niger* and barley malt α -glucosidases that also showed preference for maltose cleaved isomaltose with an *approx.* 25-fold reduction of the catalytic efficiency as compared to maltose (Chiba S. 1997; Frandsen T.P. *et al.*, 2002).

Due to the possibility for an enzyme to efficiently cleave either maltose or isomaltose, not both, which is the case for IMA proteins, there are few exceptions that could challenge this idea of adaptive conflict between maltase-like and isomaltase-like subfunctions in a single protein (Voordeckers K. *et al.*, 2012). As a counterexample, we can cite the yeast *Lipomyces starkeyi* α -glucosidase that was indeed able to use maltose and isomaltose with high and similar efficiencies (Kelly C.T. *et al.*, 1985). Likewise, the honeybee *Apis mellifera* HBG-II protein preferred maltose but was also able to use isomaltose, in contrast to the HBG-I and HBG-III isozymes that were active on maltose but almost inactive on isomaltose (Kimura A. *et al.*, 1990; Takewaki S. *et al.*, 1993; Nishimoto M. *et al.*, 2001; Ngiwsara L. *et al.*, 2011). These examples of proteins that developed activities towards both isomaltose and maltose with similar efficiencies therefore nuance to some extent the idea that the two activities cannot be fully optimized in a single enzyme and that gene duplications repeatedly spawned daughter genes in which mutations optimized either isomaltase or maltase activity (Voordeckers K. *et al.*, 2012).

With the exception of Ima3p, the three other IMA proteins displayed a measurable activity on some trisaccharides including isomaltotriose and panose, but also maltotriose (*approx.* 5% relative to isomaltose). This activity on maltotriose deeply contrasted with the inefficiency of IMA proteins to

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cleave the α -1,4 linkage of maltose. No activity could be detected with longer malto-oligosaccharides such as maltopentaose or maltoheptaose (data not shown). Moreover, despite some activity on isomaltose, all IMA proteins were entirely ineffective on longer isomalto-oligosaccharides (IMOs) contrary to some bacterial orthologs such as *Geobacillus thermoglucosidasius* and some *Bacillus cereus* isomaltases (Suzuki Y. *et al.*, 1979 and 1982). While analyzing structure-specificity relationships from already known 3-D data (MacGregor E. *et al.*, 2001), it was proposed that such preference for short IMOs may result from the pocket-like structure of the active site, as it is observed in the *B. cereus* α -1,6-glucosidase (Kizaki H. *et al.*, 1993; Watanabe K. *et al.*, 1997). This pocket-like structure may be unable to dock long-chain substrates, contrary to the shallow groove structure that is more characteristic of the active site of polysaccharide endo-hydrolases such as the isoamylase (Katsuya Y. *et al.*, 1998).

3.3.2 Impact of point mutations

Ima2p and Ima3p showed significant differences in substrates specificities and activities, despite only three different amino acids in their primary sequence (A54/T54, P240/L240 and Q279/R279 for Ima2p *versus* Ima3p, respectively). We therefore studied the two L240P and R279Q variants of Ima3p, which were already constructed and analyzed previously (thermostability chapter), in order to find which of these amino acids could explain the significant drop of activity and/or loss of activity on many sugars for the Ima3p protein. About Ima5p, despite the significant sequence divergence of this protein relative to the other members of the family (around 65% of sequence identity), the most appealing difference in the amino acid sequence was the triplet VGI in Ima5p (residues 278-280) instead of MQH in Ima1p and Ima2p (Figure 3. 7), which is the only difference when analyzing the consensus regions I - IV of the GH13 family. We then constructed, purified and

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studied the catalytic properties of the Ima5p_MQH variant, which therefore bears exactly the same consensus regions I – IV as in Ima1p or Ima2p.

Substrate	Linkage	Specific activities ($\mu\text{mol}/\text{min}/\text{mg}$)		
		Ima3p_ L240P	Ima3p_ R279Q	Ima5p_ MQH
α-pNPG	4-Nitrophenyl α -D-glucopyranoside	5.5	8.5	0
α MG	α -Methylglucopyranoside	6.5	50	0
Disaccharides (Glc \rightarrow Glc)				
Isomaltose	O- α -D-glucosyl-(1 \rightarrow 6)- α -D-glucose	2.2	29	0
Disaccharides(Glc \rightarrow Fru)				
Sucrose	O- α -D-glucosyl-(1 \rightarrow 2)- β -D-fructose	11.2	22	0
Leucrose	O- α -D-glucosyl-(1 \rightarrow 5)-D-fructose	0	1.5	0
Palatinose	O- α -D-glucosyl-(1 \rightarrow 6)-D-fructose	2.8	33	0
Trisaccharides				
Panose	O- α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose	0	3.1	0

Table 3. 10 Substrates specificities of IMA variants

[The substrate concentration was 100mM with an exception of 5mM *p*NPG. The assay was performed at 30°C in the Citrate-Potassium Phosphate buffer pH7.]

While the L240P variant of Ima3p presented almost the same characteristics as Ima3p, the single R279Q mutation essentially swapped substrate specificities to Ima2p characteristics. We indeed restored the activity on panose and leucrose, and increased specific activities on α -*p*NPG, α MG, α -1,6 disaccharides and sucrose close to Ima2p levels (Table 3.10). The result demonstrated that Ima3p_R279Q allowed recovering the substrate specificities and specific activities close to Ima2p characteristics. On the other hand, the results obtained for the Ima5p_MQH variant were totally in contradiction to our initial expectations, as it indeed failed to swap Ima5p substrates specificities and

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activities to Ima1p characteristics. Even worst, Ima5p_MQH completely abolished the catalytic activity of this isoenzyme, whatever the substrate used.

The existence of a crystal structure of Ima1p in complex with various substrates (Yamamoto K. *et al.*, 2010 and 2011) and a recent study by Voordeckers and coworkers (Voordeckers K. *et al.*, 2012), provided the first clues in structural determinants for activity of yeast isomaltase (Voordeckers K. *et al.*, 2012). Herein, Ima1p crystal structure (See Figure 2. 11) stands as a perfect model of Ima2p and Ima3p structures due to high sequence identities. It was also used to further explore the link between structural elements the clear-cut differences in the parameters (enzymological and biochemical) observed within this protein family in the present study. We therefore draw the 3D structures of isomaltase in the catalytic site (Figure 3. 10). With respect to substrates specificities and catalytic efficiency, the fundamental role of the residues Q279 and V216 for activity on disaccharides with α -1,6 bonds was very recently discussed and emphasized (Voordeckers K. *et al.*, 2012). Thus, we confirmed that the replacement of R (Arginine) by Q (Glutamine) at position 279 in Ima3p resulted in a Ima3p_R279Q variant presenting good kinetic properties similar to them of Ima2p (and Ima1p) properties. The reason of the loss of activity for the native Ima3 protein as compared to Ima1p and Ima2p probably relies on the negative interaction between the R279 and Y158 residues.

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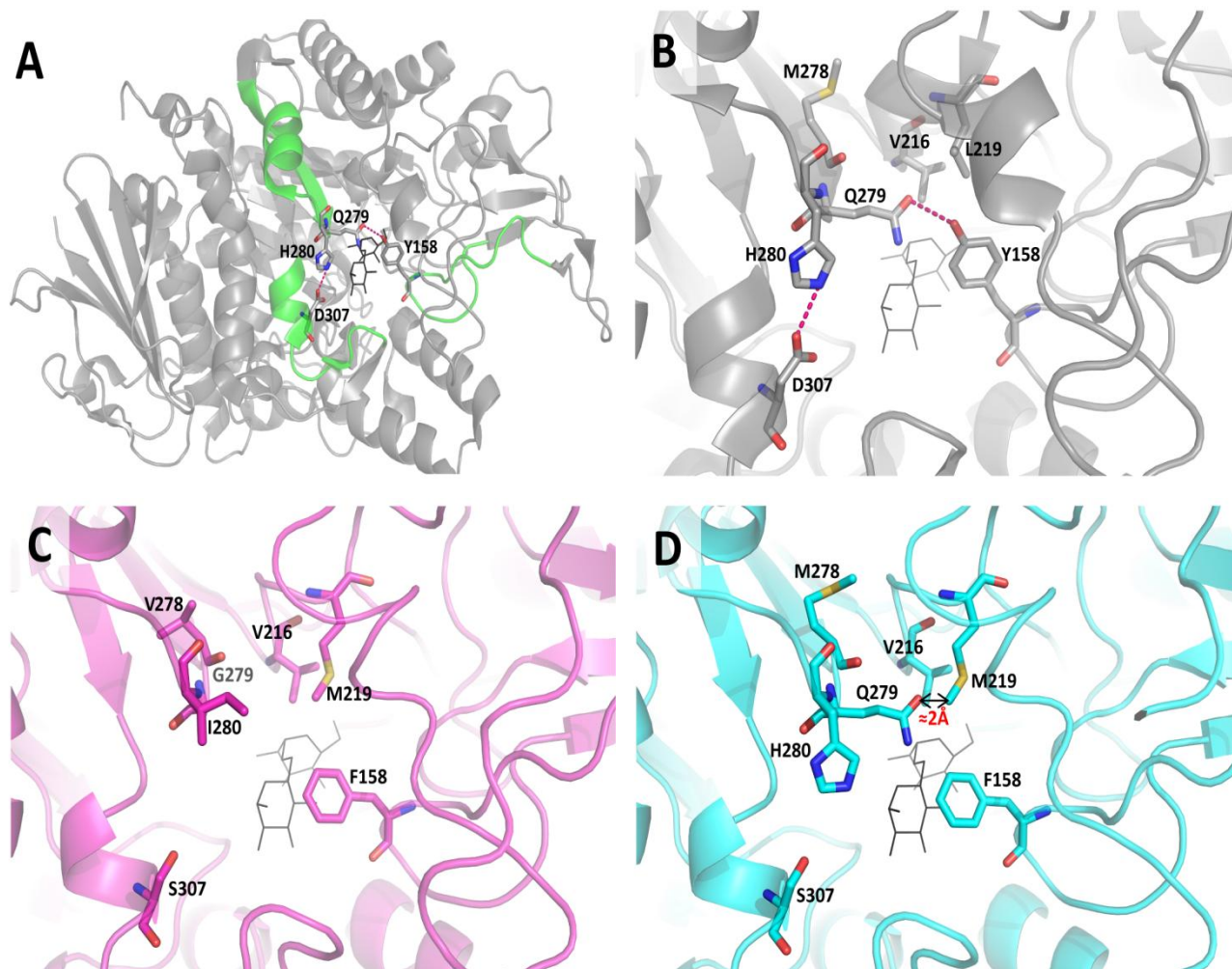


Figure 3. 10 Structure of isomaltase in the catalytic site

[A. Catalytic site of Ima1p/Ima2p; B. Catalytic site of variant Ima3p_R279Q; C. Catalytic site of Ima5p; D. Catalytic site of variant Ima5p_MQH

Important amino acid residues discussed in this work were highlighted as sticks. (A) Global x-ray structure of Ima1p (pdb code: 3AJ7) showing isomaltose (black lines) in the active site pocket. The salt-bridge between H280 and D307 (red dashed line), probably helps maintaining the vicinity of the loops (residues 273 to 292 and 303 to 314 highlighted in green) and the thermal properties of Ima1p in a pH dependent manner. The positioning of the third, green loop (residues 150 to 164) is also probably important, keeping in interaction the side chains of Q279 and Y158 through an hydrogen bond (dashed line). Panel (B) shows details of the active site of Ima1p. Panel (C) shows a similar view of the Ima1p-based model of Ima5p protein. Panel (D) highlights the steric hindrance in the active site of Ima5p after replacement of VGI by MQH (pos. 278-280), which probably explains the total loss of activity in this protein variant. This figure was generated using Pymol (Delano Scientific, <http://pymol.sourceforge.net/>).

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The Y158 residue from Ima1p was only described to constrict the entrance of the active site pocket (Yamamoto K. *et al.*, 2010). However, analysis of Ima1p crystal structure indicated that the apparent unfavorable, steric interaction between Q279 and Y158 might be actually stabilised through hydrogen bond between their side chains (Figure 3.10 A/B). This stabilization is not possible in the presence of the big, positively charged side chain of the arginine 279 found in Ima3p, leading to the probable displacement of R279 and/or Y158 side chains within the catalytic pocket of this protein. This unfavorable steric displacement is probably the cause of the observed significant drop of activity on α -1,6 substrates and loss of activity on others such as kojibiose, leucrose and all trisaccharides. Interestingly however, the ability for Ima3p to cleave substrates with α -1,3 bonds as well as the α -1,2 linkage of sucrose, suggested that the recognition and/or cleavage mechanism of these sugars can occur independently of the R and/or Q279 residue and therefore diverge from α -1,6 related substrates.

More phylogenetically distant, Ima5p displays approximately 65% sequence identity with the other three members of this family. Contrary to expectations, substitution of the triplet VGI by MQH (position 278-280) led to the complete loss of activity of Ima5p, indicating that the perfect conservation of these four core sequences could not be assigned as the sole molecular clue for determining enzymatic properties of this class of enzymes. Other amino acids were identified in or near the binding pocket (Yamamoto K. *et al.*, 2010/2011; Voordeckers K. *et al.*, 2012) and amongst them, amino acids 158, 219, 307 and 411 likely form a favorable environment for substrate recognition and cleavage. Especially the spatial cluster Q279 -V216-L219 found in Ima1-4 proteins was described as an important clue of the substrate specificity of isomaltases, with the need for one protruding residue (either the Q279 or the M219) , not both, to lead to stabilization of isomaltose-like substrates while causing steric hindrance of the maltose-like sugars (Voordeckers K. *et al.*, 2012).

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In the native Ima5p, the VGI triplet co-occurs with a methionine residue at position 219, therefore leading to the spatial cluster G279-V216-M219 that somehow respects this need of only one protruding residue. Also very interestingly, Ima1p-based modeling of Ima5p structure (Figure 3. 10 C) through the Swiss-model tool (Arnold K. *et al.*, 2006) allowed us to notice that this protruding M279 probably co-evolved with a valine at position 177, replacing the L177 that may lead to incompatible steric interaction with M219. Similarly, it can be noticed that in Ima5p the tyrosine residue at position 158 was replaced by a phenylalanine, which keeps a huge phenyl ring on its side chain, This residue now faces in Ima5p the Val278, which doesn't lead to any steric hindrance. Taking all these elements into account, it could be proposed that the loss of activity of the Ima5p_MQH variant (Figure 3. 10D) may be due to exacerbated steric hindrance in the catalytic pocket arising simultaneously from i) the presence of two protruding amino acid residues surrounding V216, *i.e.* glutamine (Q279) and methionine (M219), and ii) the lack of hydrogen bonding between Q279 and F158 in the absence of the hydroxyl group on the phenyl ring.

As a conclusion, our results on the native Ima5p together with this clear-cut result for its MQH inactive variant altogether contradicted previous conclusions that (i) the V216 and Q279 residues were largely responsible for determining the specificity of α -glucosidases for α -1,6 substrates, and that (ii) the presence of Ala, Gly, or Asn residues at position 279 could be definitely considered as determinant to get α -glucosidases with α -1,4-glycosidic hydrolyzing activity (Voordeckers K. *et al.*, 2012). For one thing, Ima5p was fully active on α -1,6 substrates while bearing a glycine residue at position 279. For the other thing, the insertion of a Q at position 279 (VGI to MQH Ima5p variant) totally eliminated the activity of this protein.

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3.3.3 Kinetics parameters of purified isomaltases

To analyze and compare the IMA proteins for their singularities on the different substrates, the kinetic parameters were determined for some of the substrates that yielded to substantial activities, *i.e.* α -*p*NPG, α -MG, isomaltose, palatinose, isomaltotriose, panose and sucrose. The assay was also performed at standard condition, in the Citrate-Potassium Phosphate buffer pH7, at 30°C. As shown in Figure 3. 11 A~D, the non-linear fitting of the velocity as a function of substrate concentration showed typical Michaelis-Menten. Deduced from these curves, the K_m , k_{cat} and K_i constants, expressed as the mean \pm SEM with 95% confidence interval between brackets, as well as the catalytic efficiency [k_{cat}/K_m ratio (Eisenthal R. *et al.*, 2007)] are summarized in Table 3.11 A~D. As a rule, K_m value for α -*p*NPG (0.58 mM) was much lower than that of its natural substrates (10 to 200 mM), and the catalytic efficiency (k_{cat}/K_m) of IMA proteins was much higher for α -*p*NPG than for natural “sugar” substrates, including isomaltose (from 3-fold for Ima1/2/5p to 50-fold for Ima3p).

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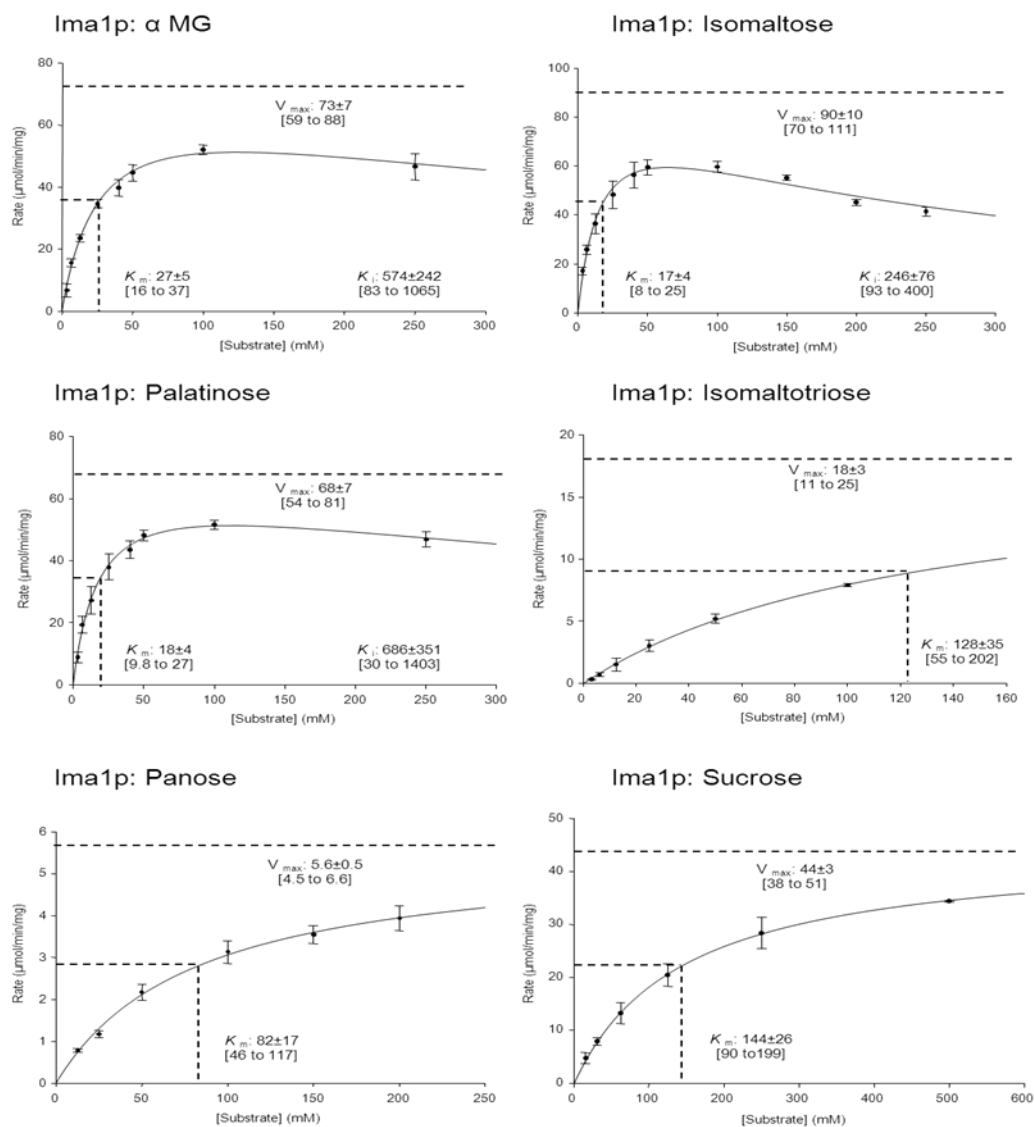


Figure 3. 11- A: Velocity as a function of substrate concentration for Ima1p

Substrate	Ima1p			
parameters	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_i (mM)
α -pNPG	0.58 ± 0.16	8.7 ± 1.0	15 ± 6	25 ± 15
α MG	27 ± 5	84 ± 8	3.1 ± 0.9	574 ± 242
Isomaltose	17 ± 4	103 ± 12	5.7 ± 0.9	246 ± 76
Palatinose	18 ± 4	78 ± 8	4.3 ± 1.4	686 ± 351
Isomaltotriose	128 ± 35	21 ± 4	0.16 ± 0.07	None
Panose	82 ± 17	6.3 ± 0.5	0.078 ± 0.023	None
Sucrose	144 ± 26	51 ± 4	0.35 ± 0.09	None

Table 3. 11-A: Kinetic parameters of Ima1p for selected substrates

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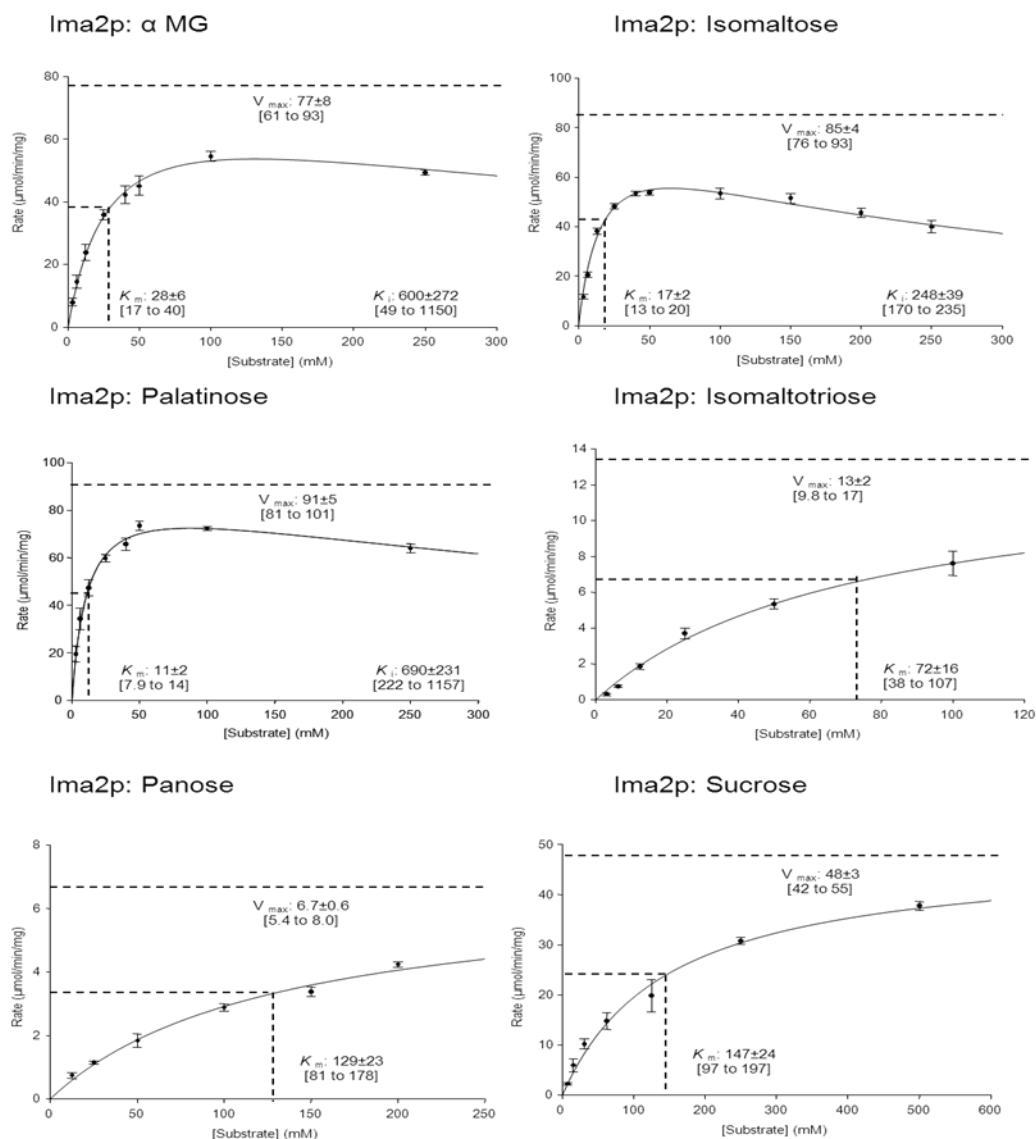


Figure 3.11- B: Kinetic parameters of purified Ima2p

Substrate	Ima2p			
parameters	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_i (mM)
α pNPG	0.89 ± 0.13	14 ± 1	16 ± 3	12 ± 3
α MG	28 ± 6	88 ± 9	3.1 ± 1	600 ± 272
Isomaltose	17 ± 2	97 ± 5	5.6 ± 0.9	248 ± 39
Palatinose	11 ± 2	104 ± 6	9.4 ± 1.8	690 ± 231
Isomaltotriose	72 ± 16	15 ± 2	0.21 ± 0.07	None
Panose	129 ± 23	7.7 ± 0.7	0.051 ± 0.004	None
Sucrose	147 ± 24	55 ± 4	0.38 ± 0.09	None

Table 3.11 B: Kinetic parameters of Ima2p for selected substrates

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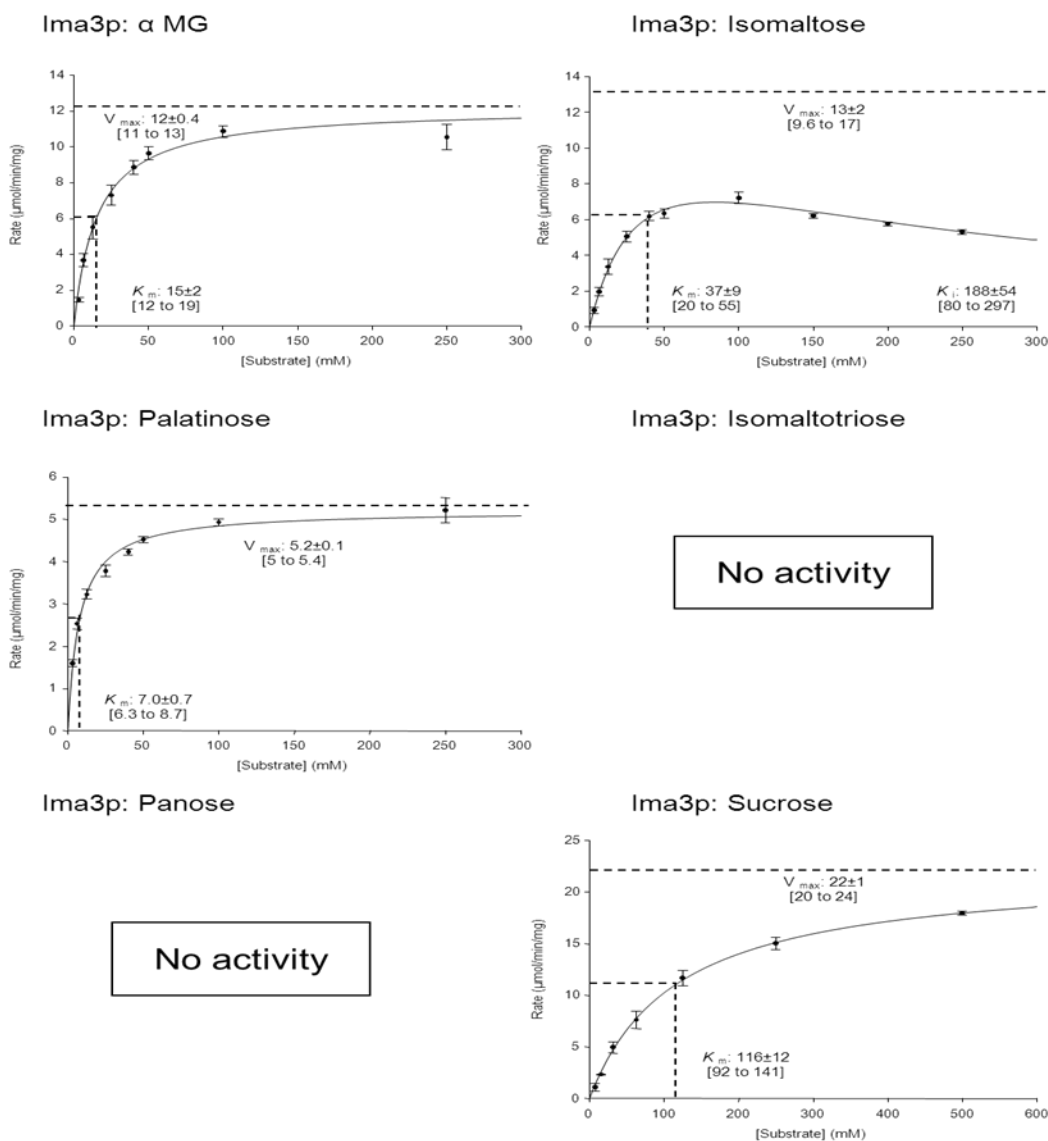


Figure 3.11- C: Kinetic parameters of purified Ima3p

Substrate	Ima3p			
parameters	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_i (mM)
α pNPG	0.35 ± 0.09	7.1 ± 0.7	20 ± 7	9.8 ± 3.2
α MG	15 ± 2	14 ± 1	0.9 ± 0.1	None
Isomaltose	37 ± 9	15 ± 2	0.41 ± 0.15	188 ± 54
Palatinose	7.0 ± 0.7	6.0 ± 0.1	0.8 ± 0.1	None
Isomaltotriose	0	0	0	None
Panose	0	0	0	None
Sucrose	116 ± 12	25 ± 1	0.22 ± 0.03	None

Table 3.11C: Kinetic parameters of Ima3p for selected substrates

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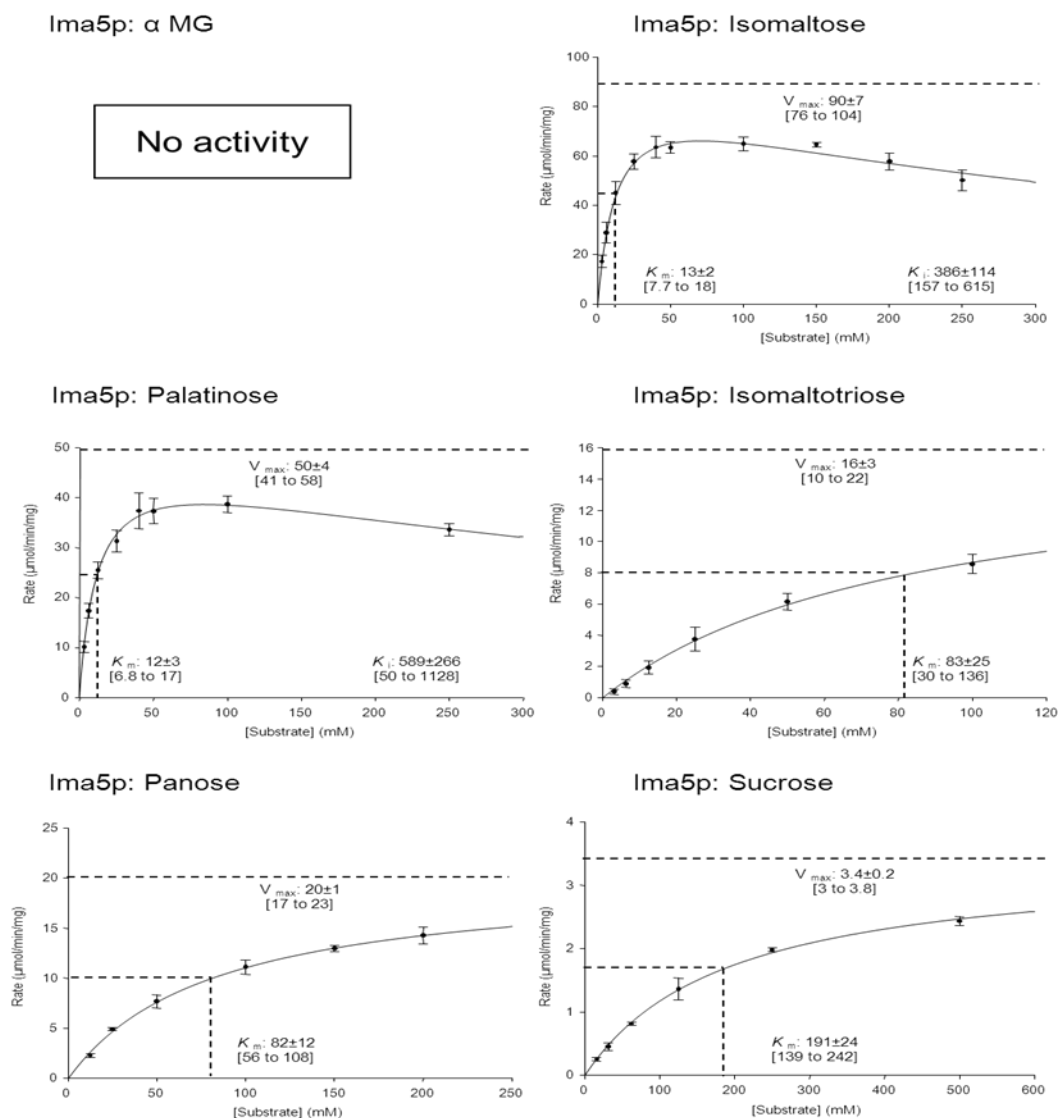


Figure 3. 11- D: Kinetic parameters of purified Ima5p

Substrate	Ima5p			
parameters	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_i (mM)
α pNPG	0.48 ± 0.11	14 ± 1	28 ± 9	33 ± 21
α MG	0	0	0	None
Isomaltose	13 ± 2	101 ± 8	8.0 ± 2.2	386 ± 114
Palatinose	12 ± 3	56 ± 5	5.0 ± 1.6	589 ± 266
Isomaltotriose	83 ± 25	18 ± 3	0.22 ± 0.10	None
Panose	82 ± 12	23 ± 1	0.28 ± 0.06	None
Sucrose	191 ± 24	3.8 ± 0.2	0.020 ± 0.004	None

Table 3. 11D: Kinetic parameters of Ima5p for selected substrates

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We also provided an overview of Velocity-Substrate profiles and relative activities Figure 3.12, with which the curves of the velocities as functions of substrate concentration were summarized.

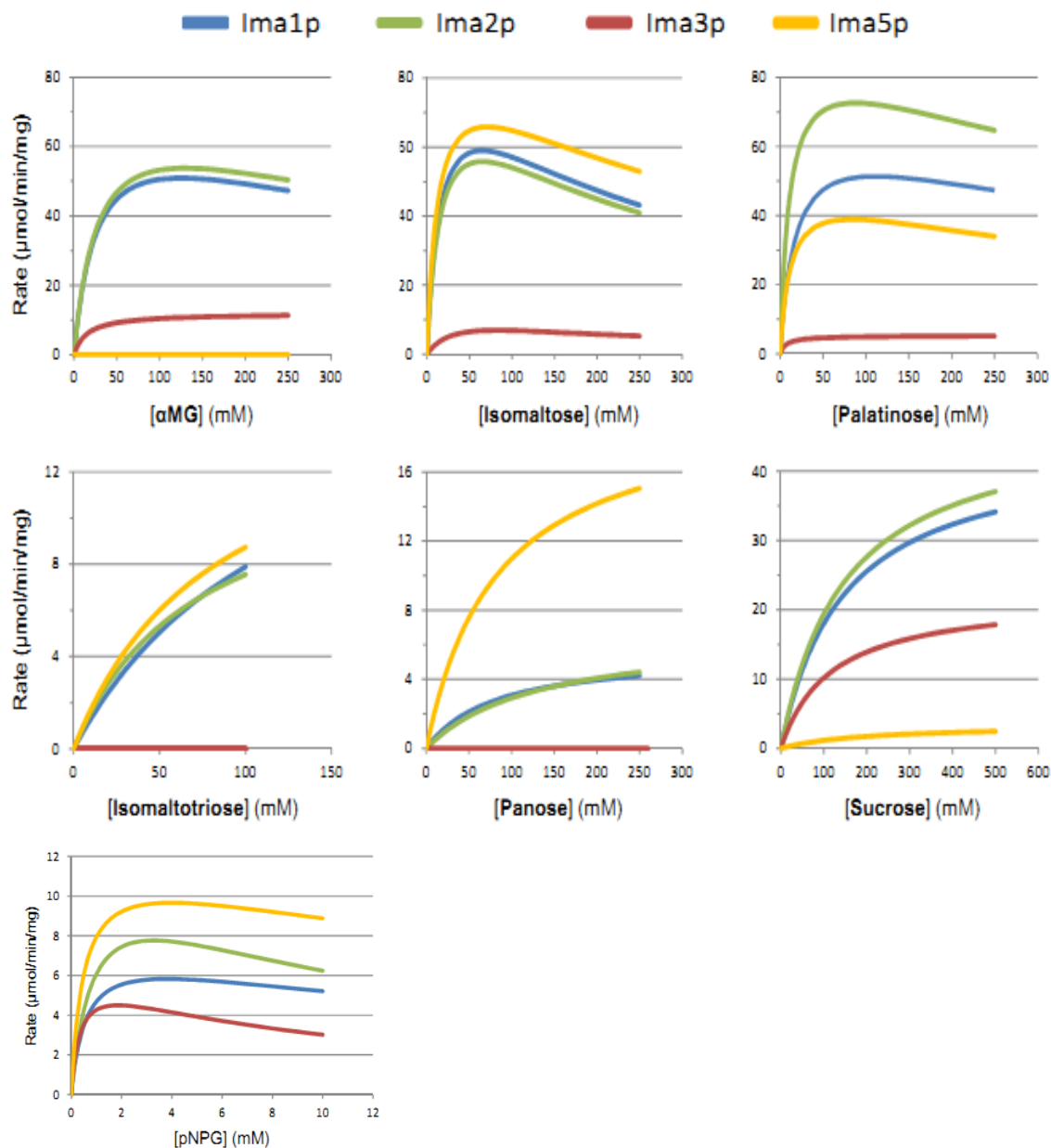


Figure 3. 12-A: Overview of velocity-substrate profiles

[Comparative plots of the four isomaltases on different substrates. Representative curves of the velocity as a function of substrate concentration, plotted from the equation: $v = (V_{\max} S) / (K_m + S(1 + S/K_i))$. For each isomaltase/substrate pair, V_{\max} , K_m and K_i values can be found on the original plots.]

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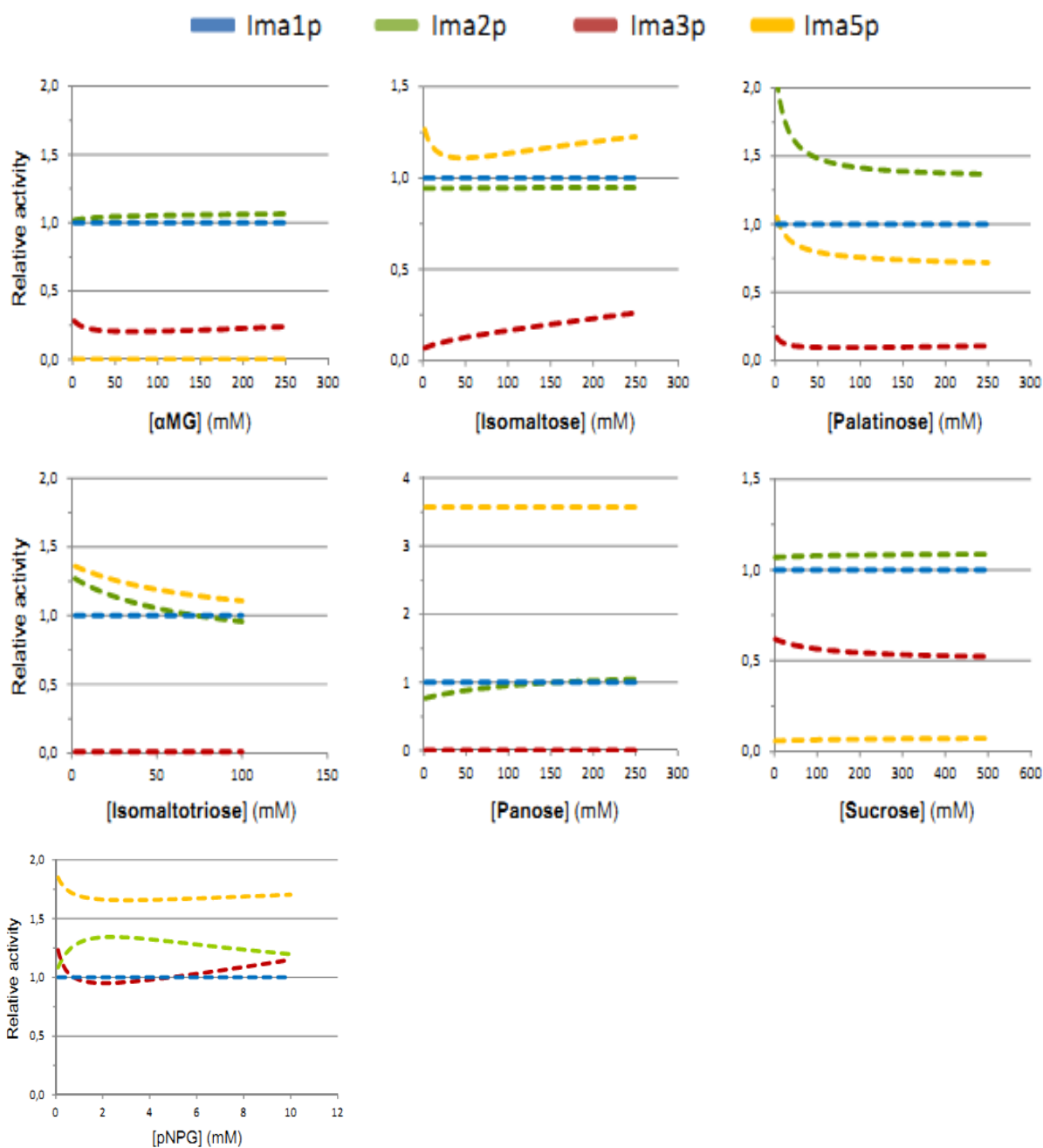


Fig. 3-12-B: Overview of velocity-substrate relative activities

[Comparative plots of the four isomaltases on different substrates.

The ratio of velocities ($v_{\text{Imaxp}} / v_{\text{Ima1p}}$, Ima1p being taken as the reference) was plotted as a function of substrate concentration.]

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Combined Figure 3. 11 with Figure 3. 12 A, these curves highlight a clear inhibition at high concentration for some of the substrates. It was particularly significant when isomaltose concentration increased from 100 to 250 mM, where the activity decreased approximately by 25%. Quite puzzling, this inhibition had not been reported previously for Ima1p (Yamamoto K. *et al.*, 2010) while this protein was studied in a similar range of concentrations, *i.e.* from 50 to 500 mM isomaltose. As can be seen in Figure 3. 12, Michaelis-Menten kinetics were recorded with other substrates including α -MG, palatinose, panose, sucrose and isomaltotriose, with evident inhibition by excess of palatinose and to a lesser extend of α -MG, except for Ima3p that did not display this behaviour (also presented in Figure 3. 11C). Similarly, all Ima isoforms were inhibited at high concentration of α -pNPG (Figure 3. 12A), with a drop of activity when the substrate concentration increased to 10 mM (10% drop of activity for Ima1p or Ima5p to 33% for Ima3p).

Figure 3. 12B provided better comparison of the four proteins on the different substrates (Ima1 enzyme as reference), and even more explicit than the sole catalytic efficiency value. This comparative analysis method was precognized by Eisenthal (Eisenthal R. *et al.*, 2007). The blue and green curves in AB were very close to each other, indicating that the catalytic properties of Ima1p and Ima2p on the various substrates were remarkably similar (comparable catalytic constants presented in Table 3. 11 A and B). In spite of notable analogy with Ima1p and Ima2p on the various substrates, the slightly higher activity of Ima2p on palatinose is the only different feature between these two proteins, especially when decreasing substrate concentration (Figure 3. 12B). The high catalytic efficiency values for isomaltose and palatinose as compared to sucrose, isomaltotriose and panose, clearly demonstrated the preference of Ima1p and Ima2p for disaccharides with α 1,6-glycosidic linkage. The low catalytic efficiencies of both proteins on sucrose could be easily explained by their high K_m values for this

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substrate (~145 mM), which nevertheless suggested that Ima1p or Ima2p could lead to significant cleavage of sucrose only when present at very high concentration.

Our results also demonstrated that Ima3p was the most atypical protein of IMA proteins, which was totally ineffective on isomaltotriose and panose (Figure 3. 11C and Table 3. 12C), as it was previously mentioned from the specific activity values for many sugars (Table 3. 9), e.g. kojibiose, leucrose and other trisaccharides. Ima3p was 5 to 10-fold less active than Ima1p on isomaltose and palatinose, which was mainly explained by a significant drop of the catalytic constant. Ima3p conserved the possibility to cleave substrates with α -1,3 bonds (see Table 3. 9) and presented the highest activity on sucrose. When referring to kinetic constants however, the catalytic efficiency on sucrose remained 2 and 4 times lower than on isomaltose and palatinose, respectively (Table 3. 11C). Besides, with only 65% sequence identity to Ima1p, Ima5p yet showed similar catalytic constants and patterns on substrates bearing α -1,6 bonds, such as isomaltose, palatinose and isomaltotriose, despite no activity on α MG. As clearly illustrated in Figure 3. 12B, Ima5p was much more active on panose than Ima1p (3.5-fold more activity whatsoever the panose concentration), but it was barely active on sucrose (10-fold lower activity than Ima1p).

It was worth noting that a significant action of IMA proteins on sucrose in addition to their great activity on disaccharides with α -1,6 linkages. This activity on sucrose was contradictory reported in the literatures despite efforts to purify isomaltase, confirming the interest to biochemically analyse separately each isoenzyme as we did in this work. With a specific activity on sucrose as high as for isomaltose, Khan and Eaton most likely purified Ima1p or Ima2p, or a mixture of both (Khan N. A. and N. R. Eaton, 1967). The so-called Glucosidase I isoenzyme purified by Matsusaka (Matsusaka K. *et al.*, 1977), which was highly active on isomaltose yet hardly active on sucrose, could look like the Ima5 isoform (1000-fold reduction of the catalytic efficiency on sucrose as compared to isomaltose in the

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present work). But Ima5p specificities besides isomaltose and sucrose did not support this possibility and reinforce the existence of yet unknown and/or uncharacterized *IMA* encoded proteins in *S. cerevisiae* strains from various origins.

Interestingly, the variability in sucrose utilisation by isomaltases also occurred in isomaltases from other yeast species. For example, while the yeast *L. starkeyi* α -glucosidase was totally inactive on sucrose (Kelly C.T. *et al.*, 1985), the enzyme purified from the yeast *T. pretoriensis* displayed the same activities on sucrose and isomaltose (Oda Y. *et al.*, 1993). . Actually, Sucrase activity appeared to be present in most α -1,4- and α -1,6-glucosidases characterized so-far in yeasts and distant fungi (Krakenaite R.P. and A.A. Glemzha, 1983; Geber A. *et al.*, 1992; Williamson P.R, *et al.*, 1993), no matter how lower was the k_{cat}/K_m ratio than those reported for their preferred substrate. This was also true for other eukaryotic systems including honeybee α -glucosidases (Ngiwsara L. *et al.*, 2011) or bacterial enzymes as in various *Bacillus* species (Schonert S. *et al.*, 1998; Noguchi A. *et al.*, 2003), even if counterexamples also exist, such as the *B. breve* glucosidases that was shown to be totally inactive on sucrose.

Maltose utilization is also very interesting, yet variable feature amongst isomaltases. While *S. cerevisiae* IMA proteins were totally unable to cleave maltose, the yeast *Lipomyces starkeyi* α -glucosidase was on the contrary capable of utilizing maltose and isomaltose with high and similar efficiencies (Kelly C.T. *et al.*, 1985). Similarly, the honeybee *Apis mellifera* HBG-II protein preferred maltose but was also able to use isomaltose with only 3-fold reduction of the catalytic efficiency (Kimura A. *et al.*, 1990; Takewaki S. *et al.*, 1993; Nishimoto M. *et al.*, 2001). These rare examples of proteins exhibiting activities towards isomaltose and maltose with similar efficiencies therefore nuance to some extent the idea that both activities cannot be fully optimized in a single enzyme.

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As a conclusion and as emphasized above for isomaltose/maltose/sucrose utilization, reports dealing with enzymes from various origins (from bacteria to eukaryotes through yeasts) presented remarkable differences in the number and nature of sugars actually cleaved by these α -glucosidases. Moreover, if the substrates were cleaved, significant variations in catalytic efficiencies could actually be measured. Using modern modeling and /or enzyme 3D structure, it might be possible to predict the substrate spectrum and specificities of isozymes of interest.

3.3.4 Inhibition of isomaltase

It has been reported that maltose act as a competitive inhibitor of Ima1p (Yamamoto K. *et al.*, 2010), which could agree with its ability to bind to the substrate-binding site (Yamamoto K. *et al.*, 2010 and 2011), without being cleaved. Nonetheless, this study by Yamamoto and coworkers on the inhibition of Ima1p by maltose was carried out using high concentrations of substrate, *i.e.* isomaltose in the range 50-500 mM, which according to our results inhibited by itself the isomaltases. Therefore, this assay was still performed under standard condition (in the Citrate-Potassium Phosphate buffer pH7, at 30°C), although reconsidering with lower, non-inhibitory concentrations of isomaltose as substrate (50 mM as the maximal concentration value to avoid inhibition by the substrate itself). We investigated maltose inhibition of Ima1p and Ima5p in the absence or presence of 50-200 mM maltose (Figure 3. 13). Classical, linear representations that complement one another plot of $1/v$ against I (Dixon plot) *versus* the plot of S/v against I , (Cornish-Bowden Athel, 1974) were applied for determining inhibition models (Figure 3. 14).

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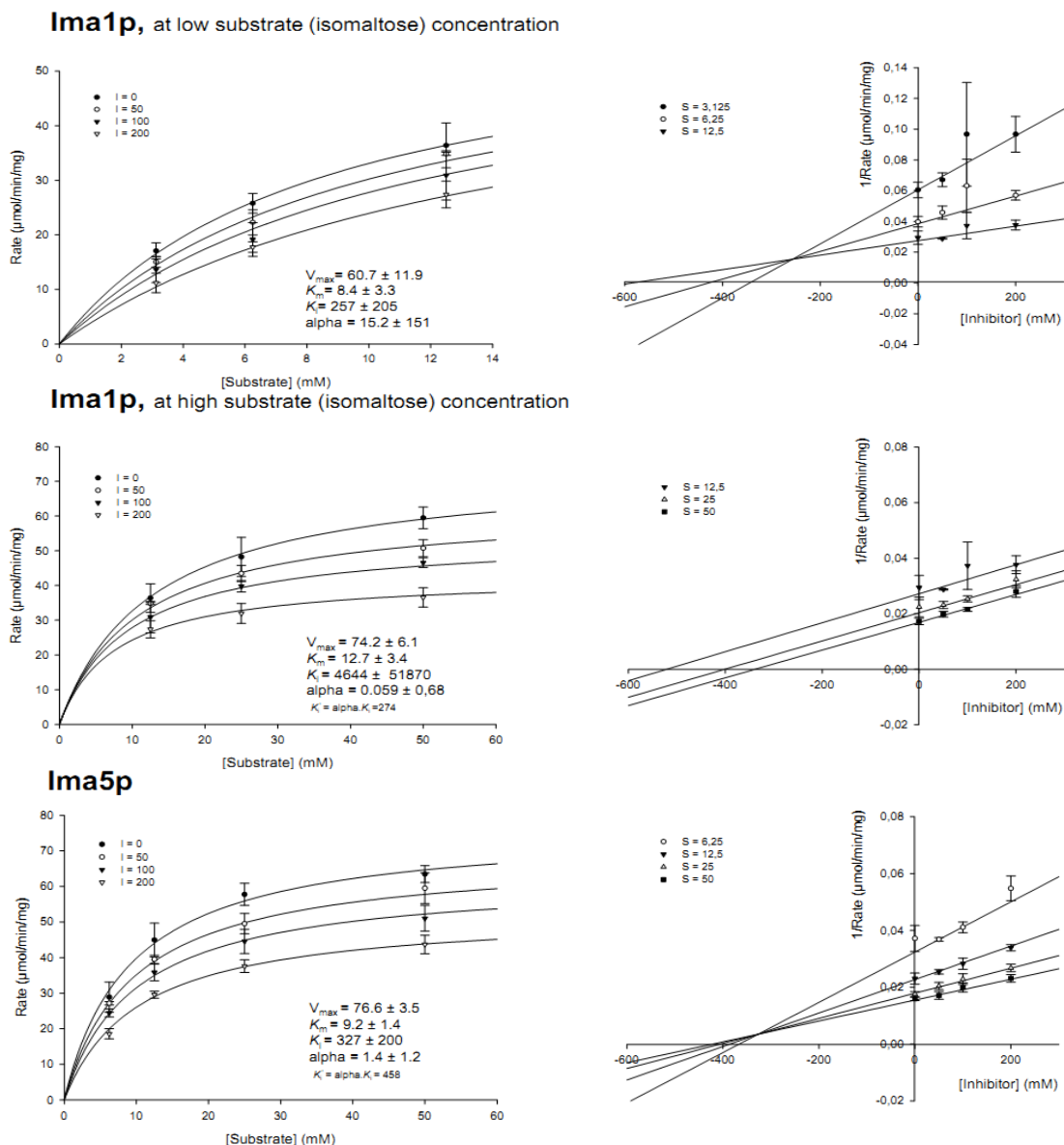


Figure 3. 13 Kinetic parameters of the inhibition by maltose

[Left column (Michaelis-Menten plots): Plot of velocity as a function of substrate concentration in the absence (●) or in the presence of 50 mM (○), 100 mM (▼) or 200 mM (Δ) inhibitor. Kinetic parameters were determined from non-linear curve fitting to the general, mixed inhibition model ($v = (V_{max} S) / [K_m(1 + I/K_i) + S(1 + I/K_i')]$), performed by using the single substrate-single inhibitor inhibition option of the SigmaPlot Enzyme Kinetics 1.3 module. V_{max} : maximum velocity; S : substrate concentration; I : inhibitor concentration; K_m : affinity constant; K_i : inhibition constant and $K_i' = \alpha K_i$. Results are from 3 independent assays.

Right column (Dixon plots): Reciprocal of velocity as a function of inhibitor concentration for substrate concentrations of (3.125 mM(●), 6.25 mM(○), 12.5 mM (▼), 25 mM (▽) and 50 mM (■). Inter-dependent lines drawn from the SigmaPlot Enzyme Kinetics module, using kinetic parameters from the non-linear curve fitting to the general, mixed model.]

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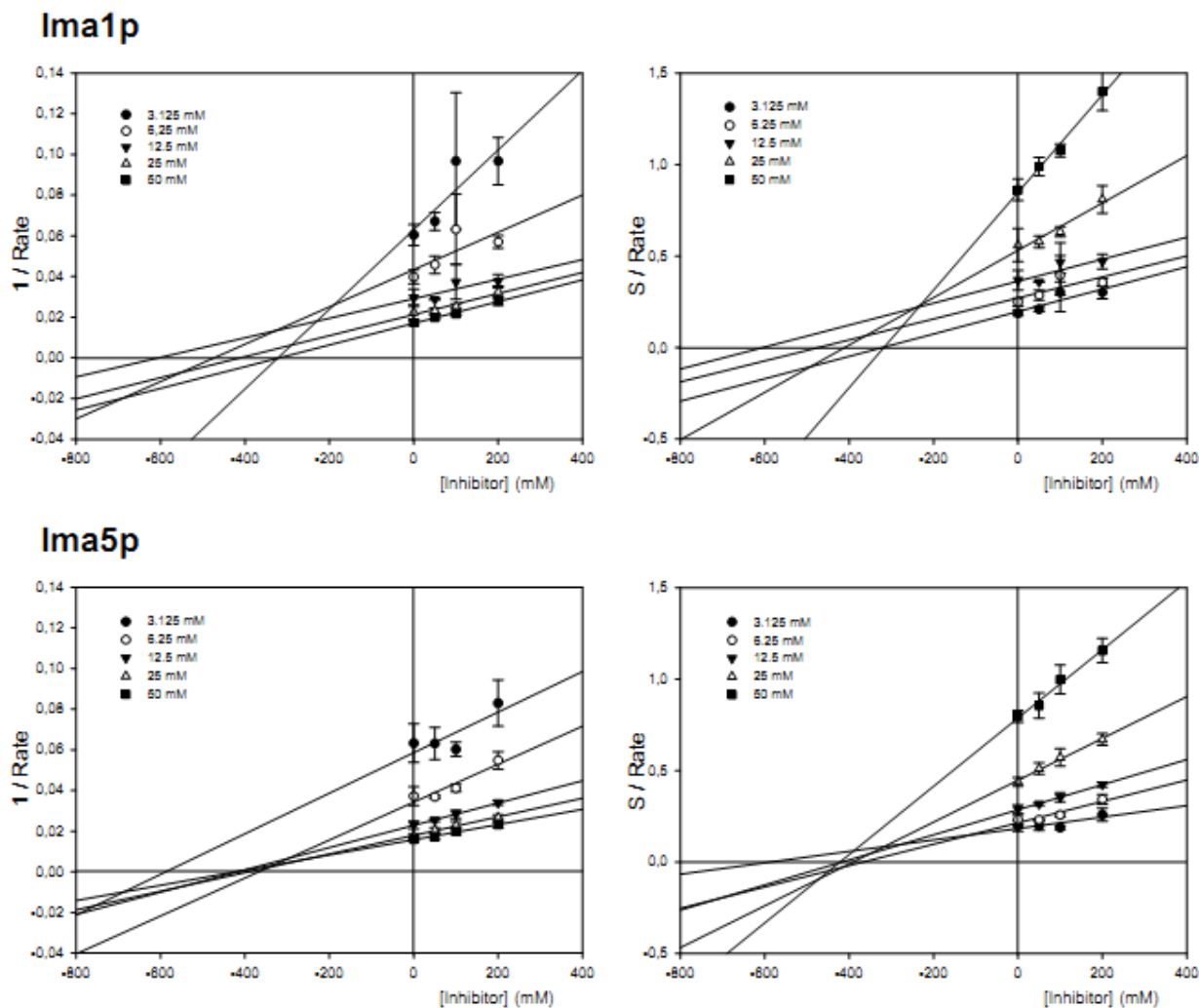


Figure 3. 14 Inhibition of isomaltases by maltose

[Plots of $1/v$ (Dixon plot, Left column) and of S/v (Right column) against inhibitor concentration (*i.e.* maltose). Linear regression was performed by SigmaPlot V.11.0 software, considering points at a fixed substrate concentration as independent series (3.125 mM(●), 6.25 mM(○), 12.5 mM (▼), 25 mM (▽) and 50 mM (■). Ima1p : Evidence for a bimodal behaviour of the inhibition type of Ima1p by maltose. At lower substrate concentrations (3.125 to 12.5 mM), the lines were pretty well concurring on the dixon plot while they appeared as parallels in the plot of S/v against I , which is the hallmark of competitive inhibition; The reciprocal was observed at higher substrate concentrations (12.5 to 50 mM), which is indicative for uncompetitive inhibition. Ima5p: Nearly perfect concurring lines in both plots, with the exception of the lower substrate concentration series (3,125 mM), which is indicative of a mixed inhibition model. The fact that the intersections of the lines occurred almost on the I axis in both plots indicated that this mixed model presented a very strong non-competitive component.]

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Our results confirmed that isomaltose utilization by isomaltases was inhibited by maltose, but the characteristics of this inhibition provided evidence for a much more complex inhibition model. It was actually worth mentioning that Ima1p exhibited different behavior due to substrate concentration. Classical, linear representations provided evidence for a bimodal behaviour of the inhibition type of Ima1p by maltose. As shown in Figure 3. 13, it was illustrated by the fact that at lower substrate concentrations (3.125 to 12.5 mM), the lines were pretty well concurring on the dixon plot while they appeared as parallels in the plot of S/v against I, which was the hallmark of competitive inhibition. The reciprocal was unambiguously observed at higher substrate concentrations (12.5 to 50 mM), which was indicative of uncompetitive inhibition. The presented non-linear fitting supported the conclusion. The linear mixed inhibition model, a general case that includes all of the common types of inhibition as asymptotic or special cases, clearly shifted towards the competitive inhibition at low substrate concentration (K_i of 257 mM, $K_i' \rightarrow \infty$, Figure 3. 13), while it stabilized close to the uncompetitive inhibition at high substrate concentration ($K_i \rightarrow \infty$, K_i' of 274 mM). About Ima5p, our data revealed a different pattern of inhibition with nearly concurring lines in both plots (Figure 3. 14), which is characteristic of a mixed inhibition model. The non-linear fitting of the data consolidated this conclusion (327 mM for K_i and 458 mM for K_i' , Figure 3. 13), and further underlined the strong non-competitive component of this inhibition as indicated by similar constants (equal constants in case of non-competitive inhibition). Ima5p sharing only 65% sequence identity highlighted a different behavior as compared to Ima1p, since it was subjected to a mixed inhibition in the presence of maltose.

As a conclusion, if maltose is definitely not a substrate of yeast isomaltases, our results confirmed that it inhibits isomaltose utilization. However, very interestingly, this inhibition followed a more complex inhibition patterns than it was previously reported for Ima1p (Yamamoto K. *et al.*, 2010). The reason of this discrepancy may be found in the fact that maltose inhibition was tested by these

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authors at high substrate concentration (from 50 to 500 mM isomaltose), which was by itself inhibitory for all IMA isoforms. Using non-inhibitory concentrations of isomaltose as substrate, *i.e.* up to 50 mM, we confirmed the competitive inhibition of Ima1p, but it was strictly restricted to low substrate concentrations (up to approximately 10 mM). This inhibition unambiguously turned out to be uncompetitive as the isomaltose concentration increased in the assay, up to 50mM.

Whatever the isomaltase and the model under consideration, this inhibition by maltose was rather weak since the presence of maltose at high concentration (200 mM) only led to approximately 30% inhibition of activity for all three enzymes, Ima1p, Ima2p and Ima5p. As a consequence and despite replicates to estimate accurately the K_i value, they all nevertheless fall in the range of several hundred mM, which deeply contrasted with K_i in the μ M range that have been reported for potent inhibitors of several α -glucosidases (including yeast maltase) (Kim M.J. *et al.*, 1999; Kimura A. *et al.*, 2004).

Many substances alter the activity of an enzyme by reversibly combining with it in a way that influences the binding of substrate and/or its turnover number, which can modify the enzyme's structure and cause loss of affinity and inhibition of activity (Chrost R. J., 1991). We therefore tested the acarbose, which is an inhibitor of α -glucosidase. Acarbose is composed of an acarviosin moiety with a maltose at the reducing terminus. Acarbose is a pseudo-tetrasaccharide that is structurally similar to the typical oligosaccharides derived from starch digestion. Acarbose was shown to inhibit α -amylase activity, and selected intestinal activity such as glucoamylase (90%), sucrase (65%), maltase (60%) and isomaltase (10%) (Bischoff H. 1994; Mooradian and J.E. Thurman, 1999). In present work, a preliminary study on the inhibition of isomaltose cleavage (50mM) was performed in presence of 0.25mM to 5mM acarbose. Interestingly, we showed that the acarbose was indeed capable to inhibit all yeast isomaltases (shown in Table 3. 13). This inhibitory activity was slightly more effective with Ima1p, which led us continuing the analysis on this sole protein.

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Enzymological characterization of IMA proteins

Acarbose (mM)	Ima1p	Ima2p	Ima3p	Ima5p
0	100,00	100,00	100,00	100,00
0,25	91,07	97,73	100,00	97,06
0,5	85,71	93,18	100,00	94,12
1	85,71	92,05	97,56	91,18
5	75,00	82,95	92,68	88,24

Table 3. 12 Inhibition of IMA proteins by acarbose isomaltose (50mM)

[The inhibitory activity was measured with 50mM isomaltose in the Citrate-Potassium Phosphate buffer pH7 at 30°C.]

Still using 50 mM isomaltose as substrate, we observed 78.13 % and 42.76 % Ima1p residual activity in the presence of only 5 mM and 20 mM acarbose, respectively (See Table 3. 14). Even if these data highlighted potent inhibition of isomaltases by acarbose as compared to maltose, isomaltases nevertheless appeared much less sensitive to acarbose than maltase. It was indeed reported by Kim *et al.*, that maltases presented only 30% residual activity when the assay was performed in the presence of only 1 mM acarbose and 30mM maltose as substrate (Kim M.J. *et al.*, 1999).

Acarbose (mM)	0	5	10	20
Residual activity %	100	78,13	61,15	42,76

Table 3. 13 Inhibition of Ima1p by acarbose

[50mM isomaltose was used as substrate in the Citrate-Potassium Phosphate buffer pH7 at 30°C.]

Prof. Laube reviewed the affinity of acarbose, and he pointed out that acarbose has little affinity for isomaltase and no affinity for the β -glucosidases. The relative affinity of acarbose for specific enzymes is as follows: glycoamylase > sucrase > maltase > dextranase (Laube H., 2002). Acarbose was reported the ability that attached to the carbohydrate binding site of α -glucosidases in a competitive manner (Luo H. *et al.*, 2001). Kimura investigated with various types of α -glucosidases obtained from

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

micro-organisms, plants, and insects. Their results suggested that two types of α -glucosidases of brewer's yeast could be clearly distinguished by isoacarbose and acarviosine–glucose (derivative of acarbose) based on their inhibition kinetic data (Kimura A. *et al.*, 2004). Biochemical data also indicated that acarbose inhibited quite effectively the activity of *Bacillus subtilis* α -amylase: the concentration needed for 50% inhibition is 0.2 or 1.6 μ M, depending on whether the enzyme was pre-incubated with acarbose (Takase K. *et al.*, 1992).

Many studies have been done to screen potential α -glucosidase inhibitors. Kim *et al.* published that acarviosine–glucose was a potent inhibitor for baker's yeast α -glucosidase, inhibiting more effectively than acarbose (Kim M.J. *et al.*, 1999). Some others reported about miglitol and voglibose as well as their inhibitory activities. Shinozaki and coworkers investigated the effect of voglibose, and identified voglibose as competitive inhibitor of α -glucosidase (Shinozaki K. *et al.*, 1996). Miglitol and voglibose also showed significant inhibitory activities (Ren L. *et al.*, 2011). Besides, nectrisine was found to inhibit α -glucosidase (Tsuji F. *et al.*, 1996). In addition, genistein was identified as an α -glucosidase inhibitor from fermentation broths of a *Streptomyces sp.*, and it was demonstrated that genistein was a reversible, slow-binding, non-competitive inhibitor of yeast α -glucosidase (Lee D.S. *et al.*, 1996). All these mentioned compounds could be further evaluated for their inhibitory activities on yeast IMA proteins.

3.3.5 Transglycosylation activity

It is known that many glycoside hydrolases exhibit transglycosylation activity (Eneyskaya E.V. *et al.*, 1997). Transglycosylation occurs through a double-displacement mechanism of retaining glycoside hydrolases (GHs) (Shown in Figure 3. 15). During the first displacement the glycosidic oxygen is protonated by this catalytic acid leading to the scission of the glycosidic bond and the

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

formation of an oxocarbenium ion-like transition state. In the second displacement step, the free hydroxyl of a sugar or other aglycone molecule attacks to the glycosyl-enzyme intermediate rather than water resulting in deglycosylation of the enzyme and release of transglycosylation product (Withers S.G, 2001).

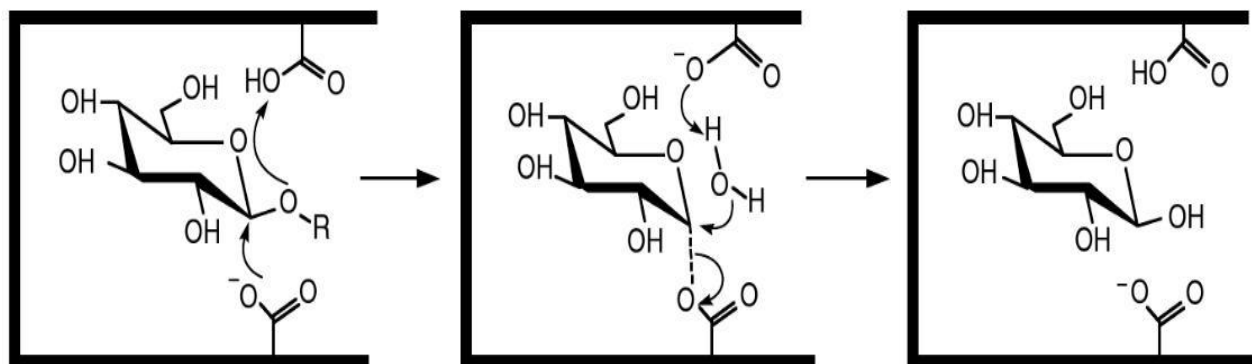


Figure 3. 15 Double-displacement mechanism of transglycosylation

(Withers S.G, 2001)

Many GH13 family members are capable to catalyze transglycosylation activity. Hung *et al.* found a GH13 α -glucosidase from *Geobacillus sp.* which showed optimal activity in alkaline (pH 9.0) and high temperature (60°C) conditions, and efficiently catalyzed a transglycosylation reaction with maltose as the sugar donor (Hung V.S. *et al.*, 2005). Shirai *et al.* found a GH13 α -glucosidase from one of the deep sea bacteria that exhibited the transglycosylation activity (Shirai T. *et al.*, 2008). This GH13 α -glucosidase accepted a wide variety of nonsugar molecules as acceptors in the transglycosylation reaction. Interestingly, the enzyme could convert 30% of curcumin into mono-, di-, or tri-glycoside products, although the chemical structure of curcumin has little in common with sugars. Labes *et al.* obtained two novel members (neopullulanase and cyclodextrinase) of glycoside hydrolase family 13 derived from environmental DNA (Labes A. *et al.*, 2008). Both enzymes showed transglycosylation activity, with combinations of 100 mM glucose, maltose, and maltotriose as substrates (either alone or in combinations of two or three sugars) and protein, pH 5.5~6, at 55°C. The cyclodextrinase could form

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Enzymological characterization of IMA proteins

maltose from glucose and maltotriose. We therefore investigated whether IMA proteins may present such an activity.

To test the transglycosylation activity, the transglycosylation products were detected by HPAEC. We therefore prepared a series of sugars as standards whose retention times were used in comparisons with potential transglycosylation product. Table 3. 15 presented the retention times of these sugars.

Standard	Retention time (min)
Glucose	3,8
Fructose	4,1
Leucrose	5,4
Isomaltose	6,3
Maltulose	6,28
Sucrose	7,1
Turanose	11,7
Melezitose	13,3
Palatinose	14,0
Erlose	21,7
Panose	24,0

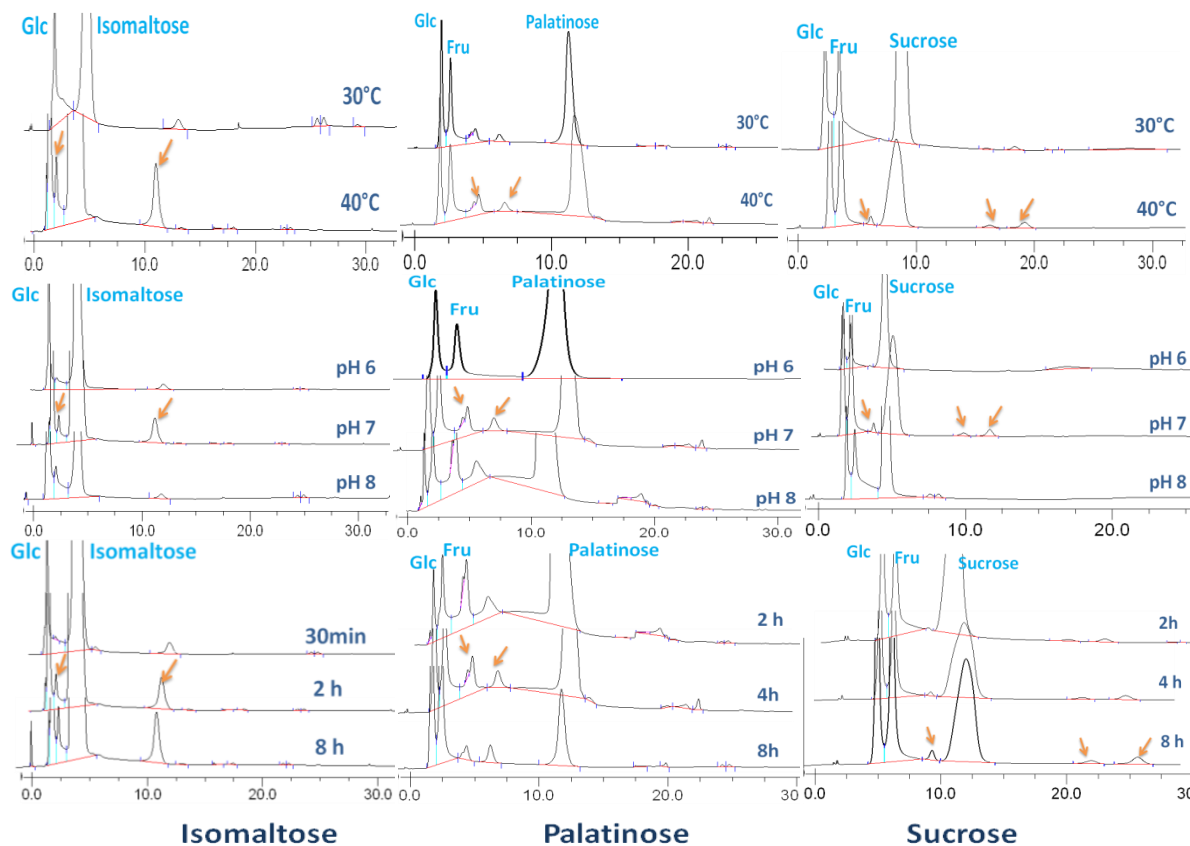
Table 3. 14 List of standards

The transglycosylation activity of purified Ima1p was tested through the putative accumulation transglycosylation products, by varying reaction parameters such as pH, temperature and reaction time. Three sugars including 200mM isomaltose and paltatinose, as well as 1M sucrose were used as substrates. The reactions were set at 30°C and 40°C respectively, following by HPAEC. The influence of pH 6, 7, and 8 was evaluated. And then, the effect of reaction time was observed from 5mins to 48 hours. The reactions under these conditions were summarized in Figure 3. 16. These Chromatograms demonstrated Ima1p formed new products, indicating that Ima1p exhibited transglycosylation activity.

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

Amongst these conditions, it was showed that Ima1p had more accumulation of transglycosylation products at 40°C. Besides, for all these substrates, Ima1p displayed better transglycosylation activity at pH 7. Ima1p exhibited some differences between three substrates. Applied isomaltose as substrate, the products of transglycosylation started to appear from 30 mins' reaction, whereas more than 2 hours for paltatinose and sucrose. The accumulation of transglycosylation products reached maximal amount after 2h, 4h and 8h incubation for isomaltose, paltatinose and sucrose respectively. In addition, we also tested with different substrate concentrations, but no significant differences were observed (no data shown).



Isomaltose Palatinose Sucrose
Figure 3. 16 analyses of transglycosylation products by HPAEC

[Applying 200mM isomaltose as substrate, the activity assays were preformed in the Citrate-Potassium Phosphate buffer at different pH and temperature as well as reaction time. The horizontal axis: Rention time (min); The vertical axis: PAD response (nC).]

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

Literatures published the optimal conditions of α -glucosidase transglycosylation activity. An α -glucosidase from fungi *Aspergillus nidulans* was published to exhibit strong transglycosylation activity with 0.5% (wt/vol) maltose at pH 5.5 and 45 °C (Kato N. *et al.*, 2002). Song *et al.* performed the transglycosylation activity assay with α -glucosidase produced by *P. pastoris* with 100mM maltose (pH 7) at 37 °C (Song K.M. *et al.*, 2013). Our results of Ima1p were comparable with the reported conditions. About the reaction time, Zhou *et al.* detected transglycosylation products of α -glucosidase from *Arthrobacter sp.* after 10min incubation; Ngiwsara *et al.* reported that the honeybee GH13 α -glucosidases started transglycosylation rapidly (in 2 minutes) (Ngiwsara L. *et al.*, 2011). However, Ima1p in the assay was slightly different. The accumulation of the transglycosylation products required a long reaction period. We observed the transglycosylation products in 30 min with isomaltose, and even longer with palatinose and sucrose. There are some other methods to optimize the condition for transglycosylation activity and increase transglycosylation products. Dols-Lafargue published the optimization of glucooligosaccharide synthesis from maltose and sucrose by dextransucrase from *Leuconostoc mesenteroides* (Dols-Lafargue M. *et al.*, 2001). They tested the some factors affecting this biosynthetic reaction: pH, temperature, the sucrose / maltose concentration ratio and the total sugar concentration. It has been reported that the transglycosylation activity was enhanced appreciably in the presence of ethanol and methanol (Kaur J. *et al.*, 2007). Further optimization of isomaltases transglycosylation activity could be carried out with other substrates, with varying concentrations and ratios. Besides, the presence of alcohols at different proportions in the reaction buffer could be an interesting issue for increasing the yield of isomaltases transglycosylation products.

On the other hand, our results consolidated the conclusion of substrate inhibition by isomaltose at high concentration (higher than 50mM). Since some researchers pointed out that an apparent decrease of substrate hydrolysis was due to substrate transglycosylation, rather than substrate inhibition

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

for the enzyme (Kawai R. *et al.*, 2004). In this case, IMA proteins might play a part of the transglycosylation activity during this hydrolysis process, which could explain the substrate inhibition itself. Therefore, we tested the transglycosylation activity under the same condition as hydrolysis assay (pH7 at 30°C). No detectable transglycosylation products formed in 15 min reaction (Maximal determining time of hydrolysis assay in present methods), indicating the decrease of hydrolysis owing to substrate inhibition. Thus, we confirmed that on some conditions, Ima1p possesses transglycosylation activity, since transglycosylation products were formed and detected, even though these products need to be further identified

Furthermore, our results illustrated that the peaks of transglycosylation products on the chromatograms (Figure 3. 16) had different retention time with the hydrolysis products and the substrates. The transglycosylation products were unknown and require more identification, especially identified by ¹H NMR spectroscopy and electrospray ionization mass spectrometry analysis. Indeed, transglycosylation activity of the α -glucosidases has been utilized in industries to produce isomaltooligosaccharides and also to conjugate sugars to biologically beneficial materials, aiming to improve their chemical properties and physiological functions (Yamamoto I. *et al.*, 1990; Murase H. *et al.*, 1997). IMOs can also be obtained from transglycosylation reactions using a neopullulanase from *Bacillus stearothermophilus*, which has been modified by site-directed mutagenesis (Kuriki T. *et al.*, 1993). The transglycosylation activity of Ima1p was preliminary investigation as an exploratory work for these isomaltases. It will be interesting to research whether isomaltases could produce IMOs by transglycosylation, and further study of their transglycosylation activities needs to be done in future.

RESULTS AND DISCUSSIONS

Enzymological characterization of IMA proteins

3.3.6 Conclusion

The enzymological analysis of the isomaltase is an important part of this Ph.D's work which completed the research of enzymological properties of isomaltases. Substrates specificities, kinetics parameters of isomaltases, inhibition and transglycosylation activity were all included. Enzymological parameters were determined and properties have been characterized. IMA protein family members exhibited clear preference for the α -1,6 bonds substrate, and most family members were able to hydrolyse a much broader substrates range, along with strong discrepancies in published data on substrate utilization by yeast isomaltase. It was further observed the similar kinetic parameters and inhibitory behaviors of Ima1p and Ima2p. All proteins could be differentiated with each other by enzymological characteristics, especially for Ima3p and Ima5p.

4 Conclusions and Perspectives

4.1 General conclusion

The presence of duplicated genes in a same species has a crucial role in evolutionary processes, especially in the rapid development of new functions (Conant G.C. and K.H. Wolfe, 2008; Innan H. and F. Kondrashov, 2010). The large ‘*Mal*’ multigene family from the yeast *S. cerevisiae* encompasses the *IMA* and related *MAL* subtelomeric genes. This family has very recently been taken as a model due to the fast expansion and evolution of these genes as compared to non-subtelomeric genes to provide experimental evidences on the mechanisms that allow such families to evolve from a common promiscuous ancestor (Brown C.A. *et al.*, 2010; Voordeckers K. *et al.*, 2012). It was proposed that gene duplications repeatedly spawned daughter genes in which mutations optimized either isomaltase or maltase activity, to circumvent adaptive constraints on a multifunctional gene by optimizing the subfunctions separately in different paralogs. This idea was perfectly supported by focusing on the α -1,4 and α -1,6 specificities in this large ‘Mal’ family (Voordeckers K. *et al.*, 2012). However, quantitative data reported in the present work showed that besides the α -1,6 glycosidase activity, relevant activities on substrates such as sucrose or α -1,3 disaccharides did persist to different extends in this *IMA* protein family. This substrate “ambiguity” probably enhances the phenotypic plasticity required for sugar utilization in microorganisms such as the yeast *S. cerevisiae*.

The comprehensive analysis of the characteristics of this entire protein family also illustrated evolutionary singularities, highlighting on how mutations on very few, key residues can lead to significant effects. As an example, the very recent duplication events within the family indeed led to two identical genes, *IMA3* and *IMA4*, which encode a protein with only 3 different amino acids relative to *Ima2p* although presenting significant alterations in both catalytic and thermostability parameters.

Conclusions and Perspectives

Typically, as the most divergent protein of the family, Ima5p could maintain most of Ima1p or Ima2p catalytic characteristics, with the notable exception for its highly altered thermostability. This later result reinforced the idea from Voordeckers *et al.* that different evolutionary routes leading to variable amino acids combinations could possibly lead to similar changes in substrate specificities and highlighted somehow the robustness of biological systems through evolution.

4.2 Perspectives

As previously mentioned, Ima1p was the only solved crystal structure of the protein family. 3D molecular structures enable sound identification and analysis of secondary structure elements, and provide more robust insights on key structural elements. Therefore, to pursue research on the remaining IMA protein family members, it would be interesting to get 3D structures of these proteins to analyse these structures and get better knowledge on the structure-function relations in these proteins. Especially with the comparison of Ima1p and Ima5p X-ray structures, the thermostabilization of IMA proteins could be studied in depth. Indeed, the total number of proline residues in Ima1p and Ima5p is approximately the same (30 vs. 29, respectively) but few differences in key positions of proline residues in Ima5p might explain its great temperature sensitivity. But the X-ray structure will also allow proposing and testing other probable mechanisms of thermostabilization.

It was observed from kinetic studies, that substrates with α -1,6 glycosidic linkage could be hydrolyzed by isomaltase, but the transglycosylation activity that was identified suggests the capability of synthesis of new products. Further research could be carried out with optimization of transglycosylation activity, products identification and insights on mechanism, to finally consider new applications of these enzymes in biotechnological processes.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

5 EXPERIMENTAL PROCEDURES

5.1 Strains and culture conditions

5.1.1 Bacteria strains

In the present study, *Escherichia coli* DH5 α (*F*, *supE44*, Δ *lacU169* (ϕ *lacZ* Δ *M15*), *endA1*, *recA1*, *hsdR17*, *thi-1*, *gryA96* λ) was used to amplify the plasmids. Bacteria *Escherichia coli* DH5 α cells were cultivated on LB medium (Tryptone 1%, Yeast extract 0.5%, NaCl 0.5%, agar 2%) solid plate at 37°C. For plasmid preservation, Ampicilline (Sigma, No. A9518) was added to LB medium to obtain a final concentration of 150 μ g/mL when temperature has dropped to < 60°C. Ampicilline was prepared as a 150 mg/mL stock solution in distilled water (steriled by filtration) and stored at -20 °C.

5.1.2 Yeast strains

The *Saccharomyces cerevisiae* strain CEN.PK113-5D (MATa *ura3-52 MAL2-8^c SUC2*) (Van Dijken J. P. *et al.*, 2000; Nijkamp J. F. *et al.*, 2012) was used to express IMA proteins,. The strain was grown on YPD solid plate (Yeast extract 1%, Bacto-peptone 2%, Glucose 2%, agar 2%) in standard conditions. After transformation with plasmid it was cultivated in liquid YNB medium (Yeast Nitrogen Base w/o amino acids and w/o ammonium sulfate 0.17%, Ammonium sulphate 0.5%, Glucose 2%).In order to induce the expression of the IMA proteins, cells were grown in YNR (YNB medium in the presence of 2 % raffinose (w/v)) at 30°C overnight (OD₆₀₀ approx. 4), and then diluted to OD₆₀₀ 0.4 in YNGal (YNB medium in the presence of 2 % galactose (w/v)) for 6 hours.

EXPERIMENTAL PROCEDURES

Molecular biology methods

5.2 Molecular biology methods

5.2.1 Primers

Forward (F) and reverse (R) primer sequences used to clone the designed *IMAx* in the pYES2.1 TOPO[®] TA vector are listed in Table 5. 1.

ORF	Primer sequences
<i>IMA1</i>	F: ACAATGACTATTTCTTCTGCACATCCAG R: TTCGCTGATATATATTCTTCCTTCC
<i>IMA2</i>	F: ACAATGACTATTTCTTCTGCACATCCAG R: TTCAGATATGTAAATTCTGCCCTCC
<i>IMA3</i>	F: ACAATGACTATTTCTTCTGCACATCCAG R: TTCAGATATGTAAATTCTGCCCTCC
<i>IMA5</i>	F: AACATGACGATCATCCATAATCCTAAATG R: CTTCAACAAGTAAAGTCTTCCTTCCC

Table 5. 1 List of primers

5.2.2 PCR amplification

PCR was performed with the Bio-Rad My cycler[™] thermal cycler. The pGEMt-*IMAx* plasmids (*IMA1/YGR287C*, *IMA2/YOL157C*, *IMA3/YIL172C*, *IMA5/YJL216C*) were used as template (Teste M.A. *et al.*, 2010). The reaction was performed in a final volume of 50 μ L by adding about 100 ng of template DNA, 5 μ L of 2.5 μ M Primers, 5 μ L of 2.5 mM dNTP, 1 \times final Phusion HF Reaction Buffer, and 0.5 μ L of Phusion DNA polymerase (FINNZYMES, No. F-530S). After initial denaturation (30sec at 98°C), the thermocycling program includes 30 cycles of 10 sec denaturation at 98 °C, following 20 sec annealing at 58°C and 1min extension at 72°C.

EXPERIMENTAL PROCEDURES

Molecular biology methods

5.2.3 Purification of DNA fragment

We used QIAquick Purification Kit (QIAGEN, No. 28706) to purify the PCR products. 5 volumes of Buffer PB were added to 1 volume of the PCR sample and mixed by pipetting. To bind DNA, we applied the sample to the QIAquick column and centrifuge for 30–60sec. Then the QIAquick column was washed with 0.75 mL of Buffer PE. After a first centrifugation, the column was centrifuged empty for an additional 1 min. To elute DNA, 40 µl of H₂O were added to the center of the QIAquick membrane and centrifuged for 1 min. To increase DNA concentration, we refilled the center of the QIAquick membrane with the first elution and performed a final centrifugation.

5.2.4 Cloning in pYES2.1/V5-His-TOPO[®] (TOPO[®] Cloning)

pYES2.1/V5-His-TOPO[®] is a plasmid vector used for regulated expression in *Saccharomyces cerevisiae*. The Figure 5.1 summarizes the features of the pYES2.1/V5-His-TOPO[®] vector. The vector is supplied linearized between base pairs 512 and 513. It provides a highly efficient, one-step cloning strategy ("TOPO[®] Cloning") by direct insertion of "Taq polymerase-amplified PCR" products.

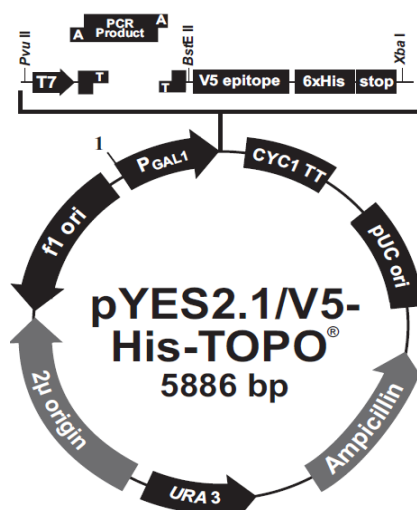


Figure 5. 1 Plasmid Map of pYES2.1/V5-His-TOPO[®] vector

EXPERIMENTAL PROCEDURES

Molecular biology methods

As Phusion DNA polymerase has proof-reading activities and generates blunt-end PCR products, the blunt-end PCR products should be A-tailed before cloning in a pYES2.1/V5-His-TOPO[®] TA vector. 7 μ L of the obtained PCR product was mixed with 1 μ L of 10 \times Thermol Pol Buffer 1 μ L of 2 mM dATP and 1 μ L of Taq DNA polymerase (M0267S, NEB) and incubated at 70°C for 30 minutes. Then 4 μ L of fresh PCR product was mixed with 1 μ L of Salt Solution and 1 μ L of TOPO[®] vector. Mixed reaction was incubated for 20 minutes at room temperature, and then placed on ice. 2 μ L of the reaction was added into a vial of Chemically Competent *E. coli* DH5 α (NEB, C2987H). The tube was kept 20min on ice and heat-shocked for 30 seconds at 42°C (without shaking). 250 μ L of SOC supplied medium was added and the tube was shaken at 37°C for 1 hour. Accordingly, 10-50 μ L from transformation were spreaded on a pre-warmed selective plate (LB + Ampicilline) and incubated overnight at 37°C. Next day, we extracted the plasmids with QIAprep spin Miniprep kit (QIAGEN, No. 27106) and checked by restriction enzyme. The obtained plasmids were named pYES2.1 TOPO/*IMA*_x and verified by sequencing.

5.2.5 Mutation construction

Ima3_L240P (CGAAAATTCAAAGTGGCAACCCAGTG; CTC \rightarrow TTC : L240P mutation; Underlined: silent mutation, *DdeI* lost), Ima3_R279Q (GAAATGCAAACACGCTACT; GAC \rightarrow CAA : R279Q mutation; Underlined: silent mutation, *NspI* lost) and Ima5_VGI-MQH (TTCCCGAGGGT(N)₂₁GAAATGCAGCACGGA; GTTGGTATC \rightarrow ATGCAGCAC : VGI to MQH mutation; Underlined: silent mutation, *AvaI* added) variants were synthesized by Eurofins MWG Operon (Ebersberg, Germany). *MfeI* mutated fragments of *IMA3* (854 bp) were inserted into *MfeI* sites of the pYES2.1 TOPO/*IMA3* plasmid yielding to the final expression vectors. Similarly, the *Tth1111/SalI* mutated fragment of *IMA5* (896 bp) was subcloned into the pYES2.1 TOPO/*IMA5* plasmid.

EXPERIMENTAL PROCEDURES

Yeast transformation by lithium acetate

5.3 Yeast transformation by lithium acetate

We performed the yeast transformation by lithium acetate as reported previously (Gietz R.D and R.H. Schiestl, 2007). Yeast strain CEN.PK113-5D was inoculated in 5 mL of YPD and incubated overnight at 30° C with shaking. Next morning the culture was diluted to 0.25 (OD₆₀₀) in 5 mL of warm YPD, and incubated at 30° C on a shaker at 200rpm until 1.0 (OD₆₀₀). 0.5 mL of culture was centrifuged at 4800 rpm for 2 min, and the cells were washed with 1 mL of sterile water. After washing, the cells were resuspended in 200 µl of 100 mM LiAc, and placed at room temperature for 5min. Following centrifugation, the cells were mixed sequentially in a final volume of 360µL containing 240 µl of PEG (50% w/v), 36 µl of 1.0 M. LiAc, 50 µl of ss-DNA (2.0 mg/mL, previously boiled 5 min and put on ice), 3 µl of plasmid DNA and 31 µl of sterile water. The tube was incubated 30 min at 30° C with shaking, and then at 42° C in a Mini dry bath for 30 min more. After centrifugation the cells were resuspended in 200 µl of sterile water and spreaded onto URA minus YNB plates for 3 days at 30°C.

EXPERIMENTAL PROCEDURES

Expression and purification

5.4 Expression and purification

5.4.1 Preparation of crude extract

To induce the expression of the protein, yeast strain CEN.PK113-5D was inoculated in 5 mL of liquid YNB and incubate with shaking overnight at 30° C. Next day, cells were transferred in YNR at 30 °C overnight (OD_{600} approx. 4), and then diluted to OD_{600} 0.4 in YNGal for 6 hours. Then, 50 mL of culture (enough to obtain 30 μ g of purified protein) was collected by centrifugation (2000 g, 5 min), and washed with 1 mL of water. The cells were resuspended in 700 μ l of ice-cold extraction solution containing 50 mM sodium phosphate buffer pH 7 and 1 mM PMSF (freshly prepared), and mixed with an equal volume of glass beads (0.4–0.5 mm diameter) to break cells. The suspension was vortexed four times each 30 seconds, and placed on ice between intervals, then centrifuged at 2000g for 5 min at 4°C. The supernatant was kept on ice for purification.

5.4.2 Purification of proteins by affinity chromatography

The first step was resin preparation (TALON[®] Metal Affinity Resin [Clontech, Ref. 635503]). 150 μ l of blended resin was added into a tube and centrifuged at 700g for 3min. The resin was washed twice by centrifugation (700g, 2 min) with 625 μ l of 1x Equilibration/Wash Buffer (300 mM NaCl in 50 mM sodium phosphate at pH 7). 500 μ l of crude extract was loaded to the equilibrated resin and shook gently for 35min on a wheel at room temperature. After centrifugation (700g for 2min), the resin was washed twice with 625 μ l of equilibration/wash buffer. The final step was elution (3 times): firstly, add 125 μ L of 1x Equilibration/Wash Buffer in presence of 150 mM imidazole and centrifuge 2min; secondly, 75 μ L of 1x Equilibration/Wash Buffer and 350mM of imidazole were used and centrifuged again 2min; finally, 75 μ L of 1x Equilibration/Wash Buffer were applied, and imidazole was up to

EXPERIMENTAL PROCEDURES

Expression and purification

500mM, along with the last centrifugation. Consequently, we merged the three elutions and we obtained 275 μ L purified protein.

5.4.3 SDS-PAGE/ Coomassie Blue staining

The polyacrylamide gel of SDS-PAGE was made by adding 60 μ l of 10% ammonium persulfate and 6 μ l of TEMED to 10mL of NEXT GEL™ 12.5% acrylamide solution (AMRESCO, No. 1027B060). 18 μ L protein samples and 6 μ L 4x of denaturing buffer were mixed and denatured 5 mins at 100°C. 20 μ L of sample were loaded and 5 μ l of PageRuler™ Prestained Protein Ladder (Fermentas, No. 26616) was used as ladder. Migration of the protein was carried out by using the migration buffer of 1 \times dilution of NEXT GEL™ RUNNING BUFFER, 20 \times (AMRESCO, No. 2487B033) at constant voltage of 150V for 1.5 hour. After migration, the polyacrylamide gel was stained with the dye PageBlue™ Protein Staining Solution (Fermentas, No. R0571).

5.4.4 Measurement of proteins concentration (Bradford assay)

Protein standards were prepared using bovine serum albumin (BSA) with concentrations of 0, 2,4, 5,8, 10, 20,30,40 50 μ g/mL. 200 μ L of each protein solution and 50 μ L of Bio-Rad protein assay dye reagent (Ref 500-0006) were mixed in microplate and incubated at room temperature for 15 minutes in dark. The standard curve was created by plotting absorbance (550 nm) versus amount of protein. For the unknown purified protein, samples were diluted 15 and 25 times respectively and the absorbance was measured in the same way as the standard curve. The concentrations of IMA protein solutions were obtained according to the standard curve.

5.4.5 Removal imidazole (The Zeba™ Desalt Spin Columns)

To investigate the influence of imidazole on specific activities of the enzymes, imidazole was

EXPERIMENTAL PROCEDURES

Expression and purification

removed from the purified protein solution with ZebaTM Desalt Spin Columns (Thermo Scientific No. 89890). The storage solution was removed by placing column in a collection tube and centrifugation at 1,000 *g* for 2 minutes. Then, 1 mL of Citrate-Potassium phosphate buffer was added to the column and centrifuge at 1,000 *g* for 2 minutes. Then, the column was placed in a new collection tube and 200~700 μ l purified IMA proteins were loaded to the center of the compact resin bed, and the column was centrifuged at 1000*g* for 2 minutes to collect the sample. .

EXPERIMENTAL PROCEDURES

Enzymatic assays

5.5 Enzymatic assays

5.5.1 Colorimetric reaction with *p*NPG

We used an artificial substrate *p*NPG for the enzymatic reaction. *p*NP (chromophore) is released after the cleavage of the *p*NPG by the enzyme (Figure 5.2). The reaction is detected photometrically using an Agilent HP 8453 UV-visible spectrophotometer (Agilent Technologies, Massy, France) which offered a control of assay temperature and online acquisition of the absorbance. The activity of the enzyme can thus be determined via the amount of liberated dye and the incubation time.

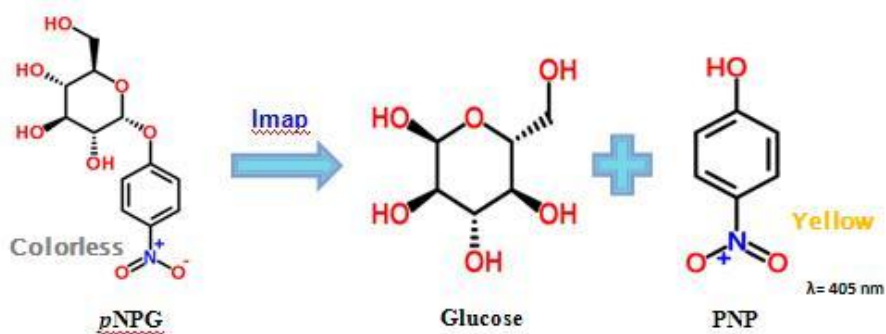


Figure 5. 2 Reaction of an isomaltase with *p*NPG (4-Nitrophenyl α -D-glucopyranoside).

First we made a standard curve with a range of concentration of *p*NP from 0 to 25 μM and plotted the absorbance against concentration of *p*NP to calculate the molar extinction coefficient in our conditions. The obtained molar extinction coefficient is $0.0681 \text{ mM}^{-1}\text{cm}^{-1}$. The enzyme was diluted in such a way to have an absorbance fitting with the standard curve. Unless indicated otherwise, all assays were carried out in 1 mL reaction mixtures containing 20 μL of IMA protein solution and *p*NPG (*x* mM) dissolved in 980 μL of Citrate-Potassium phosphate buffer pH 7 (buffer was obtained by mixing 7.06 mL of 0.5M citric acid, 65.88 mL of 0.5M K_2HPO_4 and water until 200 mL) by using 1 cm optical

EXPERIMENTAL PROCEDURES

Enzymatic assays

path length microcuvettes. Reactions were initiated by protein addition and were monitored by recording the absorption at 405 nm. .

Specific activity of isomaltase on *p*NPG is the amount of *p*NPG (μM) converted by the enzyme in a unit of time (min) under given conditions per milligram of total protein. The specific activity (SA) of isomaltase is calculated using the following equation:

$$SA = (S / \epsilon_{pNPG} \times 60) / C$$

S is the slope of the line plotted (μM); ϵ_{pNPG} the millimolar extinction coefficient at 405nm; 60 is need to give the reaction time in minutes; C is the amount of isomaltase in milligram. The absorbance was plotted as a function of time. One important factor determining the accuracy of the regression curve is the coefficient of determination R^2 (shown in Figure 5.3). The closer the R^2 value is to 1, the closer the regression curve overlaps with the actual values measured. This is particularly important for initial reaction rate determination from initial slope: this initial velocity is the initial linear portion of the enzyme reaction when these IMA proteins are still stable and less than 10% of the substrate has been depleted or less than 10% of the product has formed. The coefficients of determination are always calculated and displayed automatically with Excel.

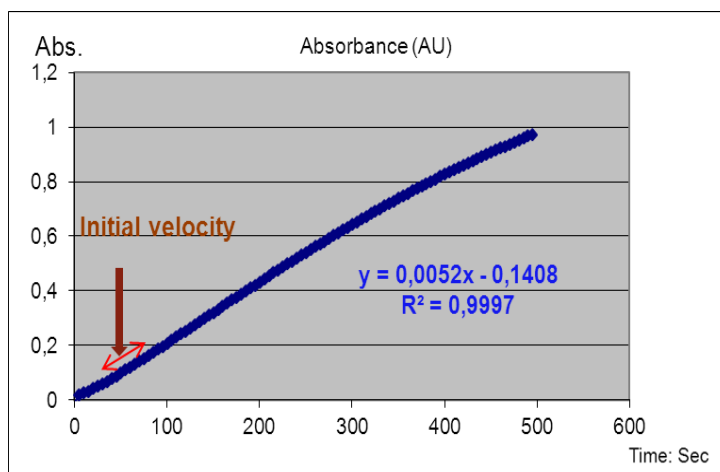


Figure 5. 3 Standard curve with non-linear regression

EXPERIMENTAL PROCEDURES

Enzymatic assays

5.5.2 Coupled enzymatic reactions for other substrates

Activity on the other substrates was measured by using a coupled spectrophotometric assay between the release of glucose and the production of NADPH at 340 nm, with the simultaneous use in the assay of hexokinase and glucose-6-phosphate dehydrogenase. This assay was based upon the following reactions:



The IMA protein converts substrate to glucose and hexokinase catalyzes the phosphorylation of glucose in the presence of ATP and magnesium ions. The resultant glucose-6-phosphate is then oxidized to 6-phosphogluconolactone with concomitant reduction of nicotinamide adenine dinucleotide (NADP). The final reduction of NADP^+ to NADPH is proportional to the glucose produced and was monitored by following the absorbance at 340 nm with the Agilent HP 8453 UV-visible spectrophotometer. Unless indicated otherwise, the reaction was started by the addition of 20 μl freshly purified protein to the 1 mL reaction mix containing 5 mM MgCl_2 , 1 mM ATP, 1 mM NADP, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase and substrate at desired final concentration. At this wavelength, the molar extinction coefficient used for NADPH was $\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$.

During the coupled enzymatic reaction, there might be some contamination of substrates by glucose. This could cause an inaccurate increase of the curve (Figure 5.4). To avoid glucose contamination artifact, IMA proteins were added after 2-3 mins when a flat baseline is reached. Thus, the enzymatic mixture should be added firstly and reaction was initiated by protein addition. Similar to

EXPERIMENTAL PROCEDURES

Enzymatic assays

colorimetric detection methods, the results of hexokinase and glucose-6-phosphate dehydrogenase coupled method can be processed by a same way (Figure 5.4).

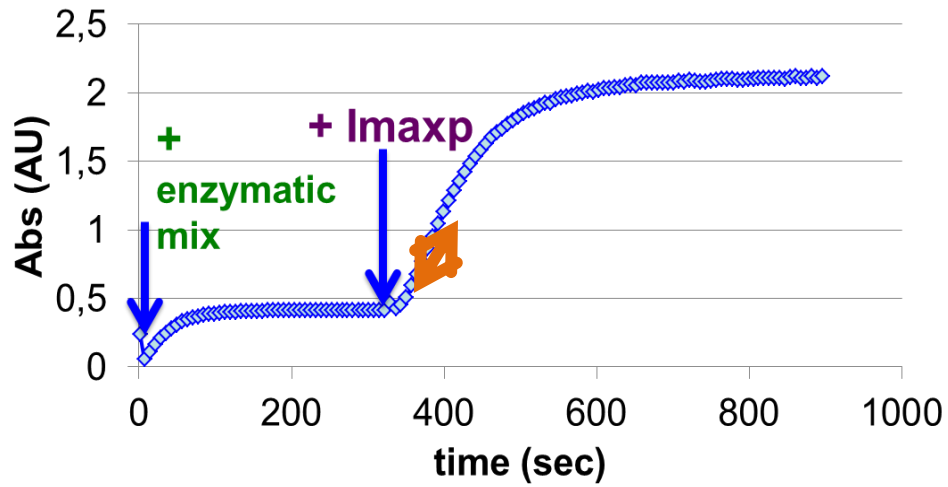


Figure 5. 4 Coupled enzymatic reaction, with initial reaction rate determination

EXPERIMENTAL PROCEDURES

Biochemical parameters

5.6 Biochemical parameters

5.6.1 Optimal temperature

The determination of the optimal temperature was carried out at pH 7 and at temperatures ranging from 20°C to 60°C, using *p*NPG at 5 mM as the substrate. IMA protein was incubated in water bath.

5.6.2 Optimal pH

The temperature was set to 30°C. The pH varied from 2.6 to 8 in Citrate Potassium phosphate buffer and in sodium phosphate buffer to extend the range of pH values to 9. The Citrate-Potassium phosphate buffer solutions pH 2 ~8 were prepared with two mother solutions: A, 0.5M citric acid solution and B, 0.5 M dipotassium phosphate solutions.

pH	vol. of solution A (mL)	vol. of solution B (mL)	vol. of H ₂ O (mL)
2	39.2	1.6	159.2
3	31,78	16,44	151,78
4	24,58	30,84	144,58
5	19,4	41,2	139,4
6	14,74	50,52	134,74
7	7,06	65,88	127,06
8	1.1	77.8	121.1

The sodium phosphate buffer solutions pH 7 – 9 was prepared with 0.1M disodium hydrogen phosphate and 0.1M HCl.

pH	vol. of phosphate (mL)	vol. of 0.1M HCl (mL)
7	756.0	244
8	955.1	44.9
9	955.0	45.0

EXPERIMENTAL PROCEDURES

Biochemical parameters

5.6.3 Half Life

In present work, half-life ($t_{1/2}$) was described as the time required for the activity to fall to half of its initial value. Half-life was determined from the derivative of the curve of pNP production as a function of time (Shown in Figure 5.5). Since IMA proteins were subjected to inactivation or denaturation, an exponential decay of enzyme activity was perfectly correlated with the irreversible thermal deactivation process. This could be modeled through the classical exponential decay law. The derivative curve was fitted using the exponential equation: $v(t) = v e^{-\lambda t}$, with λ the decay constant ($\lambda = \text{Ln}2 / t_{1/2}$), v the initial reaction rate and $v(t)$ the residual velocity at time. For this assays, the activity was followed over time by setting the reaction at a specified temperature (37°C, 40°C and 42°C)

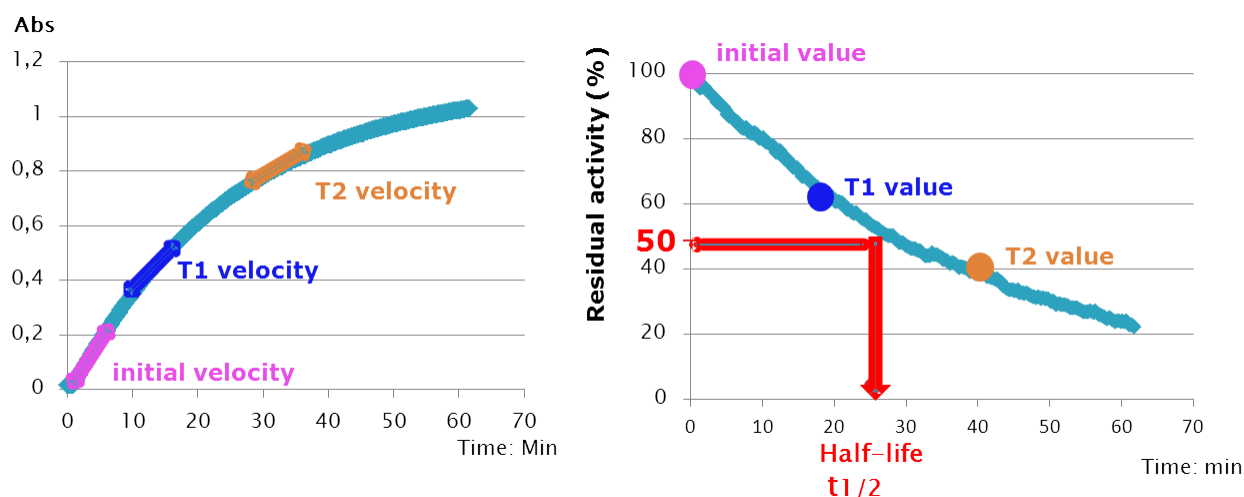


Figure 5. 5 Curves of Half life

EXPERIMENTAL PROCEDURES

Biochemical parameters

5.6.4 Melting Temperature

The melting temperature (T_m) of isomaltases was assayed by differential scanning fluorimetry (DSF), which monitors thermal unfolding of proteins in the presence of a fluorescent dye (Niesen F.H. *et al.*, 2007). DSF is a rapid and inexpensive screening method and identifies low-molecular-weight ligands that bind and stabilize purified proteins. Through unfolding of the protein, hydrophobic patches become exposed and strong fluorescent is emitted by the dye molecules. In the process, when the concentrations of folded and unfolded protein are equal, the temperature is considered as melting temperature (see Figure 5.6).

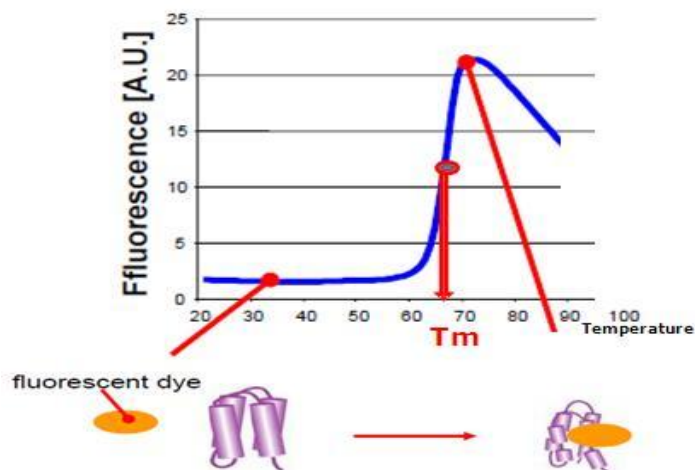


Figure 5. 6 Melting temperature curve of DSF

We concentrated IMA proteins with Amicon Ultra filter (30K) as DSF assay requires 50 μ M of purified protein. 500 μ L of protein were loaded to the column and the tube was centrifuged at 14,000 g for 15 minutes. Then we placed the Amicon Ultra filter device upside down in a clean microcentrifuge tube and we recovered the concentrated solute after centrifugation 2 minutes at 1,000 g to. We thawed Sypro-orange stock solution 5000x (Invitrogen, S6650) on ice, and diluted it to 5X. We filled a clear, non-skirted, low profile, optical reading-compatible 96 well plate with reaction components as following (Dispensing reaction components per well): 1 μ L of SYPRO orange (5X), 2 μ L of IMA

EXPERIMENTAL PROCEDURES

Biochemical parameters

protein (5 μ M final), 0/1/2 μ L of 100mM metal ion solution, 0/1/2 μ L of 1M Ligand solutions (series of disaccharides) and Citrate-Potassium phosphate (pH6, 7 or 8) buffer up to a final volume of 20 μ L. The plate was sealed and centrifuged for 1 minute at 1000rpm. Then it was incubated with an increase of temperature from 25 to 80°C (0.3°C increment every 0.3 second) in a CFX96 Real-time System (Bio-Rad).

At the end of the operation, the data were analyzed. The qPCR machine exported data (in Excel format) for both the raw fluorescence signal and the derivative of the fluorescence signal as a function of the temperature. We took the average of the three replicates (either fluorescence signal or derivative). The significant increasing slope was related to the melting of the protein (Figure 5.7-A). When the protein unfolded, more dye bound and the signal boosted. The T_m corresponded to the inflection point of the transition curve (fluorescence unit=f (T)). The derivative plots of fluorescence signals (Figure 5.7-B) illustrated the temperature at which the derivative was the lowest (most negative) was the melting temperature.

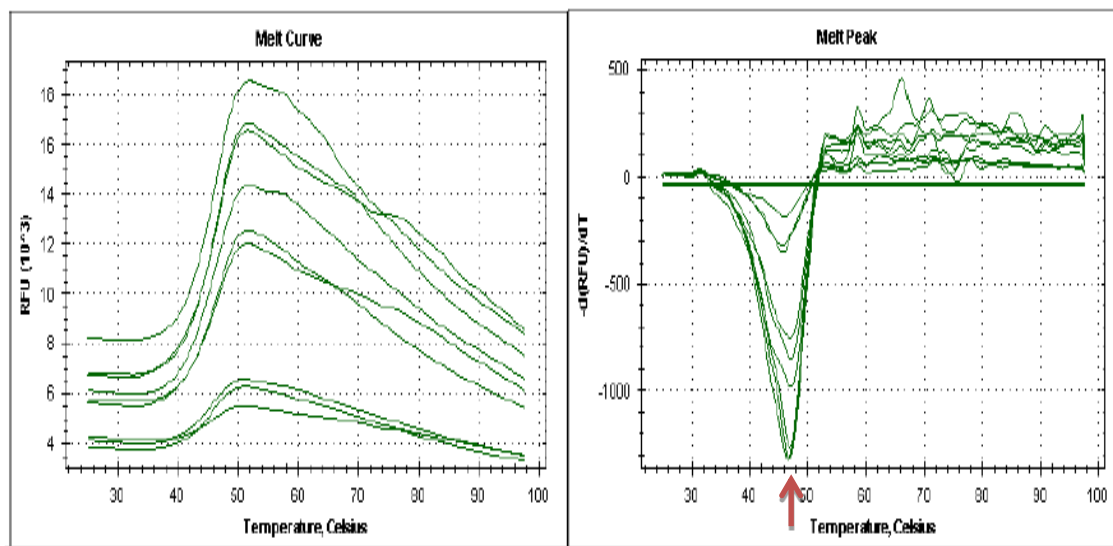


Figure 5. 7 Fluorescence signal plots (A) and derivative plots (B)

EXPERIMENTAL PROCEDURES

Biochemical parameters

5.6.5 Influence of chemical compounds and metal ions

The activity was measured under standard condition (5 mM *p*NPG as substrate in the Citrate-Potassium Phosphate buffer, pH 7, 30°C) in the absence (Control) or in the presence of different effectors. In this assay, many effectors were determined including Tris, EDTA, urea as well as glycine. All these compounds were performed at 5 mM final concentration with the exception of 1 mM of MnCl₂.

5.6.6 Kinetic parameters

The parameters V_{\max} , K_m were obtained by plotting reaction rate against substrate concentration, using nonlinear regression of the Michaelis–Menten equation, of the Enzyme Kinetics 1.3 module of the SigmaPlot 11.0 package (Systat Software, IL, USA) When inhibition occurred, the K_i was computed by nonlinear curve fitting of the velocity as a function of substrate concentration, performing the “single substrate – single inhibitor” model of the SigmaPlot Enzyme Kinetics module. In addition, the catalytic constant k_{cat} of the enzyme was calculated from $k_{\text{cat}}=V_{\max}/[E]$. The molar concentration [E] was 68.6 kDa for Ima1p and Ima2p, 68.7 kDa for Ima3p and 67.6 kDa for Ima5p.

5.6.7 Measurement of transglycosylation and analysis by HPAEC-PAD

Substrates at different concentrations (50mM, 200mM and 1M) were dissolved in Citrate-Potassium phosphate buffer (pH 6,7 and 8) and 20μl of purified Ima1p were added into 980μL of substrate solution. The tube was incubated in water bath and samples (50μl) were collected at various time intervals from 5mins to 60 hours. Temperature, pH value and substrate concentration varied to optimize reaction conditions. The enzymatic reaction was stopped by heating 5 min at 95 °C to totally

EXPERIMENTAL PROCEDURES

Biochemical parameters

denature the enzyme. Then samples were diluted to *approx.* 60 μ g/mL and filtered with 0.2 μ m Syringe Filters. In addition, 250 μ l of 22 standards were prepared. The concentration was 30 μ g/mL for each monosaccharide and 60 μ g/mL for the disaccharides. The high performance anionic exchange chromatography system used in our experiments was an ICS-3000 system (Dionex, Sunnyvale, CA, USA). This system consisted of an SP gradient pump system, a DC detector/chromatography module thermally regulated with a 25 μ L injection loop, an autosampler (Dionex, AS50, CA, USA), and an electrochemical detector equipped with an amperometric cell. The cell comprised an about 1 mm diameter gold working electrode, a glass and Ag/AgCl combination reference electrode (Dionex) and a titanium counter electrode consisting of the cell body. The chromatographic separation of the sugars was performed on a CarboPac PA100 analytical column (250 mm x 4 mm i.d. Dionex) with a CarboPac PA100 guard column (40 mm x 4 mm i.d. Dionex) at a flow-rate of 1 mL min⁻¹ at 30°C. The sample injection volume was 25 μ l. The gradient elution was performed with mobile phases A (100 mM sodium hydroxide) and B (600 mM sodium acetate trihydrate in 100 mM sodium hydroxide) using the following program: 100% of A for 10 min followed by an acetate gradient to reach a concentration of 132 mM (from 0 to 22% of solvent B) for 38 min, a 7 min cleaning step 50/50 of solvent B and C (500 mM sodium hydroxide) and finally a 10 min conditioning time.

6 APPENDIX

6.1 Enzyme biochemistry and kinetics

Enzymes are natural catalysts (protein molecules or ribozyme) that manipulate other molecules. The target molecules bind to an enzyme's active site and are transformed into products through a series of steps known as the enzymatic mechanism. These mechanisms can be divided into single-substrate and multiple-substrate mechanisms (Ebbing D.D. and M.S. Wrighton, 1996). Kinetic studies on enzymes that only bind one substrate, aim to measure the affinity of the enzyme to its substrate, evaluate the turnover rate and reveal the catalytic mechanism of an enzyme.

6.1.1 Michaelis-Menten mechanism

In the early 20th century, Leonor Michaelis and Maud Leonora Menten, proposed the model known as Michaelis-Menten Kinetics to account for enzymatic dynamics (Michaelis L. and Menten M.L., 1913). The model serves to explain how an enzyme can cause kinetic rate enhancement of a reaction and explains how reaction rate depends on the concentration of enzyme and substrate (Johnson, K.A. and R.S. Goody, 2011). The Michaelis-Menten mechanism for the catalysis of biological chemical reactions is one of the most important, simplest and best-known models in biochemistry. Besides, Michaelis–Menten kinetics have also been applied to a variety of spheres outside of biochemical reactions (Chen W.W. *et al.*, 2010),

The general reaction scheme of an enzyme-catalyzed reaction is shown in Figure 2. 16: The enzyme interacts with the substrate by binding to its active site to form the enzyme-substrate complex (ES). The reaction is followed by the decomposition of ES to regenerate the free enzyme (E), and the

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new product (P). In the reaction, k_1 and k_{-1} denote the reaction rate constants. k_1 , constants of the binding of the enzyme to the substrate forming the ES complex ; k_{-1} , constants of the reverse reaction of catalysis; k_2 , also accepted as k_{cat} , constant of the dissociation of the ES complex to free enzyme and product (Chen W.W. *et al.*, 2010). K_{cat} is the maximum number of substrate molecules converted to product per enzyme molecule per second. It is the catalytic efficiency of enzyme, which can be used to compare different enzymes or same enzyme for different substrates.

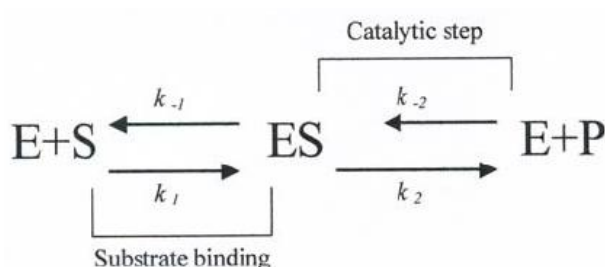


Figure 6. 1 Illustration shows the two steps in an enzyme-catalysed reaction.

[Enzyme (E) binds to substrate (S) and form a complex (ES). The ES dissociates into E + S or E + P or associates from them. Total association is governed by $k_1 + k_{-2}$ and total dissociation by $k_{-1} + k_2$]

The Michaelis–Menten model takes the form of an equation describing the rate of enzymatic reactions, by relating reaction rate v to $[S]$ - the concentration of a substrate (S). The formula is given by

$$v = \frac{d[P]}{dt} = \frac{V_{\text{max}}[S]}{K_m + [S]}$$

Here, V_{max} represents the maximum rate achieved by the system, at maximum (saturating) substrate concentrations. The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of V_{max} , and is an inverse measure of the substrate's affinity for the enzyme—as a small K_m indicates high affinity, meaning that the rate will approach V_{max} more quickly (Cornish-Bowden A.,2013). Biochemical reactions involving a single substrate are often following the Michaelis–Menten kinetics. The velocity of the reaction is proportional to the concentration of enzyme and depends of the substrate concentration $[S]$; a diagram can be plotted as shown in Figure 2. 17.

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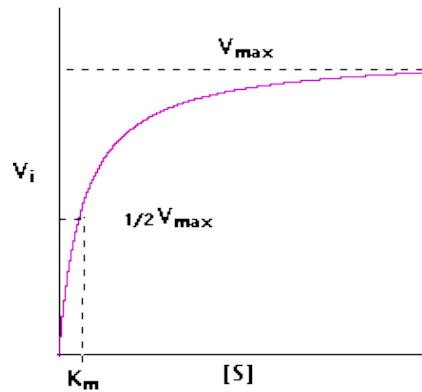


Figure 6. 2 Reaction rate versus concentration of substrate

[Typical plot of an enzymatic reaction that obeys the first order Michaelis-Menten kinetics.]

A commonly method for determining the constants V_{max} and K_m is to perform a series of enzyme assays at varying substrate concentrations, and measure the initial reaction rate. By plotting reaction rate against concentration, and using nonlinear regression using dedicated computer programs, the parameters of the Michaelis–Menten equation may be obtained (Figure 2. 18). Although discouraged when following the Guidelines of the STRENDA Commission, we could also use the reciprocal of the Michaelis Menten kinetics equation, *i.e.* the Lineweaver-Burk double reciprocal plot, which allows a simple and convenient estimation of kinetic parameters:

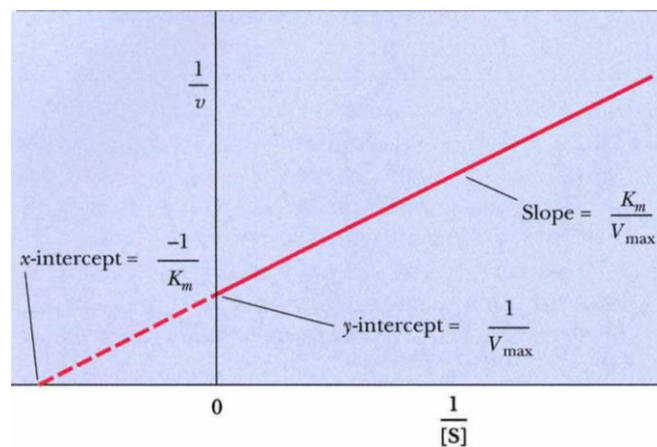


Figure 6. 3 Double reciprocal of Lineweaver-Burk equation

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(adapted from Nelson D.L. *et al.*, 2008)

6.1.2 Enzyme inhibition

Inhibitors disrupt the interaction between enzyme and substrate, slowing down the rate of a reaction in different ways (Silverman R.B, 1988; Copeland R.A, 2004). There are different types of inhibitions, including both reversible and irreversible forms. Most often inhibitors that covalently attach to the enzyme are irreversible, while inhibitors that mediate interactions through hydrophobic and ionic bonds can associate or dissociate reversibly. There are three main types of reversible inhibition: competitive, noncompetitive and uncompetitive (See Figure 2. 19) (Berg J.M. *et al.*, 2006).

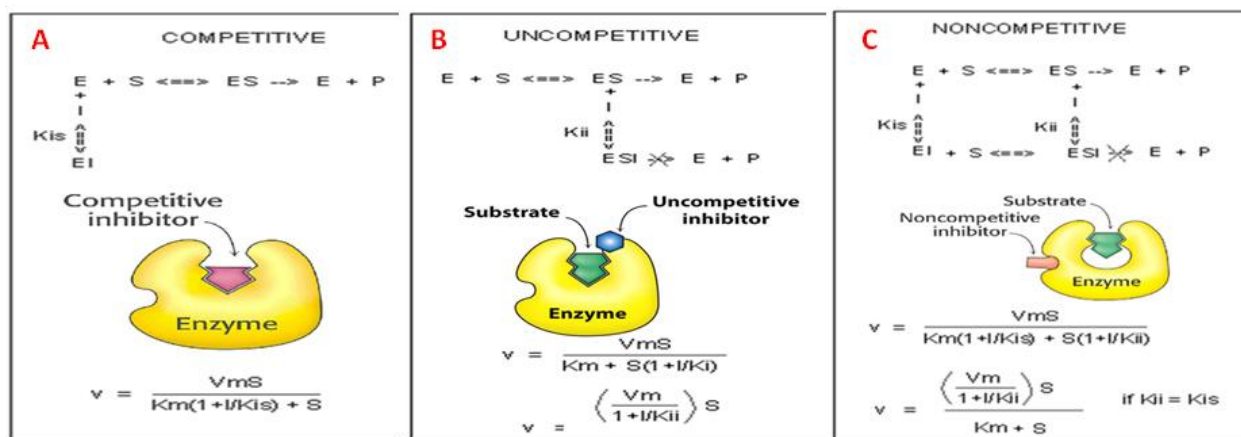


Figure 6. 4 The mechanism of the main types of reversible inhibition

[The mechanism was illustrated in at the top and the molecular image beneath. A, The signpost is that inhibition effect is lowered or removed by addition of more substrate. Often this is because they compete for occupation of the same binding site, but also allosteric binding to distant sites might lead to enzyme changes so that once one is bound, that excludes the other from binding. B, The signpost means that the inhibitor will only bind to an enzyme-substrate complex. C, The signpost is that inhibition effect is not lowered or removed by addition of more substrate, and that the inhibitor binds with same strength to free or substrate-bound enzyme (or that substrate binds equally well to free or inhibitor-bound enzyme).]

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Cornish-Bowden proposed a very robust, graphical mean to determine the type of inhibition (Cornish-Bowden Athel, 1974). Two plots actually complement each other (the plot of $1/v$ against I (Dixon plot) and the plot of s/v against I , respectively) and unambiguously allow determining the inhibition type.

i) **Competitive inhibition** occurs when substrate (S) and inhibitor (I) both bind to the same site on the enzyme and competes for access to this enzyme's active site. This usually results from the inhibitor having an affinity for the active site, the substrate and inhibitor. For competitive inhibition, the lines are parallel in the plot of s/v against i , whereas the intersection in the Dixon plot is given by $i = -K_i$, $1/v = 1/V$ (see Figure 2. 20).

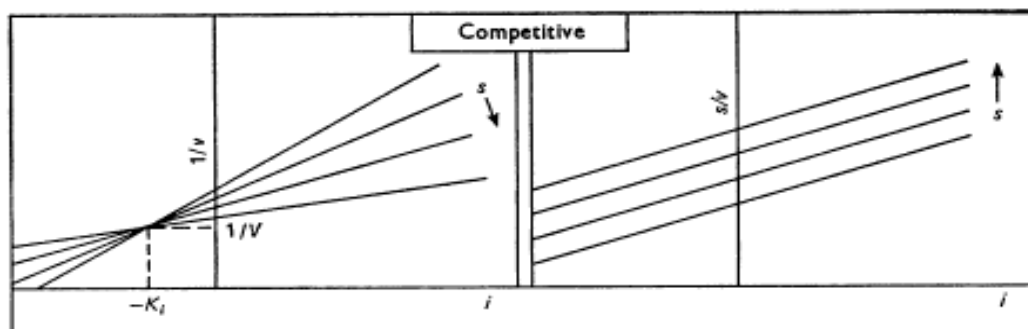


Figure 6. 5 Characteristic plots for inhibition-competitive

[Plots of $1/v$ against i (Dixon plots) and of s/v against i , where i is at two or more values of s , the substrate concentration, K_i is the dissociation constant of the EI complex, and K_i' is the dissociation constant of the EIS. The intersection point in the Dixon plot provides a measure of K_i , whereas the intersection point in the plot of s/v against i provides a measure of K_i']

ii) **Uncompetitive inhibition** appears when inhibitor (I) binds only to enzyme-substrate complex (ES) and not free enzyme (E). One can hypothesize that on binding substrate (S), a conformational change in enzyme (E) occurs which presents a binding site for inhibitor (I). For

APPENDIX

uncompetitive inhibition, the intersection in the plot of s/v against i is given by $i = -K_i$, $s/v = K_m/V$, whereas there is no intersection (parallel lines) in the Dixon plot.

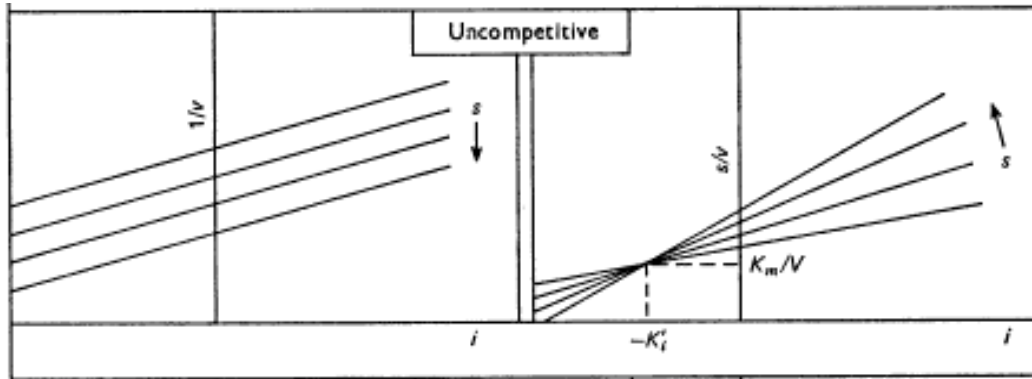


Figure 6. 6 Characteristic plots for inhibition-uncompetitive

[Plots of $1/v$ against i (Dixon plots) and of s/v against i , where i is at two or more values of s , the substrate concentration, K_i is the dissociation constant of the EI complex, and K_i' is the dissociation constant of the EIS. The intersection point in the Dixon plot provides a measure of K_i , whereas the intersection point in the plot of s/v against i provides a measure of K_i']

iii) **Noncompetitive inhibition** exists when inhibitor (I) binds to both enzyme (E) and enzyme-substrate complex (ES). It is quite rare as it would be difficult to imagine a large inhibitor which inhibits the turnover of bound substrate having no effect on binding of substrate (S) to enzyme (E). In the special case, where $K_i = K_i'$, the intersections occur on the i axis in both plots.

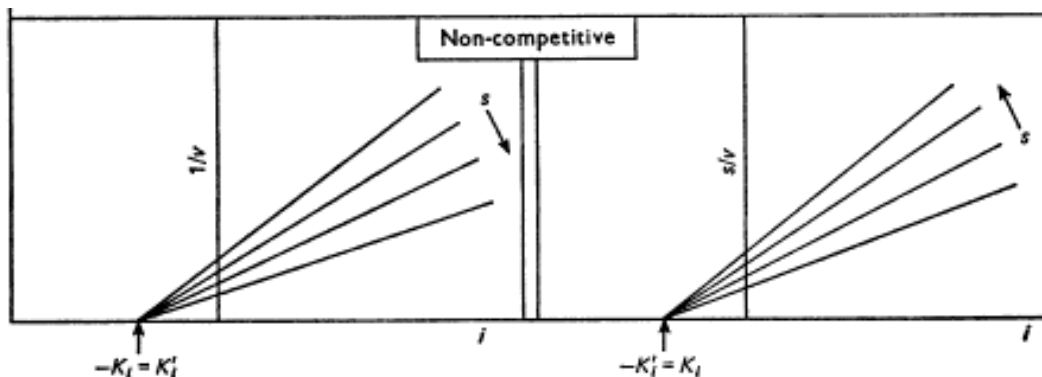


Figure 6. 7 Characteristic plots for inhibition-noncompetitive

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[Plots of $1/v$ against i (Dixon plots) and of s/v against i , where i is at two or more values of s , the substrate concentration, K_i is the dissociation constant of the EI complex, and K_i' is the dissociation constant of the EIS. The intersection point in the Dixon plot provides a measure of K_i , whereas the intersection point in the plot of s/v against i provides a measure of K_i']

The mixed inhibition is the key emphasis in present work. It is a type of enzyme inhibition in which the inhibitor may bind to the enzyme whether or not the enzyme has already bound the substrate but has a greater affinity for one state or the other (Heightman T. D. and A. T. Vasella, 1999). For comparison, the intersection of the lines of a Dixon plot for mixed inhibition occurs at a point where $i = -K_i$, $1/v = [1 - (K_i / K_i')] / V$ (see Figure 2. 23).

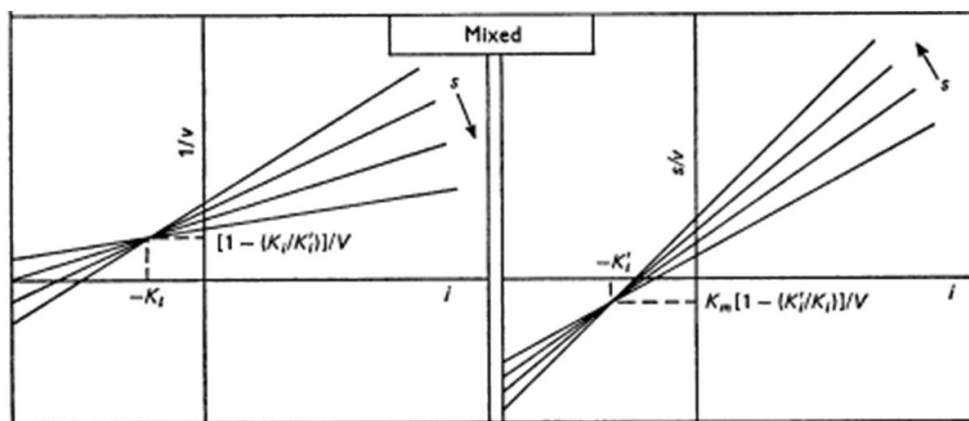


Figure 6. 8 Characteristic plots for inhibition-Mixed (Cornish-Bowden Athel, 1974)

[Plots of $1/v$ against i (Dixon plots) and of s/v against i , where i is at two or more values of s , the substrate concentration, K_i is the dissociation constant of the EI complex, and K_i' is the dissociation constant of the EIS. The intersection point in the Dixon plot provides a measure of K_i , whereas the intersection point in the plot of s/v against i provides a measure of K_i']

For the mixed inhibition, it is called "mixed" because it can be seen as a conceptual "mixture" of competitive inhibition, in which the inhibitor can only bind the enzyme if the substrate is not already bound; and uncompetitive inhibition, in which the inhibitor can only bind the enzyme if the substrate is already bound to the catalytic site of the enzyme. If the ability of the inhibitor to bind the enzyme is exactly the same whether or not the enzyme has already bound the substrate, it is known as a non-

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competitive inhibitor (Cortes A. *et al.*, 2001). The relationship between these three types of inhibition is shown in Figure 2. 24. The figure clearly illustrates that the mixed type inhibitor dissociation constant may differ between enzyme (E) and enzyme-substrate complex (ES), where the special case $K_I = K_{IS}$ is the noncompetitive inhibition.

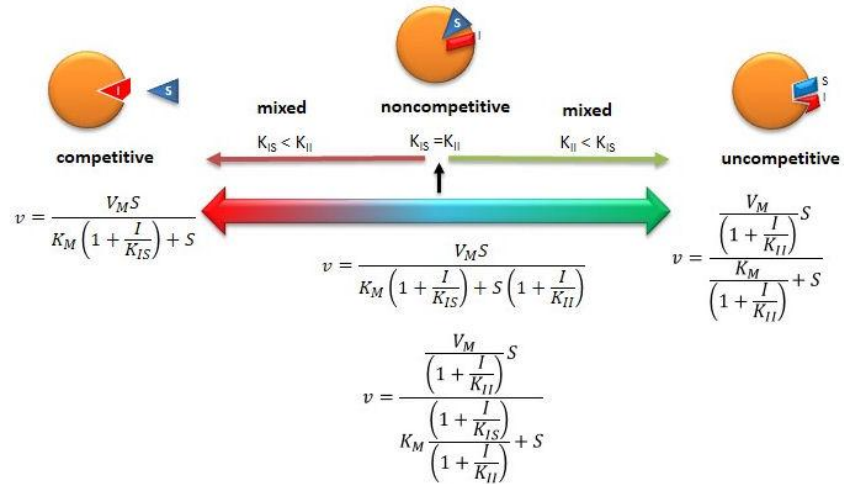


Figure 6. 9 Summary of Reversible Enzyme Inhibition

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RESUME

La levure *Saccharomyces cerevisiae* est capable d'utiliser une grande variété de sucres comme source de carbone et d'énergie. La plupart des enzymes impliquées dans l'utilisation de ces sucres sont codées par des gènes issus de familles multigéniques. Les isomaltases (EC 3.2.1.10) dont le nom systématique est oligosaccharide α -1,6- glucohydrolase, et plus généralement appelées oligo-1,6- glucosidases hydrolysent les liaisons α -1,6- glycosidiques à l'extrémité non réductrice des substrats avec rétention de la configuration anomérique. Elles appartiennent à la famille des « α – amylases », famille qui comprend près de 30 protéines avec spécificités différentes, dont par exemple, des glycoside hydrolases et des transférases qui agissent sur des substrats tels que l'amidon, le dextrane, le saccharose *etc* (MacGregor, 1988). Les isomaltases ont été classées dans la sous-famille 31 de la famille glycoside hydrolase 13 [GH13_31, (Stam M. *et al.*, 2006)] en fonction de la base de données CAZy qui classe les enzymes en fonction de leur structure et de leur mécanisme d'action (Cantarel BL, *et al.*, 2008). Les α -1,6- glucosidases sont divisées en deux types sur la base de leur préférence en fonction de la taille du substrat: (i) les glucane α -1,6-glycosidases préférant les isomaltooligosaccharides (IMO) plus longs que l'isomaltose (IG2) et actifs sur le dextrane et (ii) les oligo- α -1,6-glycosidases inactifs sur le dextrane et préférant les isomaltooligosaccharides courts avec une plus grande activité sur IG2 (Saburi W. *et al.*, 2006). Contrairement aux α -1,6- glucosidases comme la glucosidase dextran de *Streptococcus mutans* (SMDG) qui agit sur les longues chaînes, les isomaltases clivent préférentiellement les isomaltooligosaccharides courts (MacGregor, 1988).

Un premier gène codant pour une isomaltase a été isolé à partir d'une banque d'ADNc de *Saccharomyces cerevisiae* et s'est avéré être le gène *YGR287c* codant pour une protéine de 589 acides aminés capable d'hydrolyser l'isomaltose et l' α MG (α methyl glucopyranoside) mais totalement inactif sur le maltose (Yamamoto K. *et al.* , 2004). La structure cristalline de cette protéine a été résolue, soulignant l'importance de certains acides aminés dans la structure du site actif et permettant d'expliquer sa spécificité pour l'isomaltose (Yamamoto K. *et al.* , 2010 et 2011). En 2010, quatre isomaltases de la levure *Saccharomyces cerevisiae* ont été identifiées, codées par cinq gènes situés dans les régions subtélomériques de différents chromosomes composant la famille multigénique *IMA* (Teste MA *et al.* , 2010, Naumoff et Naumov, 2010) . Deux d'entre eux, à savoir *IMA3* et *IMA4* sont strictement identiques et codent la même protéine (Ima3p *id.* Ima4p). Ima2p et Ima3p se différencient seulement par 3 acides aminés, tandis que la protéine Ima5p est la plus éloignée et ne présente que 65% d'identité de séquence avec les autres membres de la famille. Toutes les protéines Imap possèdent quatre régions hautement conservées de la famille GH13 (séquences consensus I à IV) ainsi que la triade catalytique (Asp215, nucléophile; Glu277, donneur de protons et Asp352, stabilisateur de l'état de transition) et le résidu valine (Val216 chez Ima1p) identifié comme responsable de l'activité de α -1, 6 (Yamamoto K. *et al.* , 2004) .

Des travaux antérieurs sur des α -glucosidases purifiées de levure de boulangerie et de brasserie ont montré que les isomaltases hydrolysent l'isomaltose et l' α -méthylglucoside (α -MG), mais non le maltose, alors que la maltase hydrolyse le maltose mais non l'isomaltose et l' α -MG. Toutefois, des divergences fortes ont été observées dans les données publiées sur les spécificités de substrats et les paramètres cinétiques de l'isomaltase de la levure *Saccharomyces cerevisiae* et des espèces de levure étroitement liées. Ceci est probablement dû au fait que les propriétés

enzymologiques ont été déterminées à partir de différentes enzymes ou fractions purifiées qui contenaient un mélange de ces isoenzymes.

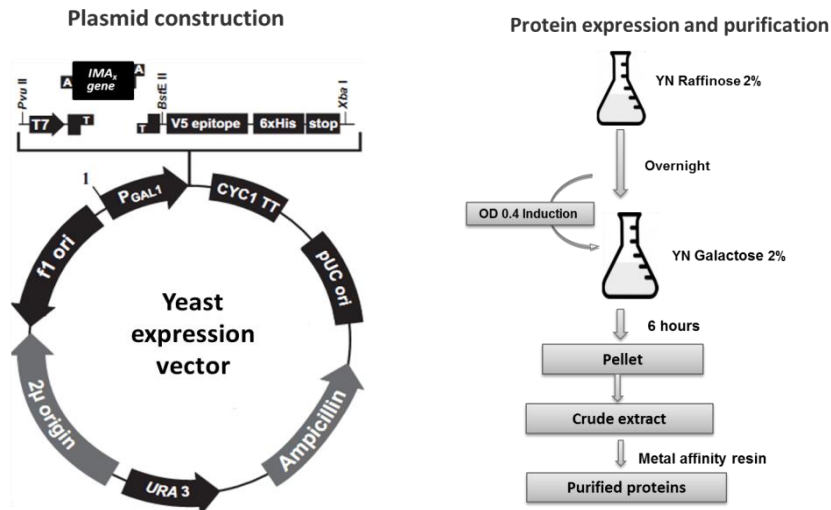
La purification des quatre protéines (Ima1p à Ima4p) de la levure *Saccharomyces cerevisiae* nous a permis de réaliser leur caractérisation biochimique et enzymologique détaillée. Des mutagenèses ciblées ont fourni des résultats clés pour comprendre le rôle des résidus d'acides aminés impliqués dans la spécificité de substrat et la thermostabilité de ces protéines. Sur la base de toutes ces recherches, nous nous sommes demandé quelle était la pertinence des différences observées entre les protéines Imap et la raison de la présence de quatre isomaltases chez *S. cerevisiae*. La présence de cinq gènes codant pour ces isomaltases provient-elle de l'évolution du génome ou a-t-elle une pertinence physiologique?

La partie bibliographique, est divisée en 4 parties qui traitent successivement de généralités sur la levure *Saccharomyces cerevisiae*, des sucres utilisés par les levures, des enzymes du métabolisme des glucides, et enfin des isomaltases de la levure *Saccharomyces cerevisiae*. La première partie est consacrée à la levure *Saccharomyces cerevisiae* comme organisme eucaryote modèle. Cette partie contient deux chapitres: des généralités et les aspects relatifs à l'évolution du génome de la levure *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* est un modèle pour expliquer les mécanismes moléculaires évolutifs, comme par exemple les duplications de gènes et des mutations (Lynch M. et al., 2001), ainsi que l'acquisition de nouveau matériel génétique (examiné par Dujon B. 2010). La deuxième partie concerne les sucres du métabolisme des hydrates de carbone chez les levures et on peut trouver une liste importante de mono-, di-, tri- et polysaccharides avec pour chacun leur voie d'utilisation dans les cellules. La troisième partie est sur

les enzymes impliquées dans le métabolisme des hydrates de carbone. Il y a quatre sections dans cette partie : la classification des hydrolases glycosides, la famille GH13, les α -glucosidases et les isomaltases. Enfin la dernière partie comprend la caractérisation de la famille multigénique *IMA*, la preuve biochimique de l'activité isomaltase, et l'étude structure-fonction en relation avec l'évolution du génome.

La plupart des systèmes enzymatiques pour l'absorption et l'assimilation des sucres s'appuient sur les familles multigéniques dans la levure *Saccharomyces cerevisiae*. La famille *IMA/MAL* a été utilisée comme un système modèle pour étudier les mécanismes moléculaires qui régissent l'évolution des gènes dupliqués. Dans ce travail, la diversité fonctionnelle de la *IMA* famille a été envisagée, en caractérisant de façon exhaustive *in vitro* leurs propriétés biochimiques et enzymologiques. Les résultats expérimentaux obtenus sont regroupés et discutés dans le deuxième chapitre.

Tout d'abord, pour la caractérisation de la famille isomaltase, nous avons exprimé et purifié les protéines recombinantes Imap de *Saccharomyces cerevisiae*. Les gènes *IMA_x* ont été amplifiés par PCR et clonés dans le vecteur d'expression de levure pYES2.1/V5-His-TOPO[®], vecteur portant le promoteur inductible *GALI* et une étiquette 6xHis-Tag. Lorsque les cellules ont été cultivées, l'expression du promoteur *GALI* a été induite avec le galactose pendant 6 heures. Les protéines Imap ont été purifiées grâce à l'étiquette His sur résine d'affinité au cobalt.



Une étape clé de la purification consiste à déterminer la pureté des protéines. Par conséquent, nous avons chargé ces protéines purifiées ainsi que l'extrait brut sur un gel SDS-PAGE. Ce gel a montré que la protéine recombinante a été bien exprimée dans le lysat cellulaire (Figure 1) et la protéine purifiée migre à un poids moléculaire apparent d'environ 70 kDa.

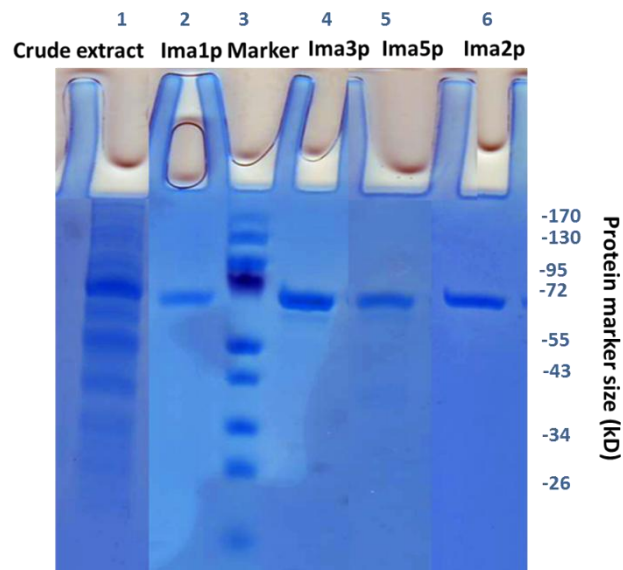


Figure 1 : SDS-PAGE de protéines de IMA recombinants chez *Saccharomyces cerevisiae*.

Ensuite, nous nous sommes concentrés sur les principaux paramètres biochimiques des isomaltases. Les pH optimaux et les températures optimales de fonctionnement sont déterminés pour les 4 isomaltases, ainsi que l'effet sur leur activité d'un certain nombre d'agents chimiques et d'ions métalliques. Ima1p et Ima2p montrent un pH optimal, une température optimale et une température

de fusion similaires. Ima3p a une température optimale identique à Ima1p mais une température de fusion beaucoup plus basse que Ima2p. Ima5p a la température optimale et de fusion les plus basses (Tableau 1).

Enzyme	Ima1p	Ima2p	Ima3p	Ima5p
Optimal pH	7	7.6	8	7
Optimal Temperature (°C)	46	43	46	36
Melting Temperature (°C)	46.6 ± 0.7	46.2 ± 0.8	38.8 ± 0.5	36.3 ± 0.4

Tableau 1: Principaux paramètres biochimiques de isomaltases

Par ailleurs, la demi-vie ($t_{1/2}$) a été déterminée par incubation de l'enzyme à la température spécifiée (37 °C, 40 °C ou 42 °C) (Tableau 2). Une température de 37 °C conduit déjà à une certaine perte de l'activité enzymatique. Alors que l'activité d'Ima5p a chuté de moitié par rapport à la vitesse initiale au bout de 3 minutes (demi-vie, $t_{1/2}$), la demi-vie d'Ima3p était de 23 min, alors que Ima2p et Ima1p étaient les plus stables avec $t_{1/2}$ d'environ 27 minutes. Lorsque la même expérience a été réalisée à 40 °C, la demi-vie est d'environ 1 min pour Ima5p, 8 min pour Ima3p et 18 min pour Ima1p et Ima2p, ce qui illustre la sensibilité dramatique des isomaltases à des températures croissantes. Lorsque la température a augmenté jusqu'à 42 °C, la demi-vie était de 11 min pour Ima2p, alors que Ima3p diminué à 3 min.

Enzyme	Half life (min)		
	37°C	40°C	42°C
Ima1p	26.1 ± 1.7	17.6 ± 1.7	nd
Ima2p	27.4 ± 1.6	18.5 ± 1.6	11.2 ± 0.7
Ima3p	23.2 ± 1.4	7.6 ± 0.9	2.9 ± 0.5
Ima5p	2.9 ± 0.4	0.8 ± 0.1	nd

Tableau 2: Demi-vie des protéines Imap à différentes températures

Dans la séquence primaire, nous avons reporté notre attention sur la proline à la position 240, car il a déjà été signalé que des résidus de proline influencent grandement la thermostabilité des protéines. Pour analyser le rôle des résidus proline dans la thermostabilisation, une seule mutation a été construite, et le variant de Ima3p L240P a été obtenu. Nous voulions savoir si ce variant de la proline gardait la thermostabilité d'Ima3p ou s'il récupérait le niveau d'Ima2p. Nous avons constaté que la substitution de la proline améliorait la stabilité thermique d'Ima3p (Tableau 3). Cependant, la demi-vie du variant L240P était légèrement inférieure à Ima2p. De plus, la température de fusion du variant L240P augmentait de 1°C par rapport à Ima3p mais était encore beaucoup plus faible que Ima2p. L'ensemble de ces résultats de demi-vie et T_m démontrent que le résidu proline à la position 240 améliore la stabilité thermique, mais n'est pas le déterminant unique pour la thermostabilité des protéines Imap.

Enzyme	Half life (min)	T_m (°C)
	42°C	pH 7
Ima2p	11.2 ± 0.7	46.2 ± 0.8
Ima3p	2.9 ± 0.5	38.8 ± 0.5
Ima3p_L240P	8.5 ± 1.3	39.7 ± 0.4

Tableau 3: comparaison de la demi vie et de la température de fusion

En conclusion de cette partie, nous avons constaté que:

1. Ima1p et Ima2p avaient des propriétés biochimiques presque identiques.
2. Ima3p était différente d'Ima2p avec seulement trois acides aminés, mais présentait une différence de thermostabilité. Le rôle central du résidu proline dans la stabilité thermique entre Ima2p et Ima3p a été confirmé.
3. Ima5p était la protéine la plus sensible à la température.

La deuxième partie porte sur la caractérisation enzymologique des isomaltases de *Saccharomyces cerevisiae*. La spécificité de substrat pour les 4 isomaltases et pour 3 variants ayant subi une modification ponctuelle par mutagenèse dirigée est tout d'abord présentée, puis les paramètres cinétiques des 4 isomaltases natives déterminés. Cette partie est enrichie d'une étude structure/fonction très intéressante dans le cas des enzymes modifiées, qui aboutit à définir des positions clés dans cette famille d'enzymes pour l'obtention d'activités d'hydrolyse de liaisons glycosidiques particulières (α -1,6 ou α -1,4). Les spécificités de substrat des isomaltases ont été déterminées en utilisant un ensemble de tri-di-saccharides ayant différentes liaisons α -glycosidiques (Tableau 4). Aucune activité n'a été observée sur β -pNPG, alors que l' α -pNPG a été parfaitement clivé. Ceci démontre que les protéines Imap ont une activité α -glucosidase. L'étude de leur activité spécifique a mis en évidence que les protéines Imap préféraient les disaccharides portant des liaisons α -1, 6. De plus, toutes les protéines Imap étaient entièrement inactives sur le maltose. Cependant, une très faible activité spécifique sur maltulose pour Ima2/5p a été observée. Enfin, les protéines Imap hydrolysaient des substrats ayant des liaisons α -1, 2 / α -1, 3/ α -1, 5 avec une faible activité spécifique, à l'exception de Ima3p sur le kojibiose et le leucrose, qui était totalement inactif. En outre, à l'exception d'Ima3p, les trois autres protéines montrent une activité mesurable sur certains trisaccharides, y compris l'isomaltotriose et le panose, mais aussi le maltotriose.

Substrate	Linkage	Specific activities($\mu\text{mol}/\text{min}/\text{mg}$)			
		Ima1p	Ima2p	Ima3p	Ima5p
Artificial compounds					
α-pNPG	4-Nitrophenyl α -D-glucopyranoside	5.8 (10)	7.7 (14)	3.9 (54)	10 (15)
β-pNPG	4-Nitrophenyl β -D-glucopyranoside	0	0	0	0
α MG	α -Methylglucopyranoside	52 (87)	55 (102)	11 (153)	0 (0)
Disaccharides (Glc \rightarrow Glc)					
Trehalose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 1)- α -D-glucose	0	0	0	0
Kojibiose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 2)- α -D-glucose	7.7 (13)	6.9 (13)	0	2.5 (4)
Nigerose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)- α -D-glucose	6.5 (11)	6.8 (13)	2.5 (35)	4.2 (6)
Maltose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)- α -D-glucose	0	0	0	0
Isomaltose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucose	60 (100)	54 (100)	7.2 (100)	65 (100)
Disaccharides (Glc \rightarrow Fru)					
Sucrose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 2)- β -D-fructose	18.6 (31)	19.7 (36)	10.2 (142)	1.2 (2)
Turanose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)-D-fructose	4.3 (7)	3.5 (6)	1.8 (25)	1.2 (2)
Maltulose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)-D-fructose	0	0.53 (1)	0	0.36 (1)
Leucrose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 5)-D-fructose	8.6 (14)	8.6 (16)	0	1.1 (2)
Palatinose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)-D-fructose	52 (87)	74 (137)	5 (69)	39 (60)
Trisaccharides					
Maltotriose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 4)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose	3.5 (6)	3.4 (6)	0	3.4 (5)
Isomaltotriose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 6)-D-glucose	7.9 (13)	7.6 (14)	0	8.6 (13)
Melezitose (Glc \rightarrow Suc)	<i>O</i> - α -D-glucosyl-(1 \rightarrow 3)- β -D-fructosyl-(2 \rightarrow 1)-D-glucose	0	0	0	0
Panose	<i>O</i> - α -D-glucosyl-(1 \rightarrow 6)- α -D-glucosyl-(1 \rightarrow 4)-D-glucose	3.1 (5)	2.9 (5)	0	11 (17)

Tableau 4 : Overview of substrates specificities

Les paramètres cinétiques des 4 isomaltases ont été déterminés pour 7 substrats, ce qui représente un travail expérimental très conséquent. Les constantes K_m et k_{cat} déduites des courbes cinétiques, ainsi que le rapport k_{cat} / K_m (Eisenthal R. *et al.*, 2007) efficacité catalytique sont résumées dans le tableau 5. En règle générale, la valeur de K_m pour l' α -pNPG (0,58 mM) a été beaucoup plus faible que celle des substrats naturels (10 à 200 mM), et l'efficacité catalytique (k_{cat} / K_m) des protéines était beaucoup plus élevée pour l' α -pNPG que pour les substrats naturels y compris l'isomaltose (de 3 fois pour Ima1/2/5p à 50 fois pour Ima3p).

Substrate	Ima1p				Ima2p			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)	K_i (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)	K_i (mM)
α -pNPG	0.58 \pm 0.16	8.7 \pm 1.0	15 \pm 6	25 \pm 15	0.89 \pm 0.13	14 \pm 1	16 \pm 3	12 \pm 3
α -MG	27 \pm 5	84 \pm 8	3.1 \pm 0.9	574 \pm 242	28 \pm 6	88 \pm 9	3.1 \pm 1	600 \pm 272
Isomaltose	17 \pm 4	103 \pm 12	5.7 \pm 0.9	246 \pm 76	17 \pm 2	97 \pm 5	5.6 \pm 0.9	248 \pm 39
Palatinose	18 \pm 4	78 \pm 8	4.3 \pm 1.4	686 \pm 351	11 \pm 2	104 \pm 6	9.4 \pm 1.8	690 \pm 231
Isomaltotriose	128 \pm 35	21 \pm 4	0.16 \pm 0.07	None	72 \pm 16	15 \pm 2	0.21 \pm 0.07	None
Panose	82 \pm 17	6.3 \pm 0.5	0.078 \pm 0.023	None	129 \pm 23	7.7 \pm 0.7	0.051 \pm 0.004	None
Sucrose	144 \pm 26	51 \pm 4	0.35 \pm 0.09	None	147 \pm 24	55 \pm 4	0.38 \pm 0.09	None
	Ima3p				Ima5p			
α -pNPG	0.35 \pm 0.09	7.1 \pm 0.7	20 \pm 7	9.8 \pm 3.2	0.48 \pm 0.11	14 \pm 1	28 \pm 9	33 \pm 21
α -MG	15 \pm 2	14 \pm 1	0.9 \pm 0.1	None	0	0	0	None
Isomaltose	37 \pm 9	15 \pm 2	0.41 \pm 0.15	188 \pm 54	13 \pm 2	101 \pm 8	8.0 \pm 2.2	386 \pm 114
Palatinose	7.0 \pm 0.7	6.0 \pm 0.1	0.8 \pm 0.1	None	12 \pm 3	56 \pm 5	5.0 \pm 1.6	589 \pm 266
Isomaltotriose	0	0	0	None	83 \pm 25	18 \pm 3	0.22 \pm 0.10	None
Panose	0	0	0	None	82 \pm 12	23 \pm 1	0.28 \pm 0.06	None
Sucrose	116 \pm 12	25 \pm 1	0.22 \pm 0.03	None	191 \pm 24	3.8 \pm 0.2	0.020 \pm 0.004	None

Tableau 5 : Les paramètres cinétiques des protéines Imap pour les substrats sélectionnés

En outre, sur la base de la séquence primaire, nous avons étudié l'effet de mutations ponctuelles. Pour le consensus III, Ima3p a un résidu arginine au lieu du résidu glutamine chez Ima2p; Ima5p diverge des autres avec les résidus VGI. Grâce aux variants Ima3p_R279Q et Ima5p_MGH, nous nous sommes demandés si le niveau d'activité d'Ima1/2p pouvait être restauré. Le résultat a montré que Ima3p_R279Q récupérait des spécificités de substrat et des activités spécifiques proches d'Ima2p. L'activité catalytique du variant Ima5p_MQH était complètement abolie (Tableau 6).

Substrate		Linkage	Specific activities($\mu\text{mol}/\text{min}/\text{mg}$)				
			Ima2p	Ima3p_R279Q	Ima3p	Ima5p	Ima5p_MQH
Artificial compounds							
α-p NPG	4-Nitrophenyl α -D-glucopyranoside		7,7	8,5	3,9	10	0
α MG	α -Methylglucopyranoside		55	50	11	0	0
Disaccharides (Glc \rightarrow Glc)							
Isomaltose	(1 \rightarrow 6)		54	29	7,2	65	0
Disaccharides (Glc \rightarrow Fru)							
Sucrose	(1 \rightarrow 2)		19,7	22	10,2	1,2	0
Leucrose	(1 \rightarrow 5)		8,6	1,5	0	1,1	0
Palatinose	(1 \rightarrow 6)		74	33	5	39	0
Trisaccharides							
Panose	(1 \rightarrow 6) and (1 \rightarrow 4)		2,9	3,1	0	11	0

Tableau 6: Résultats de la mutagénèse

Cette partie a été complétée par une analyse très rigoureuse de l'inhibition des isomaltases Imap1 et Imap5 par le maltose, qui permet de résoudre des contradictions de la littérature sur ce sujet précis. Les paramètres cinétiques ont été calculés pour Ima1p dans les gammes basse et haute concentration d'isomaltose pour trouver leur modèle d'inhibition. Nous avons obtenu cependant des constantes d'inhibition assez élevées pour Ima1p et Ima5p et bien que nous ayons confirmé que les isomaltases isomaltose étaient inhibées par le maltose, cela n'avait pas vraiment de sens physiologique.

Vient ensuite une étude préliminaire des réactions de transglycosylation qui pourraient être réalisées par l'isomaltase Imap1 en présence des substrats isomaltose, palatinose ou sucrose, et ce à différents pH, températures et au bout de différents temps de réaction. Ces travaux préliminaires conduisent à la conclusion que des produits de transglycosylation sont bien formés, mais il est dommage que des hypothèses n'aient pas été avancées quant à la nature de ces produits.

Par conséquent, nous pouvons tirer comme conclusions pour cette partie:

1. Les protéines Imap préfèrent les substrats liés en α -1, 6, mais hydrolysent également les substrats α -1, 2, α -1, 3 et α -1, 5. Elles possèdent une large gamme de substrats.
2. L'utilisation de l'isomaltose par les isomaltases a été inhibée par le maltose, mais sans réel sens physiologique.
3. Ima1p et Ima2p étaient très similaires, et Ima5p était comparable avec eux. Cependant, Ima3p a montré des activités spécifiques très faibles. L'évolution du génome a conduit à cinq gènes codant pour les isomaltases, mais cela a-t-il une pertinence physiologique.

La conclusion générale de ce travail est la suivante. La présence de gènes dupliqués dans une même espèce a un rôle crucial dans les processus d'évolution, en particulier dans le développement rapide de nouvelles fonctions (Conant GC et KH Wolfe , 2008; Innan H. et F. Kondrashov , 2010). La famille multigénique ' Mal ' de la levure *Saccharomyces cerevisiae* englobe les gènes *IMA* et les gènes *MAL* qui sont subtélomériques. Cette famille a été très récemment prise comme modèle pour fournir des preuves expérimentales sur les mécanismes qui permettent à ces familles d'évoluer à partir d'un ancêtre commun (Brown CA. *et al.*, 2010; Voordeckers K.*et al.* , 2012). Il a été proposé que des duplications de gènes permettent d'engendrer des gènes engendrés dans lesquels les mutations sont optimisées soit vers une activité isomaltase soit vers une activité maltase, permettant ainsi de contourner les contraintes d'adaptation sur un gène multifonctionnel en optimisant les sous-fonctions séparément dans différents paralogues. Toutefois, les données quantitatives rapportées dans le présent travail ont montré que, outre les α -1, 6, des activités annexes sur des substrats tels que le saccharose ou des disaccharides α -1 ,3 persistent et s'étendent à toutes les protéines de cette famille.

Cette 'ambiguïté' de substrat améliore probablement la plasticité phénotypique nécessaire pour l'utilisation des sucres dans des micro-organismes tels que la levure *Saccharomyces cerevisiae*.

L'analyse détaillée des caractéristiques de cette famille de protéines illustre des singularités évolutives, mettant en évidence la façon dont les mutations sur très peu de résidus clés peuvent conduire à des effets importants. A titre d'exemple, les très récents événements de duplication au sein de la famille en effet conduisent à deux gènes identiques, *IMA3* et *IMA4*, qui codent pour une protéine avec seulement trois acides aminés différents par rapport à Ima2p mais présente des altérations significatives dans les paramètres catalytiques et la thermostabilité. De plus, la protéine la plus divergente de la famille, Ima5p, peut maintenir la plupart des caractéristiques catalytiques d'Ima1p ou d'Ima2p, à l'exception notable pour sa stabilité thermique qui est très altérée. Ce résultat renforce l'idée de Voordeckers *et al.* que les différentes voies d'évolution menant à de variables combinaisons d'acides aminés pourraient éventuellement conduire à des changements similaires dans spécificités de substrat et met en évidence en quelque sorte la robustesse des systèmes biologiques à travers l'évolution.

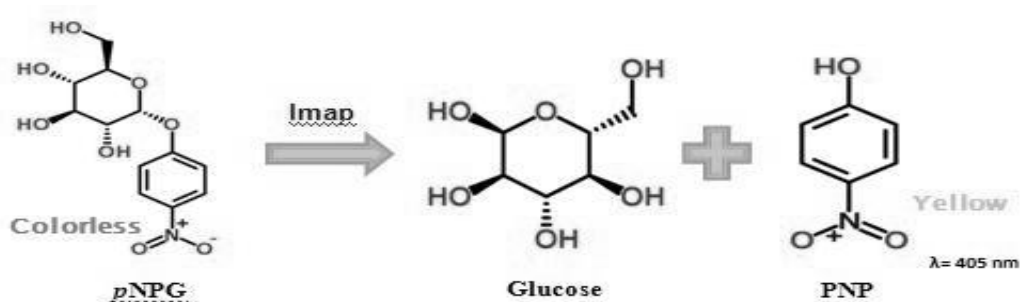
Comme il a été mentionné à propos de la structure cristalline, seule la protéine Ima1p a été seulement cristallisée. De plus amples recherches sur les membres de la famille des protéines Imap restants pourrait être poursuivies comme par exemple l'exploration plus approfondie de leur structure moléculaire 3D à comparer avec la séquence annotée, qui permettrait l'identification facile et l'analyse des éléments de structure secondaire afin de fournir des indications sur les principaux éléments structurels. Il pourrait être utile d'effectuer la cristallisation et la détermination de la structure d'Ima5p. Basé sur une comparaison des structures Ima1/5p aux X-rayons, la

thermostabilisation des protéines Imap pourrait être une autre étude intéressante. En effet, bien que le nombre total de résidus proline dans Ima1p et Ima5p soit à peu près la même (30 contre 29, respectivement), il est possible que certaines de leurs positions dans Ima5p puissent expliquer sa grande sensibilité à la température. La structure d'Ima5p et l'analyse comparative avec Ima1p pourrait fournir de nouvelles informations sur les déterminants structurels en lien avec les mécanismes impliqués lors de la thermostabilisation.

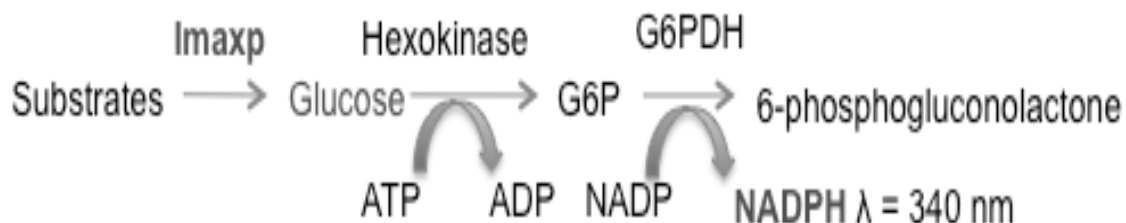
Il a été observé à partir des études cinétiques, que les substrats avec des liaisons α -1,6 glycosidiques peuvent être hydrolysés par les isomaltases ainsi qu'une capacité à synthétiser de nouveaux produits par transglycosylation. D'autres recherches pourraient être effectuées avec l'optimisation de la transglycosylation, l'identification des produits et des idées sur le mécanisme.

L'ensemble des Matériels et Méthodes utilisés dans le cadre du travail de thèse sont présentés dans le dernier chapitre. La description est précise et fournit tous les éléments pour une reproduction éventuelle des expériences réalisées. Deux méthodes ont été impliquées dans les essais enzymatiques. La première méthode est la réaction colorimétrique avec un substrat artificiel-pNPG. L'activité totale de l' α -glucosidase peut être déterminée par la quantité de colorant libéré et le temps d'incubation.

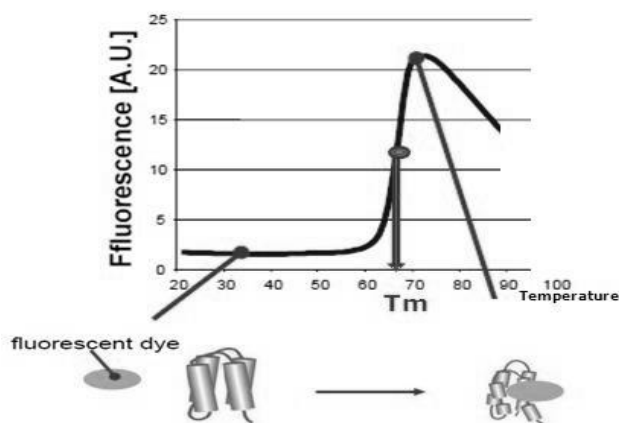
L'absorbance du PNP est mesurée à la longueur d'onde 405 nm.



L'autre méthode concerne les mesures avec des substrats 'naturels'. Ce dosage spectrophotométrique couplé a été réalisé entre la libération du glucose et la production de NADPH à 340 nm. Les deux méthodes ont donc été appliquées pour la détermination des propriétés biochimiques et enzymologiques.



La température de fusion (T_m) des isomaltases a été mesurée par fluorimétrie à balayage différentiel (DSF). La dénaturation thermique des protéines se déroule en présence d'un colorant fluorescent (Niesen FH *et al.*, 2007). La DSF est généralement réalisée avec un instrument PCR en temps réel. La DSF est une méthode de dépistage rapide et peu coûteuse et identifie les ligands de faible poids moléculaire qui se lient et stabilisent les protéines purifiées. Lors de la déstructuration de la protéine, des résidus hydrophobes deviennent exposés et une forte fluorescence est émise par les molécules de colorant. Dans le processus, lorsque les concentrations de protéine « foldée » et déstructurée sont égales, la température est considérée comme la température de fusion.



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