Supplementary Material

Hooked on α-D-galactosidases: from biomedicine to enzymatic synthesis

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1. α-Galactosides in nature: comments and structural details (see also Figure 1)

Obtaining synthetic carbohydrate derivatives for biochemical studies has been difficult in comparison to the easy access to large quantities of peptides and nucleic acids available for decades. This hurdle has been considered to be the main reason for the slow pace of progress in glycobiology. In the 70's, the interest of synthetic chemists for the production of α-galactosides was already endorsed; different strategies for the synthesis of the disaccharide α-Gal*p*-(1,3)-Gal*p*, were studied (Kronzer & Schuerch, 1974). In the same period, Lemieux and Driguez could judge, as appropriate, the chemical challenge for the synthesis of the fucosyl derivative (1, Figure 1) of the same disaccharide that is part of blood-group B antigenic determinant (Lemieux & Driguez, 1975). The regioisomeric galabiose sequence, α-Gal*p*-(1,4)-Gal*p* (2, Figure 1) is also known as a receptor of pathogens and enterotoxins but its endogenous functions remain to be assessed. In a very interesting example, Suzuki et al. investigated the species-specific expression of α-Gal*p*-(1,4)-Gal*p* on glycoproteins derived from 181 kinds of bird eggs. Galabiose-containing glycoproteins were detected in 60-70% of the birds, distributed in closely positioned branches of the phylogenetic tree. No galabiose structure has so far been found in N- nor O-glycans derived from the other classes of vertebrates except for some O-glycans in eggs of amphibians (Suzuki et al., 2003). A study in turtle egg yolk has also been recently conducted (Yagi et al., 2010).

Interest for production of α-galactosides via conventional techniques or using innovative bio-based pathways, is still supported nowadays (Sau et al., 2012). The efficient conversion of lactose to isoglobotriose (Galα-1,3-Galβ-1,4-Glc) using high cell density cultures of a genetically engineered *Escherichia coli* strain expressing the bovine gene for α-1,3-galactosyltransferase, has been described with improvements for pentasaccharide formation (Gebus et al., 2012). Globotetraose (4, Figure 1) could also be produced from exogenous globotriose. However, isoglobotriose was also enzymatically synthesized using fungi *Talaromyces flavus* α-D-galactosidase (Weignerova et al., 1999) (see also specific paragraph).

α-Galactosides of sucrose (3, Figure 1) were isolated from different natural sources and soon the stimulation of intestinal gas formation originating from legume seeds was ascribed to these compounds. As a consequence of this knowledge, research concerning α-galactosides in the food domain has been prosperous with topics such as extraction (Saini, 1988) and new analytical methods (Frias et al., 1996) still of interest today. More recently, raffinose oligosaccharides (α-(1-6) sucrose linked galactosyl-structures) have been studied as ingredients in functional foods in order to manipulate the composition of colonic microflora, contributing to human health (prebiotic oligosaccharides) (Martinez-Villaluenga et al., 2008). By virtue of these studies, different cultivars of lupin with high amounts of these oligosaccharides have been selected (Martinez-Villaluenga et al., 2005, 2006).

α-Galactose has also been found in small natural molecules such as the interesting precursor of oxalic acid, 5-O-(α-D-galactopyranosyl)-D-glycero-2-enono-1,4-lactone, (5, Figure 1); α-galactosylation of this molecule is a potential mechanism for storage, targeting and/or stabilization for transport in the phytopathogenic fungus *Sclerotinia sclerotiorum* (Keates et al., 1998).

α-Galactose is also present in polygalatenosides (compounds A and B in 6, Figure 1) isolated from *Polygala tenuifolia* Willdenow, an important herb prescribed in traditional Chinese medicine with many therapeutic (sedative, antipsychotic, cognitive improving, neuron protective, anti-inflammatory) effects on the central nervous system (Huang et al., 2008).

Furthermore, α-galactose is present in polymers of mannan forming the class of galactomannans (7, Figure 1), natural gums originating from endosperms of seeds or from microbial sources of primary

importance for food and pharmaceutical applications. Galactose is located as side groups in these structures and the ratio with mannose are highly determining rheological features. Scientific interest for galacto- gluco- and other mannans is also due to their biological activities (Tester & Al-Ghazzewi, 2013). Synthesis of similar compounds (α-D-Galp-(1-2)-α-D-Manp-(1-2)-α-D-Manp-(1-6) -α-D-Manp-O-CH_3, 8, Figure 1) has also been described in literature (Li et al., 2000).

α-Galactosylceramide (9, Figure 1) is a ligand for the activation of CD1d-mediated invariant natural killer T cells. It was developed through research about structural modification of agelasphin-9b, the main component of agelasphins, a series of glycosphingolipids isolated from the marine sponge (*Agelas mauritianus*) showing potent effects on the immune system of mice (Shiozaki et al., 2010). Promising potentials in the treatment of several diseases (cancer, malaria, and hepatitis B) has been shown by the use of α-Gal-Cer. From structure-activity relationship studies of these molecules, the importance of galactosyl moiety, of its α-linkage and of the 2-hydroxyl group in 9, Figure 1, were revealed.

2. Sources of α-D-galactosidases

The enzyme is found in legume seeds and other vegetable sources (Balasubramaniam & Mathew, 1986; Bayraktar & Onal, 2013; Bryant & Rao, 2000; Chinen et al., 1981; Feurtado et al., 2001; Kang & Lee, 2001; Kim et al., 2002, 2003; Malhotra & Dey, 1967; Marraccini et al., 2005; Zhifang & Schaffer, 1999). It is present in sweet almond (Malhotra & Dey, 1967), melon fruit (Zhifang & Schaffer, 1999) endosperm of coconut (Balasubramaniam & Mathew, 1986) and sunflower seeds (Kim et al., 2003). Two alkaline and one acid α-D-galactosidases were identified in melon fruit, including a novel alkaline form with activity toward a broader spectrum of galactosyl saccharides, particularly raffinose. This was the first alkaline α-D-galactosidase with higher affinity and activity toward raffinose than to stachyose, yet with a broad spectrum of substrates, allowing it to hydrolyze stachyose as well. An interesting three-phase partitioning system was used to concentrate and purify α-D-galactosidase from watermelon (*Citrullus vulgaris*) with a yield of 76.7% (2.7-fold purification) obtained in the interphase of the TPP system constituted by the crude extract and t-butanol in 1:1 (v/v) ratio in the presence of 50% (w/v) ammonium sulfate at pH 5.5. Gel electrophoresis analysis revealed a substantial level of purification of α-D-galactosidase from watermelon (Bayraktar & Onal, 2013). α-D-Galactosidase has been isolated from the mature leaves of pumpkin (*Cucurbita pepo*) (Brian & Webb, 1977) and from immature stalks (Chinen et al., 1981) of sugar cane (*Saccharum officinarum*). Recently, it has been isolated (Feurtado et al., 2001) from tomato (*Lycopersicon esculentum*), grape flesh (Kang & Lee, 2001) and cultured rice (Kim et al., 2002). This enzyme is also present (Bryant & Rao, 2000) in peanuts (*Arachis hypogaea L.*) and germinating seeds of coffee beans (Marraccini et al., 2005). Few fungi are included among sources (Galas & Miszkiewicz, 1996; Hashimoto et al., 1993; Ohtakara et al., 1984; Rezende et al., 2005; Wong et al., 1986). As the use of fungal α-D-galactosidase increases, it becomes important to isolate new fungal strains producing enzymes with properties more amenable to industrial applications (Krieger et al., 2004). Thermostable enzymes are of greater interest because they permit processing at elevated temperature (above 60 °C) at which rapid product formation can be achieved without the growth of mesophilic organisms. Most of the thermostable α-D-galactosidases have been purified and characterized from thermophilic bacteria and fungi (Puchart et al., 2000). Recently, Shivam and Mishra (Shivam & Mishra, 2010) reported the purification and characterization of a thermostable α-D-galactosidase secreted by *Aspergillus parasiticus* MTCC-2796. The thermophilic enzyme reported for the first time from a mesophilic fungus, catalyzed the transglycosylation reaction for the synthesis of melibiose with glucose as the acceptor. A thermostable α-D-galactosidase from *Streptomyces griseoloalbus* has recently been utilized in legume foods processing (Anisha et al., 2008). A new GH-36 α-D-galactosidase gene has been cloned from *Streptomyces* sp. S27; after expression in *E. coli*, the purified recombinant Aga-S27 exhibited optimal activity at conditions similar to the intestine of mammals and poultry (35 °C and pH 7.4). The enzyme was resistant to some neutral proteases (α-chymotrypsin, subtilisin A and collagenase) and showed hydrolytic ability to natural substrates, including melibiose, stachyose, raffinose and soybean meal. When combined with intestinal proteases, the enzyme showed higher hydrolytic ability to raffinose family oligosaccharides (RFOs) in soybean product. These favorable properties make the *Streptomyces* sp. S27 α-D-galactosidase very attractive during soybean processing for food and feed industries (Cao et al., 2010).

Many bacteria have also been reported to contain α-D-galactosidase activity. Recently, its presence has been reported in extreme thermophilic eubacterium *Rhodothermus marinus* (Blücher et al., 2000; Gomes et al., 2000), in marine bacterium *Pseudoalteromonas* sp (Bakunina et al., 1998) and lactic acid bacterium *Carnobacterium piscicola* (Coombs & Brenchley, 2000). Thermostable, neutral, extracellular α-D-galactosidase with stability in a wide range of pH, is produced by a newly isolated strain of *Bacillus stearothermophilus* grown on in expensive agricultural residues at 60°C, under submerged fermentation conditions (Gote et al., 2004). In animals, α-D-galactosidase was first reported from snails (Barnett, 1971) and then from human spleen, placenta, plasma and liver (Bishop & Desnick, 1981; Dean & Sweeley, 1979).

3. Classification and structures of α-D-galactosidases

Proteins from eukaryotes are placed in GH-27 family whereas those from most of known microbial sources are grouped into a GH-36 family. Nevertheless, it is assumed that members of GH-27 and GH-36 families have a common ancestor and form clan-D of glycoside hydrolases (clan GH-D) (Rigden, 2002). Based on bioinformatics analysis of the amino acid sequences, reported by Naumoff in 2004 (Naumoff, 2004), monophyletic origin of the GH-27 family was shown and three major subfamilies were highlighted (27A-27C). Eukaryotic proteins form five clusters on the phylogenetic tree of GH-27 family. Similar results were also obtained by R. Fernandez-Leiro et al. (2010) (Fernandez-Leiro et al., 2010). Family GH-27 members can be clustered within five groups. The results of the phylogenetic analysis are supported by experimental data on 3D-structure of representatives of every cluster. According to Naumoff (Naumoff, 2004), the GH-36 family contains four clearly separate subfamilies (36A-36D). Bioinformatics analysis of Fredslund et al. (2011) (Fredslund et al., 2011), revealed four distinct major subfamilies I, II, III and IV in GH-36, (Naumoff, 2005). Recently, α-D-galactosidases of bacteria are included in the GH-97 family (Gloster et al., 2008). The α-D-galactosidase from *Bacteroides thetaiotaomicron Bt*GH97b (PDB 3a24) is included in the GH-97 subfamily of retaining enzymes (Okuyama et al., 2009). Iterative database searches showed that GH-97 proteins have an evolutionary relationship with the GH-27 and GH-36 families as well as a related relationship with some other families of glycoside hydrolases of clan GH-D (Naumoff, 2005; Okuyama et al., 2009; Rigden, 2002). GH-4 family covers bacterial biochemically characterized α-D-galactosidase of *Escherichia coli* K12 MG65 (Liljestroml & Liljestrom, 1987; Nagao et al., 1988), as well as putative α-D-galactosidases of *Bacillus subtilis* (strain 168), *Bacillus haloduranus* (Takami et al., 2000), *Enterococcus faecium* (van Schaik et al., 2010), *Citrobacter freundii* (Shimamoto et al., 2001) whose genes are found in the complete genomes of these bacteria. GH-4 enzymes are unique in their requirement for NAD(H) and a divalent metal (Mn^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+} or Ca^{2+}) for activity and are distant homologues of dehydrogenases (EC 1.1.1.) (Anggraeni et al., 2008; Chakladar et al., 2011; Rajan et al., 2004; Varrot et al., 2005; Yip et al., 2004). Unique α-D-galactosidase, from the archaeon *Pyrococcus furiosus,* is placed in the GH-57 family (Brouns et al., 2006; Van Lieshout et al., 2003). Inverting α-D-galactosidases of *Bacteroides fragilis* are the first well characterized representatives of the GH-110 family (Liu et al., 2007, 2008).

4. Structural information about GH-27 members (Banner et al., 1975; Clark & Garman, 2009; Fernandez-Leiro et al., 2010; Fujimoto et al., 2003, 2009; Garman et al., 2002; Garman & Garboczi, 2004; Golubev et al., 2004; Henrissat et al., 1995; Koshland, 1953).

The crystal structure of free forms and their complexes with ligands (galactose and N-acetylgalactosamine) have been solved by the X-ray analysis for eight GH-27 members, including three б-N-acetylgalactosaminidases (EC 3.2.1.49) from the liver of *Gallus gallus* (PDB 1ktb) (Garman et al., 2002), and *Homo sapiens* (PDB 3hg4) (Garman et al., 2002) and from the bacterium *Bacillus haloduranus* (PDB 3cc1) (unpublished results), which catalyze the hydrolysis of terminal α-N-acetylgalactosamine from glycosylated substrate, and five □-galactosidases from: rice *Oryza sativa* (PDB 1uas) (Fujimoto et al., 2003), human *H. sapiens* (PDB 1r46) (Garman & Garboczi, 2004), fungi *Hypocrea jecorina* (*Trichoderma reesei*) (PDB 1szn) (Golubev et al., 2004), and *Umbelopsis vinacea* (PDB 3a5v) (Fujimoto et al., 2009), as well as the yeast *Saccharomyces cerevisiae* (PDB 3lrk) (Fernandez-Leiro et al., 2010). The tertiary structure of enzymes belonging to GH-27 family have the highest homology and consist of two domains: an N-terminal domain comprised of a $(\beta/\alpha)_8$-barrel as a catalytic domain and a C-terminal domain made up of eight antiparallel β-strands on two sheets in a β-sandwich containing a Greek key motif. $(\beta/\alpha)_8$-Barrel was observed earlier in a triose phosphate isomerase (Banner et al., 1975) and now represents a common motif in many glycoside hydrolases of Clan-D, functioning as catalytic domain. The C-terminal domain whose

function has not been fully understood until now is less conserved within family GH-27. The active sites of the known structures in the GH-27 family are extremely well conserved. The active site pocket was found on the C-terminal side of the $(\beta/\alpha)_8$-barrel, where the site of a D-galactose molecule binding is located. The tertiary structure of the catalytic site has provided support for the catalytic mechanism and revealed the mode of substrate binding in □-N-acetylgalactosaminidases and α-galactosidases. From 15 to 12 side-chain residues form active sites of the GH-27 enzymes. Of the 15 side-chains forming the active site of the enzymes, 12 are identical in all structures, and the dissimilar residues create substrate specificity, defining a binding site for either a hydroxyl or an N-acetyl substituent attached to the 2 position of the galactose ring. Two residues of Asp act as nucleophile or acid/base during the hydrolysis as proposed by Koshland (1953) for classic glycoside hydrolases (Koshland, 1953). Distances of 6.3-6.7 Å between these residues in various GH-27 enzymes are close to the standard characteristic distance of 5.5 Å postulated for retaining glycosidases (Henrissat et al., 1995). A disulfide bond located near the catalytic pocket takes part in formation of active center of the GH-27 enzymes. GH-27 α-D-galactosidases from rice *O. sativa* and fungi *T. reesei* are monomers (Fujimoto et al., 2003; Golubev et al., 2004); the molecules of the enzymes from chicken liver and human are made of two identical subunits, i.e. are homodimers (Clark & Garman, 2009; Fujimoto et al., 2003; Garman et al., 2002) while α-D-galactosidases from *B. halodurans*, *U. vinacea* and *S. cerevisiae* are homotetramers (Fernandez-Leiro et al., 2010; Fujimoto et al., 2009). Differences in substrate specificity must be related to the structure of loops surrounding the catalytic center, and possible with oligomerization of the GH-27 enzymes.

5. Structural information for the GH-36 members (Bruel et al., 2011; Comfort et al., 2007; Merceron et al., 2012)

Crystal structures of free forms and in complexes with galactose have been reported for six GH-36 α-D-galactosidases. The first tertiary structure was solved for α-D-galactosidase of the hyperthermophilic bacterium *Thermotoga maritima,* belonging to subfamily IV of GH-36 (Comfort et al., 2007) (PDB 1zy9). Two thermophilic α-D-galactosidases AgaA and AgaB from the bacterium *Geobacillus stearothermophilus* strain KVE39 (PDB 4fnp and 4fnq, respectively) (Merceron et al., 2012), an α-D-galactosidase domain of the unique α-galactosidase/sucrose kinase (AgaSK) from human gut bacterial simbiont *Ruminococcus gnavus* E1 (PDB 2yfo) (Bruel et al., 2011) and also α-D-galactosidases of mesophilic probiotic bacteria *Lactobacillus acidophilus* strain NCFM (PDB 2xn2) (Fredslund et al., 2011) and *Lactobacillus brevis* strain ATCC 367 (PDB 3mi6, partly described in (Fredslund et al., 2011), are published. All five last enzymes were found in subfamily I GH-36 and their 3D structures are extremely conservative (Fredslund et al., 2011). They display a large N-terminal domain so-called β-supersandwich, which is absent in the GH-27 family. The central domain presents a $(\beta/\alpha)_8$-triose-phosphate isomerase barrel fold which has high homology with N-terminal catalytic GH-27 domain common to many glycoside hydrolases. It contains the active cleft with the fundamental two catalytic residues Asp (nucleophile) and Asp (acid/base) at its bottom. The C-terminal domain presents a β-sandwich structure with a Greek key motif as in the GH-27 family. Excluding monomeric α-D-galactosidase from *Thermotoga maritima*, they exhibit a tetrameric organization and the structure provides insight concerning the recognition of monosaccharides at the active site.

6. Structural information for GH-97 α-galactosidases

In the tertiary structure of GH-97 retaining α-D-galactosidase of *Bacteroides thetaiotaomicron* BtGH97b (PDB 3a24), like α-D-galactosidase of GH-36 family, there are three domains. N-terminal domain N is a disordered β-super sandwich consisting of the 21 antiparallel β-strands and 2 short 3_{10}-helices. Domain A represents the central canonical $(\alpha/\beta)_8$-barrel, structurally homologous to the catalytic domains of GH-27 and GH-36 enzymes, but containing several 3_{10}-helices in the loops connecting the parallel main β-strands and α-helices, moreover β-strand 7 is exchanged with loop. C-terminal β-sheet domain resembles "Greek key" GH-27 family named domain C. From the analysis of crystallographic results it is considered that BtGH97b functions in solution as a monomer (Gloster et al., 2008). Calcium ion seems to play an important role for catalysis, creating calcium-coordinated water molecule in active site. Asp415 and Glu470 acts as nucleophile and acid/base catalysts, respectively.

Insert Table 2

7. Genetic regulation, biosynthesis and biological functions of α-D-galactosidases in microorganisms

Utilization of melibiose in cells of *E. coli* K12 is controlled by two inducible functions: a system of transport (melibiose-permease) of the galactoside and a hydrolyzing enzyme (α-galactosidase) (Pardee, 1957). Genes of the melibiose operon, *mel*B and *mel*A, govern both functions, respectively. In addition, two permeases, induced by melibiose, were found in *E. coli* (Prestidge & Pardee, 1965). The strain *E. coli* K12 cannot utilize raffinose as the sole carbon source, since no special inducible transport system and hydrolytic activity are present. However, the class of transmissible plasmids allows bacteria to grow on raffinose as the sole carbon source and produce hydrogen sulfide (Orskov & Orskov, 1973). Plasmids contain systems designated as *raf*, which comprise of regulatory and structural genes for raffinose-permease, α-D-galactosidase and invertase (Schmid & Schmitt, 1976). This operon is under dual control: it is negatively regulated by repressor binding RafR to twin operators O1 and O2, and positively controlled by cAMP binding protein named CRP (Muiznieks et al., 1999). Recently, a region of the genome sequence of *Streptococcus pneumoniae* comprising eight genes involved in the metabolism of raffinose, was identified. Expression of the structural gene for α-D-galactosidase (*aga*) is positively regulated by raffinose and negatively by sucrose in growth medium belonged to carbon catabolite repression (Rosenow et al., 1999). The sugar utilization system in *S. mutans* is an operon of multi sugar metabolism (msm) (Russell et al., 1992). This gene cluster contains eight genes involved in the uptake of melibiose, raffinose, isomaltotriose and the metabolism of melibiose, sucrose, and isomaltotriose. In addition, it contains a gene for the synthesis of a regulatory protein that acts as a positive effector. Recently, the galactoside utilization system, that is similar to the component of the bacterial binding protein dependent transport system in *Thermus* sp., has been studied (Fridjonsson et al., 2000; Russell et al., 1992). There are reports on the genetic characterization of α-D-galactosidase in *Lactobacillus plantarum* (Fridjonsson et al., 2000), *Lactococcus raffinolactis* (Boucher et al., 2003) and *Carnobacterium piscicola* (Coombs & Brenchley, 2000). Studies on the expression and the transcription of the structural gene for α-D-galactosidase and intended transporter of raffinose (*melA* and *rafP*) in *Lactobacillus plantarum,* showed that unlike other microorganisms *melA* was transcribed from its own promoter and is not part of an operon. Furthermore, the gene *melA* is regulated at the transcriptional level, i.e., it is induced by melibiose, but is not completely suppressed by glucose. On this basis, it was suggested that the regulation of *melA* is achieved by induction rather than by repression of catabolites (Silvestroni et al., 2002). Genetic analysis of *L. raffinolactis* showed that genes of galactoside metabolism were organized in an operon containing the genes of α-D-galactosidase (*aga*), galactose kinase (*galK*), and transporter galactose-1-phosphate (*galT*). The expression of the aga-galKT operon is modulated by the transcription regulator GalR. It is assumed that expression of the gene in the aga operon in *L. raffinolactis* is under negative control of GalR and is induced by products from galactoside metabolism (Boucher et al., 2003). The ability to use melibiose in *Saccharomyces* sp. Is dependent on the presence of the gene MEL1 encoding α-D-galactosidase (Sumner-Smith et al., 1985). MEL1 gene in *S. cerevisiae* is required to produce α-D-galactosidase for melibiose catabolism. α-D-Galactosidase production is induced by galactose and repressed by glucose and melibiose. GAL4 and GAL80 regulatory proteins are the positive and negative controls of inducibility, respectively (Post-Beittenmiller et al., 1984).

Other functions of α-D-galactosidase, related to the specific lifestyles of individual microorganisms for example Aspergillus tamarii (Civas et al., 1984), Penicillium simplicissimum (Luonteri et al., 1998), Trichoderma reesei (Margolles-Clark et al., 1996) and A. niger (Ademark et al., 2001), have been suggested, indicating the ability to catalyze the hydrolysis of different substrates and therefore their different expression patterns. Similar to plant α-D-galactosidases, fungal enzymes hydrolyze terminal α-1,6-linked galactose residues from oligomeric (e.g., melibiose, raffinose, and stachyose) as well as polymeric (galacto-glucomannans) compounds (Meier & Reid, 1982). However*, the complete enzymatic degradation of galactomannan to monosaccharides involves endo-1,4-β-mannanase (β-mannanase, EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and α-D-galactosidase (EC 3.2.1.22)* (Margolles-Clark et al., 1996; Meier & Reid, 1982). Genes encoding three different α-D-galactosidases have been cloned from A. niger (Ademark et al., 2001). Only the expression of aglA and aglB genes is modulated by sugars with the participation of the carbon catabolite repressor protein CreA, implying that different mechanisms control the expression of aglC encoding the third α-D-galactosidase AglC. Based on the expression data, it appeared that the constitutively high level of AglC production on locust bean gum indicated an important role for this enzyme in galactomannan derived oligosaccharide Gal_2Man_5 degradation together with the *A. niger* beta-mannosidase MndA (Ademark et al., 1999). The action of MndA on Gal_2Man_5 exposes terminal, nonreducing galactose groups that are readily hydrolysed by AglC. An alignment of the AglC sequence with

those of other α-D-galactosidases showed that it was highly related to T. reesei AGLII (Margolles-Clark et al., 1996) and to a number of bacterial α-D-galactosidases belonging to family 36 of the glycosyl hydrolases (de Vries et al., 1999). The report about an enzyme of *Thermus* sp. acting on the terminal galactose residues was the first one to consider the substrate specificity of bacterial α-D-galactosidase toward galactomanno-oligosaccharides as well as those of fungal α-D-galactosidase I (Ishiguro et al., 2001). Since the second α-D-galactosidase isoform AgaB in *Geobacillus stearothermophilus* with new specificities including transglycosylation has been expressed (Merceron et al., 2012), it has been suggested that it was a result of mutations originated from AgaA gene evolutionary adapted to more complex substrates that could require the development of altered specificities. *Pseudoalteromonas* sp. KMM 701 α-D-galactosidase PsGalA was found to be a single continuously expressed psychrophilic enzyme independently on the sugar characteristics in the growth medium (Balabanova et al., 2010). The strain has a unique α-D-galactosidase operon located downstream of the α-gal gene, the gene that encodes cold-adapted helicase (Balabanova, unpublished data). These findings suggest that the α-D-galactosidase production may represent an adaptive facility for *Pseudoalteromonas* sp. survival under conditions of constantly extreme low temperatures within the environment and the α-gal gene cluster consists of the housekeeping genes.

The transcript level of HvSF23 encoding a second α-D-galactosidase in barley, *Hordeum vulgare* L., stayed also constant during dark-induced leaf senescence and was not influenced by sugars (Chrost et al., 2004). Recent studies indicate that under certain conditions, e.g. freezing, drought, temperature stress, desiccation, cold acclimation and fruit ripening, various plants synthesize and accumulate raffinose-family oligosaccharides (RFOs) in the senescent plant cells (Pennycooke et al., 2004; Sou et al., 2006; Taji et al., 2002). Plants are able to survive sugar starvation by switching from sugar catabolism to protein and lipid catabolism. The mechanism controlling gene expression in response to sugar starvation involves hexokinase as sugar sensor (Fujiki et al., 2000). In contrast, the α-D-galactosidase genes such as HvSF23 on which transcript level stays rather constant during leaf senescence, may be categorized as a housekeeping gene (Buchanan-Wollaston, 1997).

It has been suggested that a similar mechanism of α-D-galactosidase biosynthesis regulation may be also targeted in bacteria to facilitate the execution of their biological role depending on how they are associated with eukaryotic organisms or survive in the extreme environment. A major component of the *Vibrio cholerae* surface-associated communities, known as biofilm matrix, are the *Vibrio* polysaccharides (VPS), containing glucose, galactose, *N*-acetylglucosamine and mannose, that facilitate environmental survival and persistence of the facultative human pathogen *V. cholerae* in natural ecosystems and its transmission during seasonal cholera outbreaks (Fong et al., 2010). In aquatic habitats, *V. cholerae* can be isolated from surfaces of phytoplankton, zooplankton, aquatic plants, crustaceans and insects, as well as sediments. The *vps* genes are clustered in two regions contributing to VPS biosynthesis, biofilm formation and virulence in *V. cholerae*. Bacteria belonging to the *Pseudoalteromonas* genus are also commonly isolated from marine environments, particularly from various mussels, pufferfish, tunicates and sponges as well as from a range of marine plants (Davey & O'Toole, 2000; Mai-Prochnow, 2006; Shnit-Orland et al., 2012). The relationships between *Pseudoalteromonas* species and their hosts have been described as symbiotic or pathogenic and realized through the biofilm developmental pathway. Complex polysaccharides, an important component of the bacterial outer membrane, play a role in initial surface attachment and then colonization for defense against other potential competitive pathogens (Davey & O'Toole, 2000; Ryu et al., 2004; Shnit-Orland et al., 2012). Recently, it has become clear that biofilm dispersal can also be an active process used by bacteria to colonize new surfaces when conditions have become unfavorable. The factors influencing on biofilm dispersal include cell signalling systems, quorum sensing, and production of endogenous enzymes (Crossman & Dow, 2004; Kaplan et al., 2003; Rice et al., 2005). It is evident that the presence or induction of microbial α-D-galactosidases as hydrolytic enzymes is related to both carbon catabolism and recycling of galactomanno-oligosaccharides in bacteria as well as their hosts (Bakunina et al., 2013; Ensor et al., 1999; Ryu et al., 2004).

8. Immobilization of α-D-galactosidases

This industrially important protein drug marketed for the treatment of Fabry's disease has been used as an example to explore the influence of magnetic particle size and the impact of standard conjugation methods on the activity, stability and robustness of the enzyme. The magnetic particles used based on polystyrene beads coated with a polyurethane layer were tosyl activated by reaction with p-toluen-sulphonyl chloride.

The resulting sulphonyl ester reacted covalently with proteins or other ligands with amino groups. The authors proved the efficient immobilization and performance of the enzyme on different types of particles keeping the enzymatic activity comparable or even higher than the soluble counterpart, and with an improved thermal and operational stability. In particular, using a galactosidase site-specific immobilization through C-terminal hexahistidine tag of the protein, resulted in a better performance with respect to the results obtained with simple covalent attachment via amino groups. This difference could be explained by a better arrangement of the enzyme molecules together with a lower steric hindrance in the presence of the hexahistidine tag acting as a spacer between the enzyme and the surface (Corchero et al., 2012).

Enzyme from coffee beans was covalently bound to dextran activated with cyanogen bromide (Kuo & Goldstein, 1983). α-D-galactosidase from *Pycnoporus cinnabarinus* was immobilized on chitin dispersions with glutaric dialdehyde (Ohtakara & Mitsutomi, 1987). An enzyme from *Aspergillus oryzae* was immobilized on Eupergit C containing epoxy groups, which provided covalent bonding of the protein by amino, SH, and carboxy groups (Hernaiz & Crout, 2000) or into calcium alginate gel, in which the enzyme was additionally entrapped with glutaric dialdehyde (Prashanth & Mulimani, 2005) or in polyacrylamide gel (Tippeswamy & Mulimani, 2003). Many other papers appeared in literature concerning the enzyme from tomato fruit on polymeric beads containing galactose (Okutucu et al., 2010), the enzyme from *Cicer* which was immobilized on chitosan or Amberlite (Singh & Kayastha, 2012), the enzyme from jack bean seeds entrapped in calcium alginate (Shankar et al., 2011) and a very stable enzyme from *Thermus* sp. T2 that has been immobilized on different supports activated with glyoxyl, epoxy or glutaraldehyde groups (Filho et al., 2008). The stability of these preparations can be increased by post-immobilization techniques (Pessela et al., 2008) to avoid the release of enzyme to the reaction medium with food contamination.

α-D-galactosidase from marine bacterium *Pseudoalteromonas* sp. KMM 701 was immobilized in a tetrakis-(2-hydroxyethyl) orthosilicate (10 wt %) matrix containing galactomannan (0.25 and 0.75%). The enzyme preserved its catalytic activity for a long time and its thermal stability increased by 10˚C. The immobilized α-D-galactosidase exhibited maximal activity at the same pH as the free enzyme. The Km with p-nitrophenyl-α-D-galactopyranoside was not substantially changed (Bakunina et al., 2006).

9. Synthesis

To improve molecular diversity of branched derivatives of cyclodextrins one or more sugars as side chains were added thus improving the solubility and possibly developing new applications (e.g. forming inclusion products with various kind of compounds) (Okada et al., 2011). D-Galactose can be easily modified by D-galactose oxidase to introduce a reactive aldehyde group further improving molecular diversity. 6-O-α-D-galactosyl α-cyclodextrin and minor 2-O-α-D-galactosyl α-cyclodextrin were formed along with transgalactosylated derivatives of the melibiose donor also present in the reaction mixture (entry 1 **Table 3**) (Kitahata et al., 1992; Koizumi et al., 1995). The coffee bean enzyme was also used to synthesize chemoenzymatically α-galactosides for PEG conjugation (entry 3 **Table 3**) (Matsuo et al., 1997). Enzymic galactosylation was also reported using *Thermomyces lanuginosus* enzyme obtaining conversion of 15-22% (**entry 5 Table 3**) (Biely et al., 2001); digalactosylation products were observed in the reaction mixture using as acceptor a cyclodextrin-like compound derived from alternan. Product analysis showed that the major product contained galactopyranosyl moiety linked by α-1,6 linkage to one of the two α-1,3-glucosylated residues of the cyclic tetramer used as acceptor. With the aim of obtaining monoglycosylated derivatives, the introduction of a short spacer was reported. It was hoped that the additional hydroxyl group would act as a selective acceptor in a transglycosylation catalyzed by coffee bean enzyme and PNP-α-galactopyranoside (7 eq) as donor. A 30% yield of isolated monogalactosylated derivative with galactose linked to the spacer was indeed obtained. Cyclodextrins branched with galactosyl units on other ring hydroxyls were not detected thus demonstrating the preference of the galactosidase for the outer hydroxyl group (Bonnet et al., 2003). Other synthetic examples using aryl donors with coffee bean enzyme or enzymes from different sources for galactosylation of more complex acceptors, were successful. Of particular interest is the study of the autocondensation of p-nitrophenyl α-D-galactopyranoside (Spangenberg et al., 1999). Two reactions were studied using NMR spectroscopy in deuterium oxide and water, discovering some differences in the kinetic profiles. However, apart the significance of these differences, due to isotopic presence, the interest is due to the possibility of using NMR spectroscopy to assess fine details of transglycosylations for an easy optimization of the reaction conditions and for stopping the reaction before competitive hydrolysis of products. The formation of all three examples of PNP-α-

galactobioses as a result of the analysis of thirty-one extracellular α-D-galactosidases of fungal origin, was also studied (**entry 4 Table 3**) (Weignerova et al., 2001). The regioselectivity of some enzyme was assessed and the formation of α-1,2 galactobiose link was recognized as uncommon.

Synthesis was obtained also by reverse hydrolysis using *A. oryzae* enzyme and galactose as donor **(entry 2 Table 3**). In the examples reported, α-galactosides of a diol and a unsaturated alcohol were formed in a solution of 80-90% acceptor with product yield reaching 37-50% (Vic et al., 1996). The enzyme catalyzing the reaction was also purified from commercial sources and the authors considered this an alternative to traditional cultivation techniques for having quick access to the biocatalyst (Scigelova & Crout, 2000).

Other fungal enzymes such as α-D-galactosidases isolated from *Aspergillus terreus* CCM55, *Aspergillus commune* CCM 2969, *Penicillium vinaceum* CCM 2384 or *Penicillium brasilianum* 2155 were used (**entry 7 Table 3**) both in transglycosylation or reverse hydrolysis for the formation of three α-galactosides of allyl-, 2-nitroethyl- or (2',2',2'-trifluoroacetamido)-ethyl- aglycones (Casali et al., 2002). The same group of authors studied the dependence of fungal activity on the presence of organic cosolvents (**entry 10 Table 3**) for the synthesis of acylated glycosides in a publication reporting the preparation of 4-nitrophenyl α-D-galactopyranosyl-(1,3)-6-O-acetyl-α-D-galactopyranoside catalyzed by α-D-galactosidase from *Talaromyces flavus* (Simerska et al., 2003). Also, the use of the same enzyme (**entry 11 Table 3**) with a series of sterically hindered alcohols (primary, secondary and tertiary) as galactosyl acceptors, demonstrating the broad synthetic potential of the *T. flavus* α-D-galactosidase (Simerska et al., 2006). In addition, *T. flavus* α-D-galactosidase was also used to prepare isoglobotriose as previously discussed (Weignerova et al., 1999).

α-Galactopyranosyl fluoride was used as a donor by α-D-galactosidase (**entry 6 Table 3**) in a study aimed to reduce the self-condensation plaguing the use of nitrophenyl activated substrate donors (André et al., 2001). Thus, methyl α-D-galactopyranosyl-(1,3)-α-D-galactopyranoside (**entry 8 Table 3**) is obtained with a 51% yield in ice while only 29% is synthesized at 37 °C (Spangenberg et al., 2002). Authors commented that their results appear to be a general property of hydrolases in that they already found the same behaviour with β-galactosidase. In another reaction (**entry 9 Table 3**) using *Bifidobacterium adolescentis* enzyme (Nieder et al., 2003), nucleotide sugars such as UDP-Glc and UDP-Gal were novel acceptors. The reactions were conducted for 21 days in the frozen state (reduced water concentration), to promote higher yields. However, authors speculate that an increased concentration of substrates in the small remaining liquid phase ("freeze-concentration") promote yields which were below 10%.

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