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REVIEW

Functional and biotechnological insights into diglycosidases*

LAURA S. MAZZAFERRO & JAVIER D. BRECCIA

INCITAP-CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa (UNLPam), Santa Rosa, La Pampa, Argentina

Abstract

β-Glucosidases are reported to act in an exo manner and so are unable to hydrolyze the bond if another sugar is attached to a non-reducing terminus of glucose. However, endo-β-glucosidases recognizing the heterosidic linkage have been known to plant physiologists for eight decades, although they have been described in detail only recently. Because of the ability of these enzymes to split off a disaccharide they were named disaccharide-specific glycosidases or 'diglycosidases'. In contrast to the sequential mechanism of two monoglycosidases, the transformation of some secondary metabolites in one step was reported as responsible for the production of toxic compounds involved in plant defense mechanisms against herbivores, such as hydrogen cyanide. The current focus of interest is on the application of their unique substrate specificity for biotransformation of plant-based foods. Four activities have been described and characterized so far, recognizing the following disaccharidic sugar moieties: primeverose, acuminose, rutinose and vicianose. Moreover, three of these proteins have been fully sequenced and mutants of one of them constructed by site-directed mutagenesis, in order to elaborate the molecular basis of substrate recognition. The present paper reviews the role of these enzymes in plant and filamentous fungi, as well as their prospects for technological applications.

Keywords: *vicianin hydrolase, furcatin hydrolase,* β-*primeverosidase,* β-*rutinosidase,* α-*rhamnosyl*-β-*glucosidase*

Introduction

Glycoside hydrolases (EC 3.2.1.–), commonly referred to as glycosidases, are a widespread group of enzymes which hydrolyze glycosidic bonds. From a biotechnological point of view these enzymes find extended applications for industrial biotransformations (Polaina & MacCabe 2007). Historically, the major interest has been in polysaccharidases such as amylases and pectinases for food processing, xylanases for bleaching of pulp and paper and cellulases for biofuel production (Breccia et al. 1998; Juge et al. 2006; Yeoman et al. 2010). Recently, enzymes hydrolyzing glycoconjugates have gained interest for the biotransformation of several plant-based foods. An important fraction of aroma precursors of tea, wine and other foods is constituted by glycosylated molecules (Hemingway et al. 1999; Wang et al. 2001). The flavonoids in citrus have a significant impact on nearly every aspect of citrus fruit production and processing, contributing to the bitter taste and also to juice clouding (Manthey & Grohmann 1996). An enzymatic deglycosylation can be carried out, with different levels of efficiency, by adding commercially available preparations to hydrolyze aroma precursors as well as flavonoids, releasing volatile compounds and debittering and clarifying fruit juices, respectively (Puri et al. 1996).

The major sugar moieties of the compounds mentioned above and other secondary metabolites of plants are disaccharides such as α-L-arabinofuranosyl-, α-L-rhamnopyranosyl-, β-D-xylopyranosyl- and β-D-apiofuranosyl-β-D-glucopyranose. Several enzymes have been reported to catalyze their hydrolysis. The most common mechanism for their deglycosylation involves two enzymes from microbial sources (*Aspergillus* or *Penicillium* spp.), which act sequentially (Sarry & Gunata 2004). First, an enzyme recognizing the linkage between the two sugar moieties splits off a monosaccharide. Examples include α-rhamnosidases (EC 3.2.1.40), α-arabinosidases (EC 3.2.1.55)

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Correspondence: J. D. Breccia, Depto. de Química-FCEyN, Universidad Nacional de la Pampa (UNLPam), Av. Uruguay 151, (6300) Santa Rosa, La Pampa, Argentina. Tel: +54 2954 436787 ext 26. Fax: +54 2954 432535. E-mail: javierbreccia@exactas.unlpam.edu.ar

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and β-xylosidases (EC 3.2.1.37) (Manzanares et al. 2001; Orrillo et al. 2007). These enzymes are known to be highly specific for the first sugar moiety but rather promiscuous for the second, and they also accept a non-glycosidic moiety. Then, a β-glucosidase (EC 3.2.1.21) hydrolyzes the heterosidic linkage between the glucose moiety and the aglycone (Barbagallo et al. 2004). β-Glucosidases are reported to act in an exo manner and therefore are unable to hydrolyze the bond if another sugar is attached to a non-reducing terminus of glucose. However, a number of endo-β-glucosidases recognizing the heterosidic linkage have also been described. Based on the ability of these enzymes to split off a disaccharide they were named disaccharide-specific glycosidases or 'diglycosidases' (Sakata et al. 2003), a term which must not be confused with 'disaccharidase', applied to enzymes which hydrolyze disaccharides to the corresponding monosaccharides, common examples being invertase and lactase.

Physiological role of plant diglycosidases

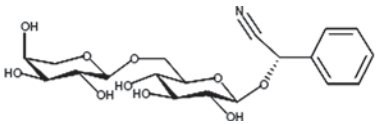
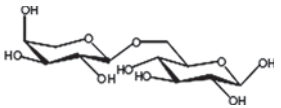
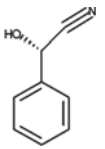
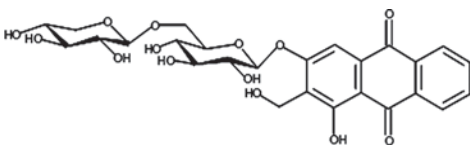
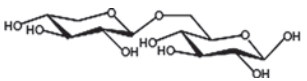
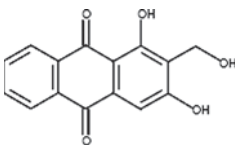
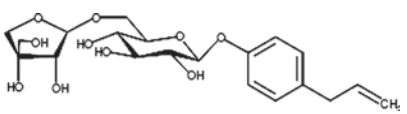
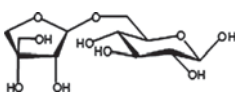
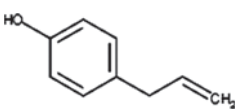
Bridel and Charaux published the first reports on diglycosidases, all of which were isolated from plants. A β-primeverosidase was described which hydrolyzed a β-primeveroside to a disaccharide (primeverose) and an aglycone (anthraquinone) (Bridel 1925). Additionally, a 'rhamnodiastase' that splits rutin into quercetin and rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) was reported by Bridel and Charaux (1926). These enzymes were thought to be widespread in the plant kingdom but despite purification attempts by several authors (Suzuki 1962; Bourbouze et al. 1975), none of the enzymes was demonstrated to be a single glycosidase until the late 1980s (Lizotte & Poulton 1988).

Studies on the physiological function of plant diglycosidases have focused on the activation of secondary plant metabolites. Cyanogenesis, the production of cyanhydric acid by living organisms, has been recognized in many plant species. Secondary metabolites responsible for this property are the so-called cyanogenic glycosides, which include compounds such as linamarin, dhurrin and amygdalin. Their toxicity arises from enzymatic degradation to produce hydrogen cyanide, usually performed in a sequential manner by β-glycosidases, which can be endogenous or not, e.g. they may arise from gut microflora in the herbivores. Lizotte & Poulton (1988) were the first to purify a diglycosidase from squirrel's foot fern (*Davallia trichomanoides* Blume). The fern was found to produce vicianin hydrolase (recommended name vicianin β-glucosidase, EC 3.2.1.119) which hydrolyzed vicianin into vicianose and cyanohydrin (*R*)-mandelonitrile. Vicianin hydrolase was shown to share several features common to other cyanogen-specific β-glycosidases such as acid optimum pH and isoelectric point; but its distinguishing characteristic was the ability to liberate cyanohydrins from (*R*)-linked cyanogenic disaccharides in a single step (Table I). As a common strategy, the substrate and the enzyme(s) are located in different cellular compartments. When plants are attacked and cells are damaged, substrate and enzyme come into contact resulting in hydrolysis and release of the toxic aglycone.

More recent studies have reported several diglycosidases from different plants, representative examples of which are shown in Table I. The enzyme furcatin hydrolase (recommended name β-apiosyl--β-glucosidase, EC 3.2.1.161) from *Viburnum furcatum* was found to be involved in defense against hervibory (Ahn et al. 2004). Furcatin hydrolase is a unique disaccharide-specific acuminosidase, which hydrolyses furcatin into acuminose and *p*-allylphenol. The enzyme was located in the chloroplasts and, although the intracellular localization of furcatin is still unknown, it is likely that the compound is located in a separate compartment as the *p*-allylphenol concentration increased by rubbing or scratching the leaves. This toxic aglycone acts as an insect antifeedant. A β-apiosyl-β-glucosidase activity has subsequently been detected in the seeds of *Dalbergia nigrescens* Kurz, but its physiological role was not investigated (Chuankhayan et al. 2005). Regarding its substrate specificity for the sugar moiety, the enzyme was shown to be promiscuous, since it was able to hydrolyze isoflavonoid 7-*O*-β-glucosides, 7-*O*-β-apiosyl-glucosides and 7-*O*-β-malonyl-glucosides.

Diglycosidase activities are present not only in leaves and seeds but also in roots. Nakanishi et al. (2005) reported lucidin formation in hairy roots of *Rubia tinctorum* L. from lucidin *O*-β-primeveroside when the tissue was squashed. The enzyme activity responsible for deglycosylation 'LuP-glycosidase' (recommended name β-primeverosidase, EC 3.2.1.149) was found to be a glycosidase able to recognize and split off the disaccharide primeverose (Table I). Apparently, lucidin *O*-β-primeveroside is accumulated in the vacuole and LuP-glycosidase pre-exists in a different cellular compartment, which explains that lucidin accumulates after mechanical disruption. Lucidin is not only a strong genotoxin but it is converted by endogenous enzymes to produce nordamnacanthal, an antifeedant (Morimoto et al. 2002). Thus, LuP-glycosidase triggers the production of diverse defensive chemicals at the site of wounding in roots of *R. tinctorum*.

Table I. Representative examples of diglycosidase activities found in plants.

Secondary metabolite		Sugar		Aglycone	Organism, enzyme name synonym, physiological role and reference
Vicianin β-glucosidase (EC 3.2.1.119)					
	$+ H_2O \rightarrow$		+		*Davallia trichomanoides* Blume vicianin hydrolase Poisoning, present in seeds (Lizotte & Poulton 1988)
Vicianin Mandelonitrile 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside		vicianose		mandelonitrile	
β-Primeverosidase (EC 3.2.1.149)					
	$+ H_2O \rightarrow$		+		*Rubia tinctorum* L. LuP-glycosidase Involved in the formation of an antifeedant, present in roots (Nakanishi et al. 2005)
Lucidin primeveroside Lucidin 3-*O*-(6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside)		primeverose		lucidin	
β-Apiosyl-β-glucosidase (EC 3.2.1.161)					
	$+ H_2O \rightarrow$		+		*Viburnum furcatum* Blume furcatin hydrolase Insect repelling, present in leaves (Ahn et al. 2004)
Furcatin *p*-Allylphenyl 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside		acuminose		*p*-allylphenol	

Diglycosidases for modification of plant-based foods

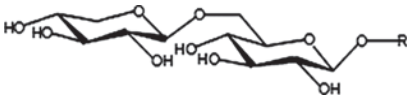
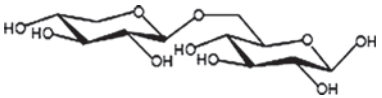
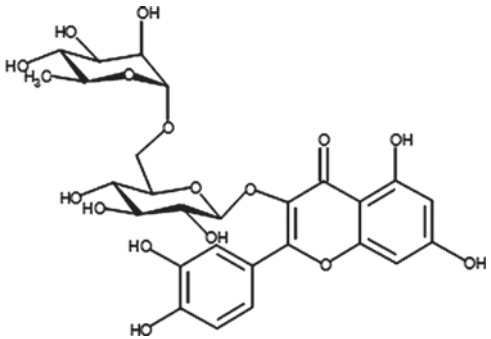
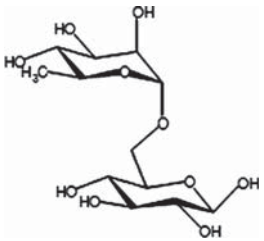
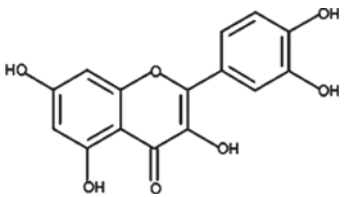
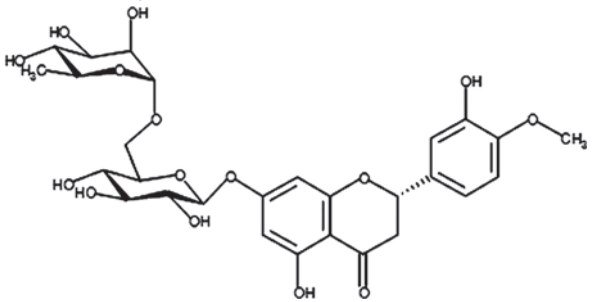
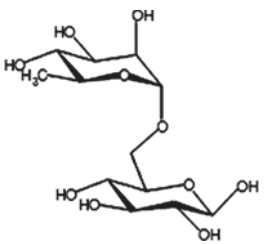
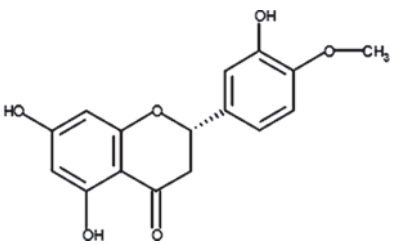
From a biotechnological point of view, plant diglycosidases were being exploited by humans a long time before knowledge of their existence was registered. During black tea manufacturing, the leaves of the tea plant (*Camellia sinensis*) are mechanically macerated. Rupture of the cell walls begins the so-called fermentation process, where enzymatic oxidation takes place giving tea the characteristic color and flavor. Besides oxidation, hydrolysis plays a vital role in tea aroma formation (Ma et al. 2001; Mizutani et al. 2002). Hundreds of potentially volatile compounds are present in the leaves as non-volatile glycosides, mostly β-primeverosides. Compounds such as linalool and geraniol are liberated by endogenous glycosidases all through the process. Among these, a β-primeverosidase is the key enzyme in aroma formation in addition to its role in defense against fungal infection and herbivore feeding (Ma et al. 2001; Mizutani et al. 2002).

After this discovery, considerable effort has been made to obtain β-primeverosidases from microbial sources for application in industry; for instance, to promote aroma formation in foods. Screening for microorganisms using the artificial substrate *p*-nitrophenyl-β-primeveroside led to identification of the filamentous fungus *Aspergillus fumigatus* AP-20, which produces an endo-cleaving enzyme (Yamamoto et al. 2002) (Table II). Since enzymes derived from pathogenic microbes are strictly regulated in their use as food-processing aids, the same research group screened again using GRAS (Generally Recognized As Safe) microorganisms. Eight fungal strains were shown to produce β-primeverosidase activity, of which the *Penicillium multicolor* enzyme was studied in detail (Tsuruhami et al. 2006) (Table II). The purified enzymes of both *A. fumigatus* AP-20 and *P. multicolor* were shown to preferentially hydrolyze the heteroglycosidic bond of *p*-nitrophenyl -β-primeveroside. When hydrolysis rates were measured for different substrates, specificity was found to be dependent on both sugar and aglycone (Table III). Eugenyl-β-primeveroside was the most rapidly hydrolyzed substrate among naturally occurring alcoholic aglycone aroma precursors. However, the hydrolytic rate was 50-fold lower than with *p*-nitrophenyl-β-primeveroside. In contrast, tea β-primeverosidase preferably hydrolyzed eugenyl -β-primeveroside. Therefore, while the screening strategy successfully identified endo-β-glycosidases their hydrolysis of natural aroma compounds was poor, and their natural substrates remain unknown. *A posteriori*, the authors obtained a patent describing a method to produce the enzyme, a gene which encodes the enzyme and the use of the enzyme (Yamamoto et al. 2006). The addition of these enzymes to fruit juices, tea and wine effectively resulted in aroma enhancement.

Challenges for diglycosidases in the citrus industry

Searches for microbial diglycosidases are gaining interest in the citrus industry for hydrolysis of flavonoids. Common flavonoids include hesperetin, naringenin, eriodictyol and quercetin, but they rarely occur as free aglycone in the fruit itself (Peterson et al. 2006a,b). Sugar moieties, particularly rhamnosyl-glucosides, are usually attached. α-Rhamnose can be linked 1–6 or 1–2 to glucose, giving rise to the disaccharides rutinose and neohesperidose, respectively. Citrus such as oranges, lemons and grapefruit contain considerable concentrations of flavonoids (up to 20 mg of aglycone per 100 g of edible fruit) which are, therefore, ingested as constituents of the human diet. Health benefits have been associated with flavonoid consumption, including reduced risk of some cancers and stroke. Grapefruit, for instance, is rich in naringin (naringenin 7-*O*-neohesperidoside), which imparts the tangy or bitter taste. Hesperidin (hesperetin 7-*O*-rutinoside) is abundant in lemons and oranges, and contributes to juice clouding. Enzymatic hydrolysis with monoglycosidases has been employed to remove the unpleasant taste or for juice clarification. However, a search for relevant diglycosidases has been motivated by the desire to complete the deglycosylation in a single step and selectively hydrolyze some flavonoids in the presence of others. Two fungal diglycosidases have been reported to act on flavonoid glycosides, both recognizing the rutinose moiety, but with distinctive specificity regarding the linkage to the aglycone. *Penicillium rugulosum* IFO 7242 produces an enzyme called rutinosidase, which is able to hydrolyze rutin (Narikawa et al. 2000). *Acremonium* sp. DSM24697 (Mazzaferro et al. 2010) produces an α-rhamnosyl-β-glucosidase which hydrolyzes hesperidin (Table II). Glycosidase-catalyzed reactions usually take as an epithet the name of the sugar moiety. In this case, the names of the enzymes are synonyms and they were assigned arbitrarily to distinguish one enzyme from the other. Rutinosidase is active against 3-*O*-flavonoid rutinosides while α-rhamnosyl-β-glucosidase only hydrolyzes flavonoids with 7-*O*-linked rutinose. Both enzymes were shown to release rutinose, a compound with high added value, from raw sources, giving these enzymes industrial potential. Rutinose production by a variety of chemical approaches has

Table II. Filamentous fungi diglycosidases.

Secondary metabolite	Sugar	Aglycone	
β-Primeverosidase (EC 3.2.1.149)			
 + H_2O →	 +	R	*Aspergillus fumigatus* and *Penicillium multicolor*
Primeveroside 6-*O*-β-D-Xylopyranosyl-β-D-glucopyranoside	primeverose		
β-Rutinosidase[a]			
 + H_2O →	 + 		*Penicillium rugulosum*
Rutin Quercetin 3-*O*-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside)	rutinose	quercetin	
α-Rhamnosyl-β-glucosidase[a]			
 + H_2O →	 + 		*Acremonium* sp. DSM24697
Hesperidin Hesperetin 7-*O*-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside)	rutinose	hesperetin	

[a]These enzymatic activities still do not have an EC-IUBMB recommended name and EC number.

Table III. Fungal β-primeverosidases substrate specificity.

Substrate	–2	–1	+1	Hydrolysis
Subsite				
p-Nitrophenyl-	*Xyl* –	*Glc*	– pN	High
β-primeveroside		*Xyl*	– *Glc* – pN	Low–undetectable
p-Nitrophenyl-	*Glc* –	*Glc*	– pN	Medium–low
β-gentiobioside		*Glc*	– *Glc* – pN	Low–undetectable
Eugenyl-	*Xyl* –	*Glc*	– E	Low
β-primeveroside		*Xyl*	– *Glc* – E	Low–undetectable

pN and E denote *p*-nitrophenyl- and eugenyl- substitutions.

been examined (Kamiya et al. 1985; Quintin & Lewis 2005) and recently it has been reported as a free sugar in *Datisca glomerata* (Schubert et al. 2010). Enzymatic production was previously only possible by reverse hydrolysis using an α-rhamnosidase and starting from rhamnose and glucose (Martearena et al. 2007). In particular, hesperidin is the flavonoid with highest concentration in many citrus varieties, and is usually precipitated during peel processing, constituting an inexpensive by-product of the citrus industry. Neither rutinosidase nor α-rhamnosyl-β-glucosidase hydrolyzes naringin, e.g. they do not recognize the neohesperidose moiety, which has a rhamnose on the C-2 hydroxyl group of glucose. The term 'naringinase', usually found in literature, refers to a multienzyme complex containing at least two monoglycosidases: α-rhamnosidase and β-glucosidase.

Understanding of the molecular basis of substrate recognition

The glycoside hydrolase sequence-based families proposed by Henrissat (1991) proved extremely useful for predicting the reaction mechanism (Henrissat & Davies 1997). Within a given sequence-derived family, three-dimensional (3D) structure is conserved. Since the mechanism is governed by the spatial orientation of the catalytic residues in their 3D template and structure itself is dictated by sequence, the stereochemical outcome of the reaction is conserved within any given family of glycoside hydrolases (Henrissat et al. 2001). Up to now, three plant diglycosidases have been sequenced: *V. furcatum* furcatin hydrolase (Ahn et al. 2004), *C. sinensis* β-primeverosidase (Ma et al. 2001) and *Vicia angustifolia* vicianin hydrolase (Ahn et al. 2007). All of them were found to belong to glycoside hydrolase family 1 (GH1), together with several monoglycosidases. Phylogenetic analysis suggested that diglycosidase activity has been independently acquired at least twice during the evolution of the family.

Differences between mono- and diglycosidases are not believed to reside in their reaction mechanism. Since sequenced diglycosidases were shown to belong to GH1, a classical Koshland (1953) retaining mechanism is expected as for the rest of the glycosidases within the family. A general trend observed for diglycosidases is their substrate specificity for disaccharide glycosides bearing a pentose or hexose unit on the C-6 hydroxyl group of glucose. However, glycosidases are often stringent with regard not only to the identities of their glycone but also to their aglycone (Blanchard & Withers 2001; Verdoucq et al. 2004). Aglycone recognition for diglycosidases was found to vary from highly specific for *V. furcatum* furcatin hydrolase to rather promiscuous for *C. sinensis* β-primeverosidase (Ahn et al. 2004). Complete accommodation of the substrate in the binding pocket seems to be responsible for diglycoside hydrolysis at the heterosidic bond. Thus, a three-subsite binding pocket has been proposed for the recognition: subsite + 1 for the aglycone moiety, subsite –1 for the glucose moiety and subsite –2 for the second sugar moiety (see Table III). Molecular and computational analysis performed with *V. furcatum* furcatin hydrolase supported this hypothesis (Daiyasu et al. 2008). A model structure was built for furcatin hydrolase by homology modeling using *Trifolium repens* cyanogenic β-glucosidase – a monoglycosidase – as a template. This model structure was specifically constructed to represent the substrate binding mode (Figure 1). The structural comparison between the di- and monoglycosidases suggested that the difference in the substrate specificity of these enzymes was not caused by global conformational changes. The carboxylate groups of the catalytic Glu residues, Glu238 and Glu447, were ~5 Å apart from each other. This location is suitable for catalysis via the retaining mechanism. Two residues next to the substrate, Ala419 and Ser504, were specifically conserved in plant diglycosidases. Mutations at these sites severely decreased the diglycosidase activity. It was suggested that Ala419 is involved in aglycone recognition while Ser504 binds to the external saccharide. Monoglycosidases were not found to posses a conserved counterpart of Ser504 (Daiyasu et al. 2008). Molecular studies of other diglycosidases will bring new clues for substrate recognition and, it is hoped, the possibility to obtain tailored diglycosidases for specific purposes.

Prospect for synthesis of diglycoconjugates

Glycoconjugates are central to numerous biochemical events. Recognition of pathogens has been associated with the disaccharide moieties of glycoconjugates (Andersson et al. 1983; Haseley et al. 2001). Some

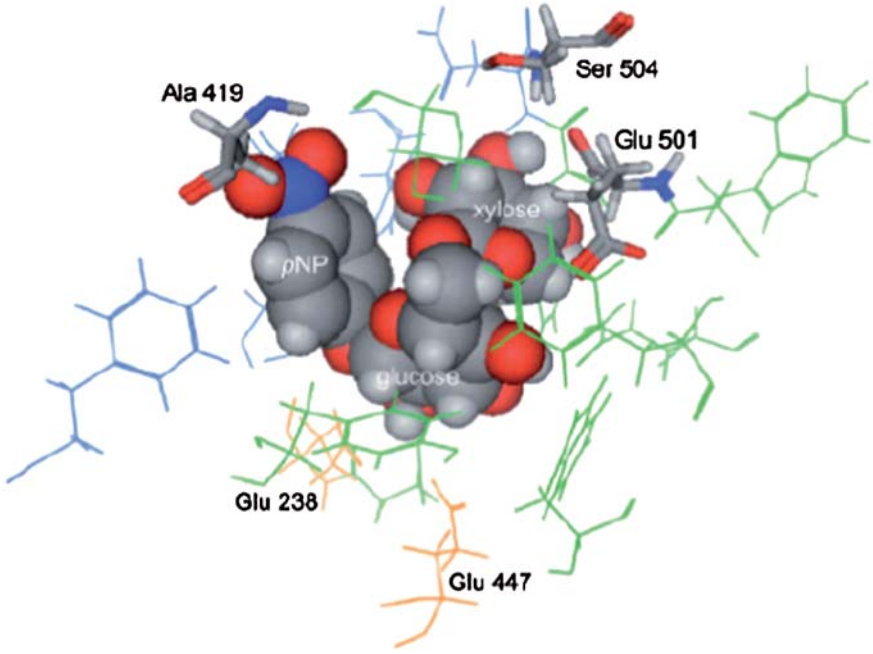


Figure 1. Active site of the furcatin hydrolase model structure (Daiyasu et al. 2008). The three mutated sites are indicated by stick models. The residues within 3.5 Å from the substrate atoms are indicated by line models, which include two catalytic Glu residues (yellow), the conserved residues within family GH1 (green) and others (blue). *p*-Nitrophenyl β-primeveroside is indicated by a space filling model, colored by atom type.

glycosidic moieties can interact with receptors or lectins on the cell surfaces followed by their active uptake (Kren & Rezanka 2008). In that sense, the diglycosidases are promising tools for the synthesis of tailored bioactive glycosides. GH1 family members have a retaining hydrolysis mechanism and are, therefore, potential transglycosylating enzymes (Cantarel et al. 2009). Although none of the fungal diglycosidases has yet been demonstrated to belong to GH1, some of them are able to transglycosylate.

Several authors have reported the synthesis of diglycoconjugates using glycosidases which catalyze the transfer of a monosaccharide unit from a polysaccharide to the corresponding monoglycoside (Zeng et al. 2000; Borris et al. 2003; Kadi & Crouzet 2006). Thus, Kadi and Crouzet (2006) synthesized phenyl-β-primeveroside using a xylanase starting from xylan and phenyl-glucoside. A different approach is to use a diglycosidase to transfer the entire disaccharide at once. *P. multicolor* β-primeverosidase was used for the synthesis of primeverosidic aroma precursors as standards for food analysis (Tsuruhami et al. 2004). The corresponding alcohols were used as sugar acceptors instead of the glucosylated acceptors needed for the previous case. The bioprocess was carried out efficiently in an aqueous–organic biphasic system with yield up to 70% for 3-hexyl-β-primeveroside.

Fagopyrum tataricum β-rutinosidase and *Acremonium* sp. DSM24697 α-rhamnosyl-β-glucosidase were shown to synthesize alkyl-rutinosides in aqueous media (Figure 2) (Zhou et al. 2009; Mazzaferro et al. 2010). The transglycosylation potential of the latter has enabled the synthesis of the diglycoconjugated fluorogenic substrate 4-methylumbelliferyl-rutinoside (unpublished results). This was performed in one step from the corresponding aglycone, 4-methylumbelliferone, and hesperidin as rutinose donor. The synthesis of rutinosides starting from 4-methylumbelliferyl-glucoside would only be possible by reverse hydrolysis since all reported α-rhamnosidases have been inverting enzymes and thus cannot catalyze transglycosylation (Martearena et al. 2007). 4-Methylumbelliferyl-rutinoside differs from 4-methylumbelliferyl-glucoside in the rhamnosyl substitution at the C-6 of glucose, which raises the possibility of exploring the natural occurrence of diglycosidases recognizing the rutinose moiety by zymographic analysis.

Concluding remarks

From the first report of diglycosidases in 1925, six decades passed until the demonstration of a pure protein responsible for such activity and systematic study is in its infancy. Diglycosidases hydrolyze the same bond as β-glucosidases and four activities, which recognize the disaccharides primeverose, acuminose, rutinose and vicianose, have been described. The diglycosidases sequenced up to now were demonstrated to belong to family 1 GH, and

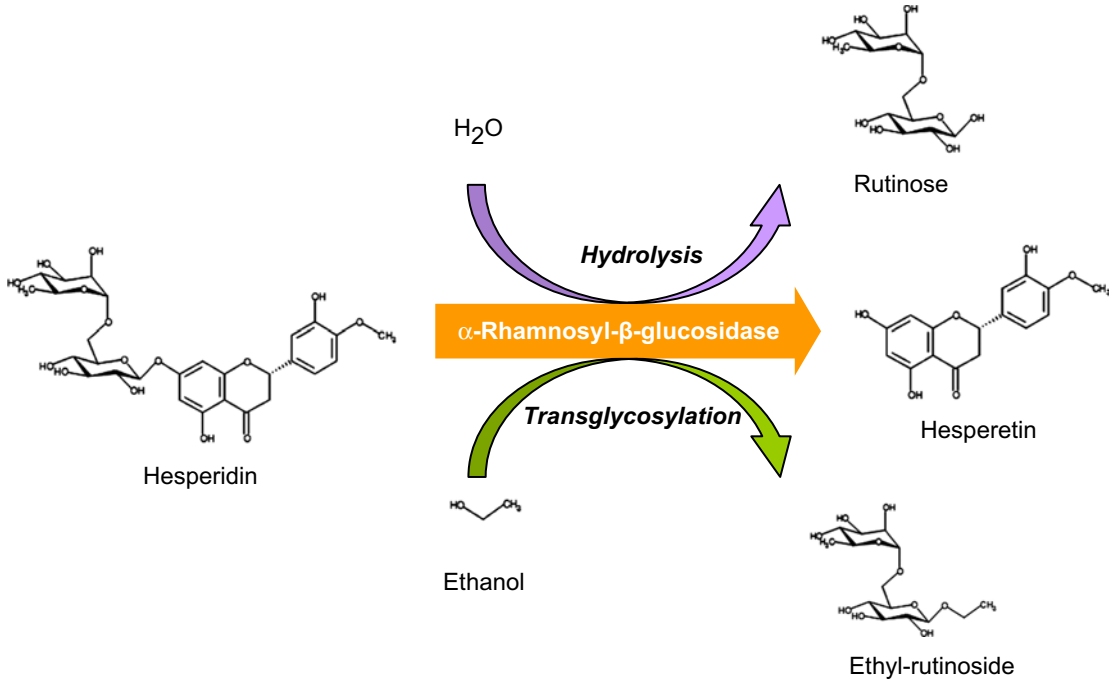


Figure 2. Synthesis of ethyl rutinoside by α-rhamnosyl-β-glucosidase.

share the conserved catalytic Glu residues, but what makes these enzymes unique is their substrate specificity which allows the liberation of a diglycosidic unit in one step. This characteristic, combined with their transglycosylation ability, makes them promising tools for biotechnology. A wide range of applications have been proposed from bulk hydrolysis of plant-based foods to the synthesis of fine chemicals.

The new findings bring about some fundamental questions. Do fungal diglycosidases possess a defensive role besides the catabolic one? Did plant and fungal diglycosidases evolve independently? Is the production of diglycosidases widespread in bacteria? Why do plant diglycosidases prefer to hydrolyze aroma precursors while fungal diglycosidases are fairly ineffective, being more suitable for their synthesis?

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