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Glycoside hydrolases for extraction and modification of polyphenolic antioxidants

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Antioxidants are important molecules that are widely used by humans, both as dietary supplements and as additives to different types of products. In this chapter, we review how flavonoids, a class of polyphenolic antioxidants that are often found in glycosylated forms in many natural resources, can be extracted and modified using glycoside hydrolases (GHs). Glycosylation is a fundamental enzymatic process in nature, affecting function of many types of molecules (glycans, proteins, lipids as well as other organic molecules such as the flavonoids). Possibilities to control glycosylation thus mean possibilities to control or modify the function of the molecule. For the flavonoids, glycosylation affect both the antioxidative power and solubility. In this chapter we overview results on *in vitro* deglycosylation and glycosylation of flavonoids by selected GHs. For optimal enzymatic performance, desired features include a correct specificity for the target, combined with high stability. Poor specificity towards a specific substituent is thus a major drawback for enzymes in particular applications. Efforts to develop the enzymes as conversion tools are reviewed.

Keywords: glucosidase, cellulase, amylase, glycosynthase, GH, flavonoid, quercetin

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1. Introduction

The increased concern about scarcity of fossil resources has lately put the use of renewable resources by biotechnological methods in focus, as these are predicted to have an increased importance in production of food, additives and chemicals. Antioxidants can be foreseen to play a role as bio-based ingredients in food (as well as other) products, both as preservatives, replacing agents with negative health aspects, and as nutraceuticals. Flavonoids are polyphenolic compounds and a class of secondary metabolites that are widely distributed in nature. The beneficial properties of flavonoids are mainly referred to their ability to counteract oxidative stress caused by oxygen species and metal ions (Lin and Weng 2006, Havsteen 2002) and they are shown to play a protective role against neoplasia, atherosclerosis and neurodegenerative diseases (Lee and Lee 2006, Boudet 2007). Because of these exclusive properties, flavonoids have received great attention and the industrial interest is growing rapidly. Apart from this role, antioxidants can also be added to food and other types of products to prolong their shelf-life. Currently over 6500 flavonoids have been identified (Corradini et al. 2011) and they are commonly found in plants, fruits, vegetables, ferns, stems, roots, tea, wine and also from bark (Nijveldt et al. 2001). Their role in plants is to protect against UV-radiation diseases, infections and insect invasion (Corradini et al. 2011). The content of flavonoids varies, dependent on the source, but is normally in the mg-range per kg rawmaterial. For example, the content of the flavonoid quercetin is around 300 mg/kg of onion (Griffiths et al. 2002), 100 mg/kg of broccoli, 50 mg/kg of apples, 40 mg/kg of blackcurrants and 30 mg/kg of green tea (Hollman and Arts 2000).

Problems with many flavonoids are, however, low solubility and poor stability (in both polar and non-polar media) which makes their uses difficult in many formulations of food, pharmaceutical and nutraceutical products (Ishihara and Nakajima 2003). Improvement of the hydrophilic nature, biological properties and stability of flavonoids can be achieved by enzymatic structural modification (Haddad et al. 2005). In nature, enzyme function has however evolved according to the conditions in the living cells and may not be perfect in specific biotechnological applications. In this chapter, we review the current use of glycoside hydrolases (GHs) in flavonoid extractions and conversions along with efforts to develop GHs

(especially β-glucosidase and endo-glucanase) for deglycosylation and glycosylation of these polyphenolic compounds.

2. Structural overview of Flavonoids and different Flavonoid glycosides

The core structure of a flavonoid is 2-phenylbenzopyranone, also known as 2-phenyl-1, 4-benzopyrone (Figure 1), in which the three carbon bridge between phenyl groups is cyclised with oxygen (Corradini et al. 2011). Flavonoids are divided into flavones, isoflavones, flavonols, flavanones, flavan-3-ols and anthocyanidins based on their degree of unsaturation and oxidation of the three-carbon segment (Hughes et al. 2001) (Table 1). They are generally found as glycosidic conjugates with sugar residues and sometimes they can also exist as free aglycones (Stobiecki et al. 1999). For example quercetin, exists mostly in the form of glycosides (Figure 1). The addition of the glycoside conjugates or glycosylation makes the flavonoid less reactive and more polar, leading to higher water solubility. Hence, this is the most important modification occurred in plants to protect and store the flavonoids in the cell vacuole (Cuyckens et al. 2003). The development of flavonoid-O-glycosides includes one or more of the aglycone hydroxyl groups bound to a sugar with formation of an O-C acid-labile acetal bond. The glycosylation does not occur in each hydroxyl groups but in certain favoured positions: 3-, and 7-hydroxyls are common glycosylation sites but glycosylations are also reported at 5- hydroxyls in anthocyanidins and 4′- hydroxyls in the flavonol quercetin (Cuyckens et al. 2003, Iwashina 2000, Robards et al. 1997). The most encountered sugar is glucose, followed by galactose, arabinose, rhamnose and xylose, while glucuronic and galacturonic acids are quite rare. Further, some disaccharides are also found in conjugation with flavonoids, like rutinose (6-O-L-rhamnosyl-D-glucose) and neohesperidose (2-O-L-rhamnosyl-D-glucose) (Robards et al. 1997).

3. Glycoside hydrolases as extraction aids

By-products from agriculture, food and forest industry have the potential to become a major source of flavonoids. Isolation of the polyphenolic compounds from the plant sources is

usually done by using different extraction methods (Figure 2). In processing of renewable resources such as agricultural by-products or bark, enzymatic hydrolysis can be coupled with the extraction process, and GHs (sometimes also termed glycosidases) are commonly used for these purposes. These enzymes are generally easy to handle, as they do not require co-factors and they can be used at an early stage on the readily available material found in the forest and agricultural sectors (Turner et al. 2007). GHs are hydrolases responsible for the transfer of glycosyl moieties from a donor sugar to an acceptor, and have either an inverting or retaining (Figure 3) reaction mechanism, and in hydrolysis the acceptor is water (Ly and Withers 1999). The hydrolysed glycosidic bond can be located between two or more carbohydrates (*e.g.* polysaccharides) but also between a carbohydrate and a non-carbohydrate moiety (*e.g.* glycosylated antioxidants). In these types of applications, enzymes can (dependent on their specificity) thus be used both in pretreatment of the raw materials – acting on the polysaccharide fibres to simplify release of the secondary metabolites (the antioxidants) in the following extraction (Figure 2), but also to change the glycosylation pattern (described in more detail in section 4) on the polyphenolic products. Pre-treatment with different types of polysaccharide-degrading glycoside hydrolases [cellulases, hemicellulases (*e.g.* xylanases and mannanases), and pectinases] before the extraction have for example been reported to promote release of the desired secondary metabolite flavonoids from matrices of different sources containing complex polysaccharides. (Fu et al. 2008, Kapasakalidis et al. 2009, Landbo and Meyer 2001, Lin et al. 2009, Maier et al. 2008, Zheng et al. 2009). Sources investigated include fruits and berries e.g. apples (Zheng et al. 2009), black currants (Landbo and Meyer 2001) and grapes (Maier et al. 2008) but also agricultural products such as pigeon peas (Fu et al. 2008) or products from forestry, such as pine (Lin et al. 2009).

3.1 Development of thermostability - a means to improve GHs as extraction aids

Thermostable GHs have been well-documented for use in low value, high volume applications, such as starch degradation and the conversion of lignocellulosics (Turner et al. 2007) but they are still relatively rarely used in extractions/conversions of glycosylated

flavonoids. Extractions from biomass often benefit from high temperature processing, as this aids in loosening recalcitrant polysaccharide fibre structures. A step in this direction is also taken in flavonoid extractions, in which novel technologies striving to increase the environmental performance have been used that replace traditionally used extraction solvents (e.g. methanol and where deglycosylation is made by acid) with pressurized hot water where deglycosylation is made in an enzymatic step (Turner et al, 2006, Lindahl et al 2010). The high temperature extraction method, puts in a need of a thermostable enzyme, which in this case was obtained from a thermophilic microorganism (*Thermotoga neapolitana*), but which also can be developed from enzymes originally active at ambient temperatures by mutagenesis. In the latter case, both rational and random methods have been utilized, but due to relatively straight forward screening possibilities (often relying on incubations and activity assays at the desired temperature) different random strategies are frequently utilized (Figure 4). Successful combinatorial designs for enhanced (thermal) stability development have for instance been reviewed by Bommarius et al (2006).

4. Glycoside hydrolases in flavonoid conversions

Use of GHs as specific catalysts to modify the substituents on the target product is currently also gaining attention. Taking advantage of the possibilities to utilize retaining enzymes for both synthesis and hydrolysis, GHs can be used to either remove glycoside substituents (by hydrolysis using water as acceptor) or to add substituents (using in this case flavonoid acceptor molecules) (Figure 3). Hydrolases express catalytic activity also in media with low water content such as organic solvents (resulting in less competition with water as acceptor molecule), and may under these conditions catalyse new reactions (Klibanov 2001). GHs however work rather poorly in organic media (compared to other hydrolytic enzymes *e.g.* lipases) due to the requirement of higher thermodynamic water activity (Ljunger et al. 1994). The reasons for this are largely unknown, but indicate that water molecules have a role in interactions between substrate and enzyme. Use of thermostable GHs, when organic media are used, may again be advantageous as these enzymes are often resistant to denaturation by organic solvents, especially when run below their temperature optima for activity.

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Enzymatic hydrolysis of flavonoid glycosides, is dependent on the aglycone moiety, type of sugar and linkage, and is *e.g.* used to obtain uniform flavonoid molecules with often higher antioxidizing power than their glycosylated counterparts (Turner et al. 2006, Lindahl et al, 2010, Ekman et al, unpublished). On the other hand, glycosylation of flavonoids is one of the predominant approaches by which the biological activity of these natural compounds is regulated in living organisms (Yang et al. 2007), and will also increase water-solubility of the molecule. Many well-designed chemical glycosylation methods are available but due to limitation of acceptor it is not possible to obtain regioselective glycosylation by using those methods (Davis 2000, Kong 2003). The delicate selectivity of biocatalysts can instead be used for this purpose and, as stated above, GHs provide versatile tools for both glycosylation and de-glycosylation. Below, a few examples of GHs used (i) for hydrolysis of glycosidic groups and developed to improve deglycosylation of flavonoids and (ii) for synthesis (introducing new glycosidic groups) and developed to increase glycosylation on flavonoid backbones are given. For hydrolysis, the examples focus on β-glucosidases, while for the synthesis the examples shown mainly concerns endo-glucanases, but also mention use of α-amylase.

4.1 Deglycosylation of flavonoids using β-glucosidases

Glycosylated flavonoids are the favoured forms for uptake in the human intestine, but are in the body converted to the aglycone and free carbohydrates in hydrolysis reactions. The hydrolysis reactions are mainly catalyzed by β-glucosidases (Walle 2004). The β-glucosidases are also helpful catalysts in analysis of flavonoid content in food, and act by releasing the non-reducing terminal glucosyl-residue leaving a uniform aglycone that is easier to quantify. The β-glucosidases (EC 3.2.1.21) are widely distributed in nature and found in all domains of living organisms, and are classified under six GH-families: GH1, 3, 5, 9, 30 and 116, of which GH1, 5 and 30 display related $(\beta/\alpha)_8$ barrel-structures and are classified as GH-clanA (Ketudat Cairns and Esen 2010). Five of the families host enzymes with retaining reaction mechanism (only GH9 holds inverting enzymes), but examples of β-glucosidases used for deglucosylation of flavonoids mainly include enzymes from GH1 and 3.

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From GH3 a few examples of enzymes used to deglycosylate flavonoids are published [including thermostable enzymes from *T. neapolitana* (termed *Tn*Bgl3B) and *Dictyoglomus turgidum* (Turner et al. 2006, Kim et al. 2011)], showing that glycosidic groups at position 7 and 4′ on the flavonoid backbone (Figure 1) were readily hydrolysable.

From GH1 which includes the largest number of characterized β-glucosidases, examples include enzymes from different domains of life, such as another enzyme from the prokaryotic thermophile *T. neapolitana* (*Tn*Bgl1A) (Turner et al. 2006), but also eukaryotic enzymes of fungal as well as human origin. *Tn*Bgl1A is a GH1 enzyme, which (like the GH3 candidates) efficiently hydrolyses glucosylations at the 4′- and 7-positions but in this case hydrolysis of glucosides at the 3-position was also recognizable, although less efficient (Lindahl et al. 2010). The two intracellular GH1 β-glucosidases from the fungus *Penicillium decumbens* named G_I and G_{II} also displayed low activity towards the 3-glucoside, and were most active towards flavonoids glycosylated at the 7-position (Mamma et al. 2004). The human β-glucosidase (hCBG) also preferred deglycosylation at the 7-position, but in this case glucosides located at the 3-position were not hydrolysed (Berrin et al. 2003). Comparison of the 3D-structures (Figure 5) showed that this difference between *Tn*Bgl1A and hCBG can be attributed to the wider opening of the active site cleft in *Tn*Bgl1A, making it possible for the 3-glucoside to fit (Khan et al. 2011).

4.1.1 Increased flavonoid hydrolysis in GH1 by structure-based site-directed mutagenesis.

The catalytic domain regions in GH1 are well conserved but the enzymes in the family have varying substrate specificities, with some enzymes very specific for only one sugar (*e.g.* true cellobiases) or one aglycone (*i.e* aryl- β-glucosidases) while others have a broad range of specificities for the glycones, the aglycones or both and are broad substrate specificity enzymes (*e.g.* using cellulose, and β-glucan as well as flavonoids as substrates) (Bhatia et al. 2002). These differences make enzymes from GH1 interesting models for studies of the relationship between structure and activity (Chuenchor et al. 2008).

Most genetic engineering studies done on the GH1 enzymes in relation to flavonoid hydrolysis have thus far focused on identifying residues of importance for activity, and mainly involve *Tn*Bgl1A (Khan et al. 2011) and the human cytosolic β-glucosidase hCBG

(Berrin et al. 2003), while a multitude of GH1 enzymes have been studied and mutated for other purposes. The studies on *Tn*Bgl1A and hCBG are site-directed mutagenesis studies, focusing on residues identified by analysis of enzymes with known 3D-structure. In our laboratory, the aim was to also improve the hydrolysis of quercetin-3-glucosides using *Tn*Bgl1A as model enzyme. To start this work, bioinformatic analysis of *Tn*Bgl1A was made; comparing this broad specificity enzyme with GH1 enzymes active on other bulky phenol-containing substrates (*e.g.* isoflavonoids and the alkaloids strictosidine and raucaffricine) and oligosaccharide specific enzymes. The analysis was made to identify differences between specificity groups in regions around the +1 and +2 sugar binding subsites in GH1 enzymes. To locate these sub-sites, 3D-structure determined enzymes were included in the analysis and the structure of *Tn*Bgl1A was determined (Khan et al. 2011, Kulkarni et al. unpublished). From the comparison, non-conserved amino acid residues located in β-strand 5 (spanning the +1 and +2 subsites) were mutated to residues occurring in the enzymes identified to use polyphenolic substrates. Different flavonoid glucosides like quercetin-3-glucoside, quercetin-3,4´-diglucoside, and quercetin-4´-glucoside were also docked in the active site *Tn*Bgl1A in order to identify putative interactions of the amino acid residues chosen for mutagenesis. For example, the GH1 sequences showed variability at positions 219, 221, and 222 (*Tn*Bgl1A numbering), and the mutations F219L, N221S, G222Q, and G222M were made on the basis of residues found in enzymes hydrolysing the bulky phenol-containing substrates. The site-directed mutagenesis methodology used was a ligation independent whole plasmid amplification methodology (Figure 4) utilizing a methylated template that was selectively degraded by a methylation specific restriction enzyme after PCR-amplification. After introducing the mutations, it was revealed that mutant N221S led to a significant increase in conversion of quercetin-3-glucosides to quercetin, while no effect was observed for F219L, and a limited increase was seen after the G222-mutations. The effect of the N221S-mutation was suggested to occur via a loss in backbone carbonyl interactions that resulted in an increased flexibility of the parallel β-sheets, which could be a reason for the observed increase in catalytic efficiency towards quercetin-3-glucosides (Khan et al. 2011).

Mutation of a neighboring residue in the human enzyme (hCBG, F225S) resulted in almost complete loss of activity of the enzyme (Berrin et al. 2003, Tribolo et al. 2007). To elucidate the role of the corresponding residue (N220 in *Tn*Bgl1A), this was also selected for

mutagenesis and was mutated to S as well as to F (which was originally present in the human enzyme). In case of *Tn*Bgl1A, the N220S mutation increased the catalytic efficiency towards quercetin-3-glucosides compared to the wild-type enzyme (a result of a combined drop in K_M value together with 3-fold increase in the turnover number). Moreover, replacement of the hydrophilic amino acid residue N220 by the aromatic hydrophobic residue F resulted in drastic drop in the hydrolysis of both flavonoid glycosides as well as smaller model substrates like *para*-nitrophenyl-β-D-glucopyranoside (Kulkarni et al. unpublished). A similar effect was also seen at position 221. The mutation N221F, which introduced small local changes in the range of 0.4-0.7Å, led to loss of catalytic activity compared to the wild type. This confirmed that also N220 plays a role in hydrolysis, but that the interactions vary between specific GH1 enzymes and that the neighbouring residues N220 and N221 in *Tn*Bgl1A may display a similar rearrangement upon a single mutation of the respective residue. The corresponding N245 (at the +2 sugar binding site) in the homologous rice β-glucosidase Os3BGlu7 was mutated to M, and resulted in 6.5-fold loss of catalytic efficiency towards laminaribiose and 17-30 fold loss of catalytic efficiency for cellooligosaccharides with degree of polymerization >2. On the other hand, the corresponding mutation M251N in Os3BGlu6 led to 15-fold increase in the catalytic efficiency for laminaribiose and 9-24 fold increase in catalytic efficiency for cellooligosaccharides with degree of polymerization >2 (Sansenya et al. 2012). These observations show this position to be important for substrate interactions in GH1 enzymes. Presence of a bulkier hydrophobic group in the local area around the amino acids 220 and 221 in *Tn*Bgl1A was not favoured neither for the hydrolysis of long chain cello-oligosaccharides nor for the bulkier flavonoid glucosides.

4.2 Glycosylation of flavonoids using cellulase and amylase

Glycosylation of flavonoids is one of the predominant approaches by which the biological activity of these natural compounds is regulated in living organisms (Yang et al. 2007). Many well-designed chemical glycosylation methods are available but due to limitation of acceptor it is not possible to obtain regioselective glycosylation by using those methods (Davis 2000, Kong 2003). However, to overcome this problem the delicate selectivity of a biocatalyst can be used and glycosyltransferases and GHs can assist as useful tools for

synthesis of defined glycosylated flavonoids (Hancock et al. 2006). In terms of substrate specificity a glycosyltransferase is usually strict and requires a complex sugar nucleotide as the donor for the catalysis reaction (Wang and Huang 2009). On the other hand, GHs can also catalyse transglycosylation reactions. If the water molecule in the hydrolysis reaction is replaced by another acceptor molecule (*e.g.* a sugar molecule or a flavonoid) the double displacement mechanism of retaining GHs will result in transfer of the covalently bound glycosyl group from the donor substrate to the acceptor molecule. Transglycosylation is kinetically controlled and during the reaction it is assumed that there is competition between the nucleophilic water and the acceptor substrate at the glycosyl-enzyme intermediate (Nakatani 2001, Park et al. 2005, Hancock et al. 2006).

Cellulases are GHs that catalyze the hydrolysis of the 1,4-β-D-glycosidic linkages in cellulose (and other related substrates *e.g.* lichenan and cereal β-glucans). The name cellulase, can refer to different types of enzymes acting on cellulose, but is most commonly used for endoglucanases (endo-1,4-β-D-glucanases, EC 3.2.1.4), which can be found in at least 17 different GH families (see: http://www.cazy.org/Glycoside-Hydrolases.html).

For the synthesis of flavonoid glycosides, a cellulase from *Aspergillus niger* has been used to add a fucose sugar moiety to a cathecin backbone (Figure 6). Cathecin is an antioxidant that is for instance found in bark, a renewable resource of significant volume. During the reaction *para*-nitrophenyl-β-D-fucopyranoside was acting as a donor while catechin monohydrate acted as acceptor resulting in a 26% yield of catechin-β-D-fucopyranoside (Gao et al. 2000).

In another study, an α-amylase from *Trichoderma viride* was reported to show transglycosylation activity towards both catechin and epigallocatechin gallate using dextrin (α-1,4-linked oligosaccharides resulting from starch degradation) as donor substrate (Noguchi et al. 2008). α-Amylases (EC 3.2.1.1) are enzymes classified under the large glycoside hydrolase family 13, and their main activity is hydrolysis of α-1,4-bonds of starch and glycogen.

4.2.1 Glycosynthases: application of a nucleophile mutated cellulase in flavonoid glycosylation

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The application of GHs in synthesis of carbohydrate or non-carbohydrate substrates has two major limitations: mainly low transglycosylation yield and secondary hydrolysis of the product. In order to overcome this problem the invention of glycosynthases was a major development (Ly and Withers 1999). Glycosynthases is a class of unique GH-mutants (mutated in the catalytic nucleophile) that can promote glycosidic bond formation in the presence of an activated glycosyl donor and there is no further hydrolysis of the newly formed glycosidic linkage (Ly and Withers 1999, Wang and Huang 2009). Drawbacks with this methodology are however the necessity to produce activated glycosyl-donors, with sometimes limited stability. Glycosynthases have been studied for the synthesis of complex glycoconjugates and in a recent report it was shown that a glycosynthase mutant E197S of the *Humicola insolens* cellulase Cel7B was able to glycosylate flavonoids (Yang et al. 2007). Cel7B is a GH7 retaining endoglucanase and the predominant activity of the wild-type enzyme is hydrolysis the α-1, 4-linked glucosidic bond in cellulose, which in the mutant is circumvented by replacing the catalytic nucleophile (E197) with S. In this study a high-throughput MS-based method was used to screen 80 different acceptors and more than 20 glycosyl donors for substrate activity. In this screening process a subclass of flavonoids was identified as an acting acceptor for Cel7B-E197S during transglycosylation reaction (Figure 7). According to kinetic studies the rate of glycosylation by Cel7B-E197S was comparable to glycosyltransferases which is very promising for the synthesis of glycosylated flavonoids (Yang et al. 2007).

5. Conclusions

To increase extraction yields and allow modification of antioxidants - new biocatalysts have the potential to adapt the compounds to different applications. Biocatalysis also has the potential of being a sustainable technology – something that is given increased attention today with current concerns about climate change and scarcity of fossil resources. In this chapter, we have reviewed emerging attempts to use and develop GHs as biocatalysts for modification of polyphenlic antioxidants classified under the flavonoid group. One aspect is the use of the GHs as extraction aids, and for this purpose an important property of the

enzyme is its stability. Concerning the flavonoid product, both the antioxidative power and stability are affected by glycosylation. Currently studies to specifically modulate this (both remove and add glycosidic groups) have started. Although work in this field is just emerging, positive results have been published, where the action of GHs allow modification at uncommon positions or with new sugar moieties. Further development in this field is expected, allowing increased development and use of novel ingredients and bio-active compounds from poylphenolics.

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Figures and tables.

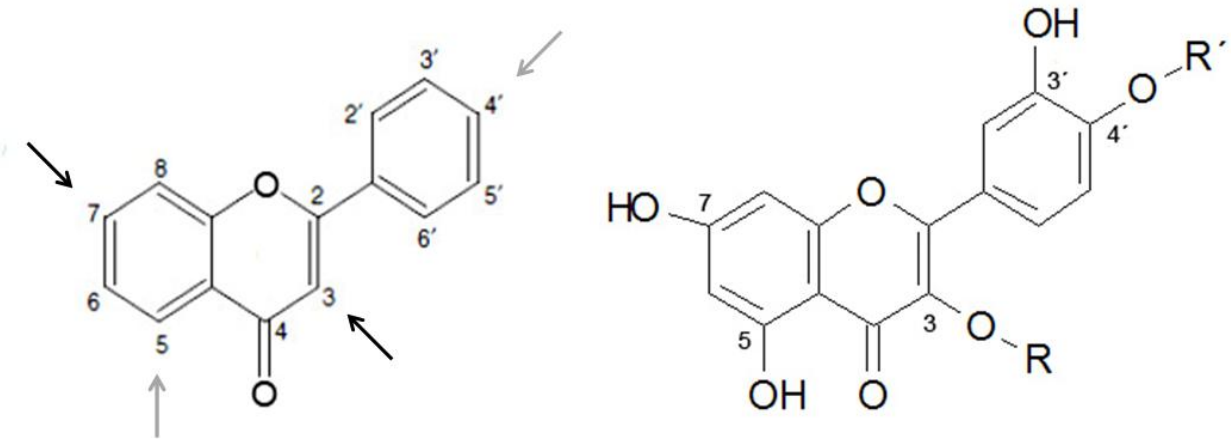

Figure 1. General structure of the flavonoid backbone (left), shown with backbone numbering. The most common hydroxyl-positions for glycosylation (3 and 7) are indicated with black arrows, and the 5 and 4′ hydroxyls that are sometimes glycosylated are indicated with grey arrows. A quercetin molecule (right) is also shown with the substituents present in this type of flavonoid. R and R′ are hydrogens in the deglycosylated form. In glycosylated forms isolated from onion, R and/or R′ represent glucosyl groups.

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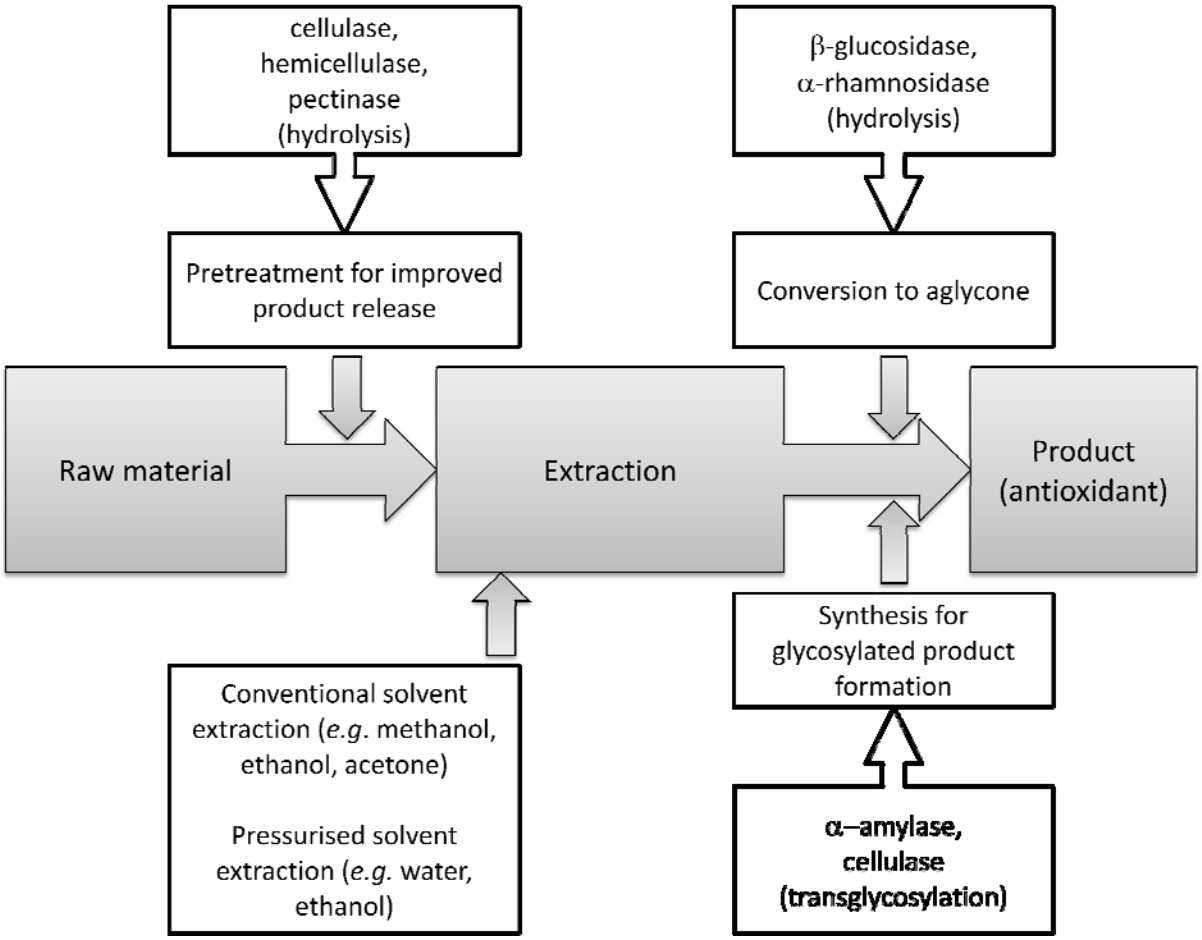

Figure 2. Schematic overview of an extraction process to obtain antioxidants with desired glycosylation patterns. The possibilities to use glycoside hydrolases in pretreatment and in conversions to modify the glycosylation are indicated.

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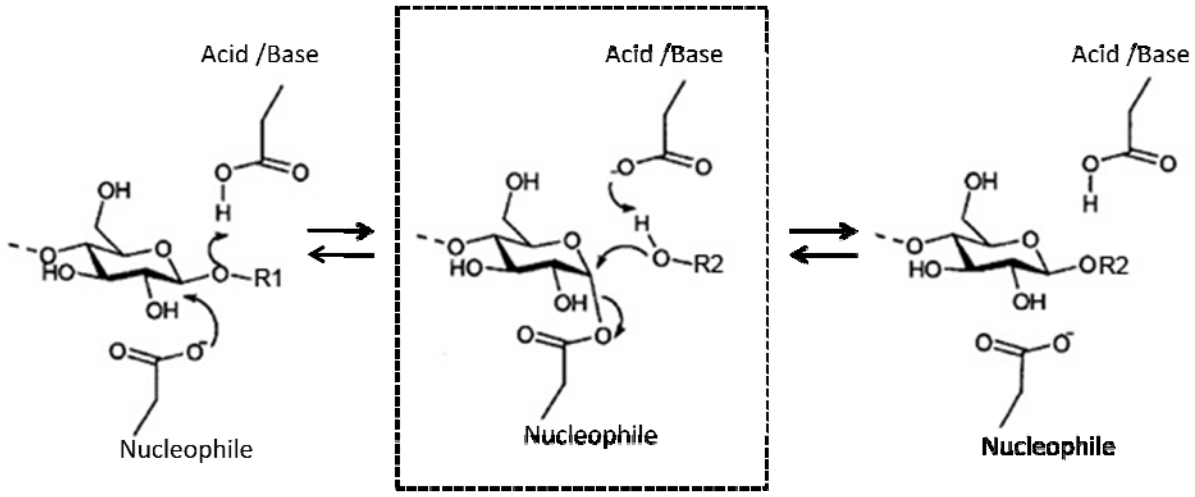


Figure 3. The double displacement mechanism of retaining glycoside hydrolases. HO-R1 represents the group cleaved from the donor substrate, while HO-R2 represents the acceptor molecule. The covalent glycosyl-enzyme intermediate is boxed. For hydrolysis reactions HO-R2 is a water molecule and R2=H.

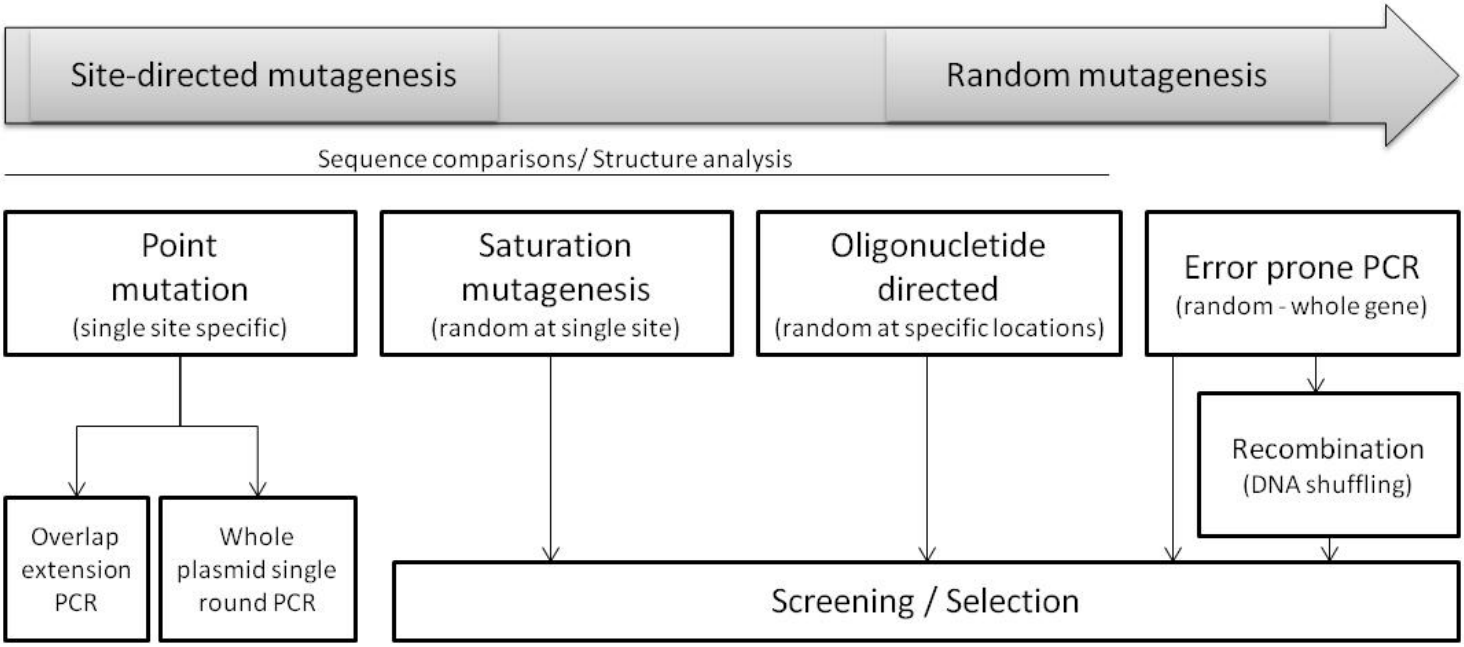


Figure 4. Strategies for mutagenesis of enzymes by rational and random methodologies.

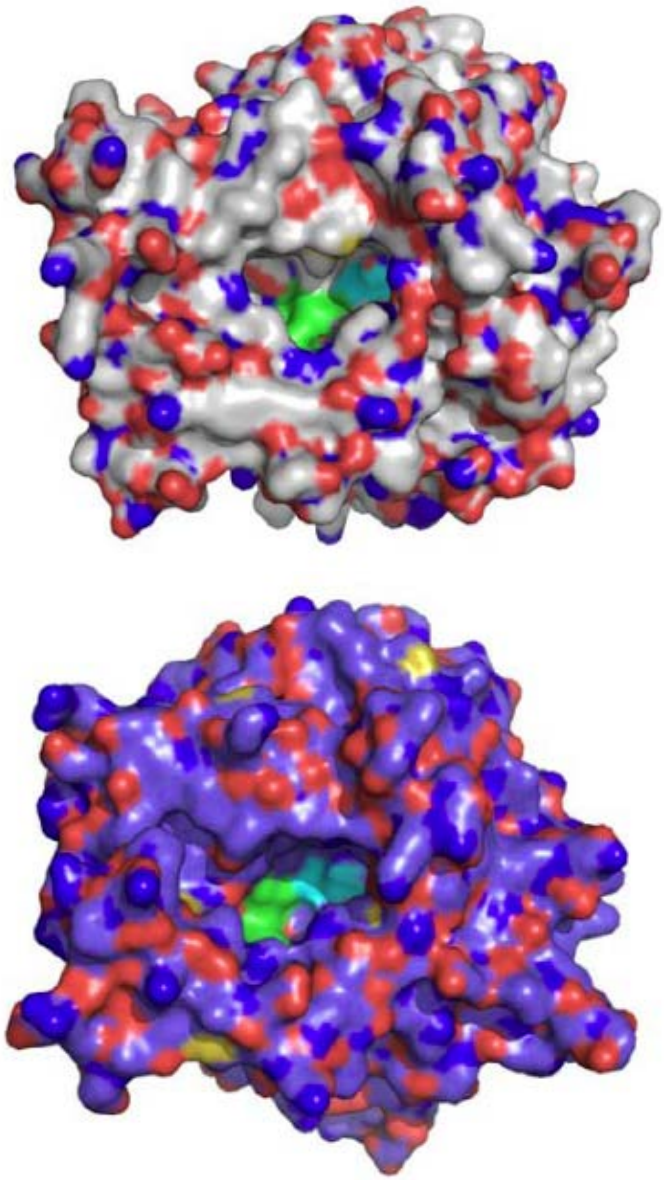


Figure 5. Spacefill representations of the human cytosolic β-glucosidase (top) and the thermostable β-glucosidase from *Thermotoga neapolitana* (bottom) facing the active site. The the wider active site opening of the flavonoid 3-glucoside hydrolyzing *T. neapolitana* enzyme is clearly visible.

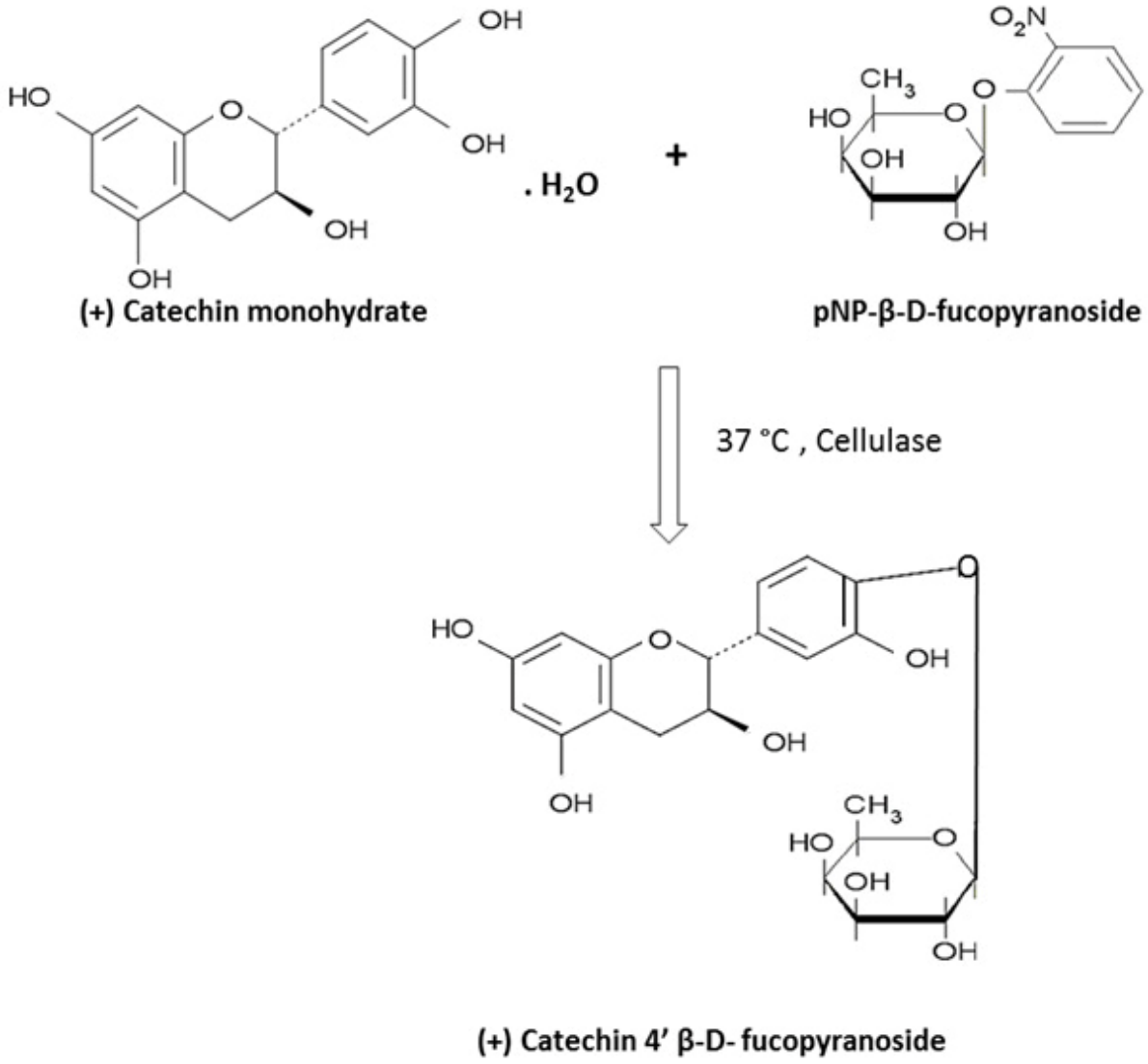
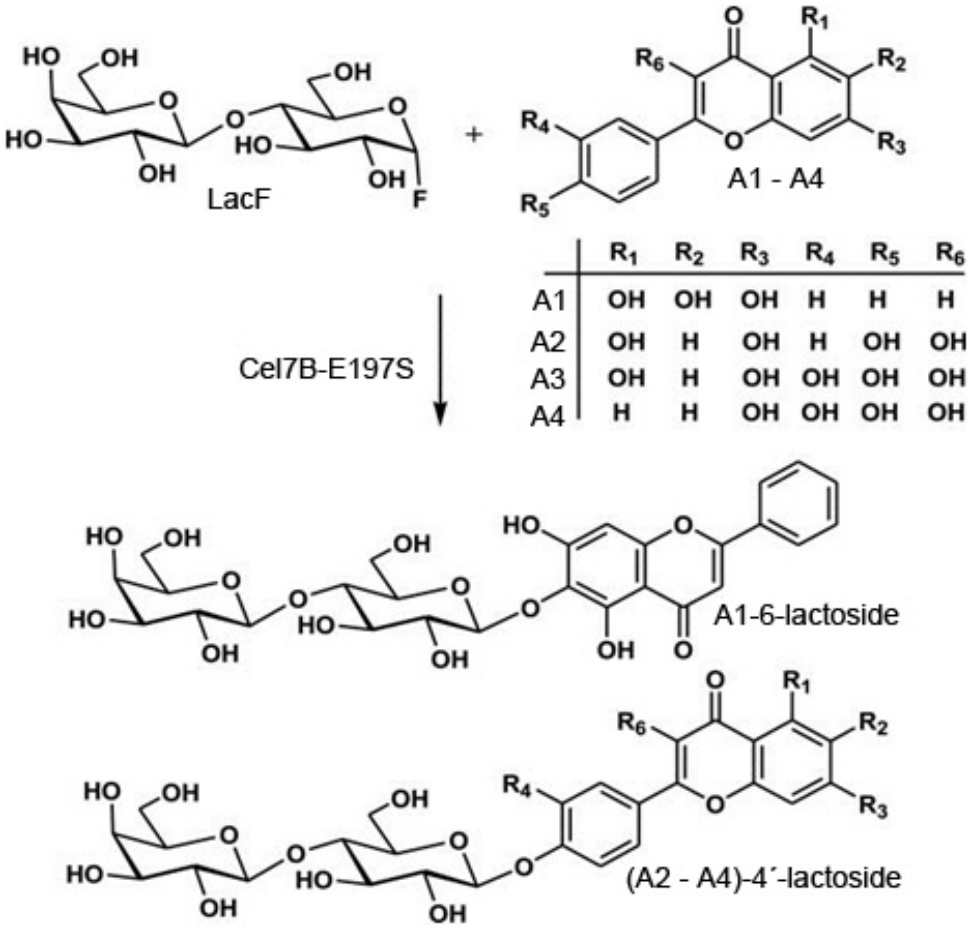


Figure 6. Glycosylation of (+) catechin monohydrate. Synthesis of (+) catechin-β-D-fucopyranoside in the presence of cellulase at 37 °C while using pNP-β-D-fucopyranoside as donor (Modified from Gao et al. 2000).



	R_1	R_2	R_3	R_4	R_5	R_6
A1	OH	OH	OH	H	H	H
A2	OH	H	OH	H	OH	OH
A3	OH	H	OH	OH	OH	OH
A4	H	H	OH	OH	OH	OH

Figure 7. Glycosylation of flavonoids by Cel7B-E197S glycosynthase (adapted from Yang et al. 2007, Wang and Huang 2009). The nucleophile (E197) of the GH7 cellulase from *Humicola insolens* is mutated to S. The lactosyl fluoride (LacF) was the disaccharide donor and transfer of lactosyl from LacF to a number of flavonoids was catalysed by the Cel7B-E197S mutant in yields of 72–95%. The synthesis was stereoselective (only β-glycosides) and regioselective for the glycosylation using the hydroxy group at 4' (as in A2-A4) while in absence of the hydroxyl-group at this position (as in A1) the 6-position was glycosylated instead. A1=baicalein, A2=luteolin, A3=quercetin, A4=fisetin.

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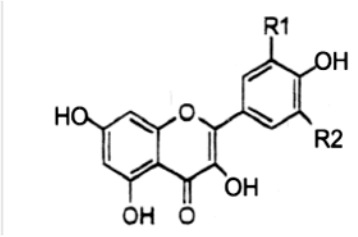
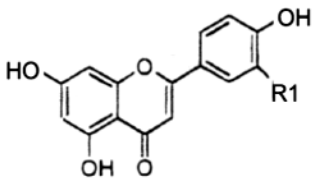
Table 1: Chemical structures of subclasses of flavonoids

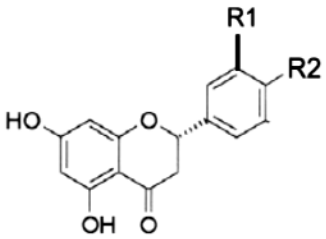
Flavonol	**R1**	**R2**
Quercetin	OH	H
Kaempferol	H	H
Myricetin	OH	OH
Isorhamnetin	OMe	H



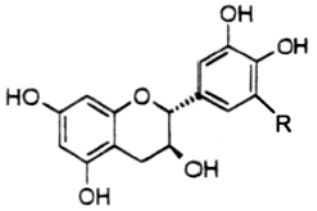
Flavone	**R1**
Apigenin	H
Luteolin	OH



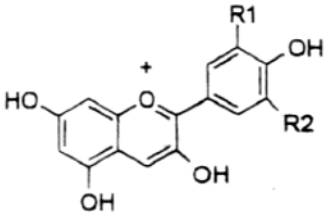
Flavanones	**R1**	**R2**
Eriodictyol	OH	OH
Hesperetin	OH	OMe
Naringenin	H	OH



Flavan-3-ols	**R1**
(+) Catechin	H



Anthocyanidin	**R1**	**R2**
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OMe	OMe



Petunidin	OMe	OH