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REVIEW

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Enzymatic synthesis of oligosaccharides by two glycosyl hydrolases of *Sulfolobus solfataricus*

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Abstract The importance of carbohydrates in a variety of biological functions is the reason that interest has recently increased in these compounds as possible components of therapeutic agents. Thus, the need for a technique allowing the easy synthesis of carbohydrates and glucoconjugates is an emerging challenge for chemists and biologists involved in this field. At present, enzymatic synthesis has resulted in the most promising approach for the production of complex oligosaccharides. In this respect, the enzymological characteristics of the catalysts, in term of regioselectivity, substrate specificity, and operational stability, are of fundamental importance to improve the yields of the process and to widen the repertoire of the available products. Here, two methods of oligosaccharide synthesis performed by a glycosynthase and by an α-xylosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus* are briefly reviewed. The approaches used and the biodiversity of the catalysts together are key features for their possible utilization in the synthesis of oligosaccharides.

Key words Glycosyl hydrolase · Thermostable enzymes · Archaea · Oligosaccharides · Glycosynthase · α-Xylosidase

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Introduction

Carbohydrates can serve as structural components and energy source of a cell. In addition, these biomolecules are involved in a variety of biological functions such as cell adhesion, differentiation, development, and regulation (Varki 1993). For these reasons, great interest arose in carbohydrate-based compounds and the development of techniques for the analysis and synthesis of oligosaccharides. However, despite the advances achieved with both regioselectivity and stereoselectivity (Toshima and Tatsuta 1993), the synthesis of complex carbohydrates for large-scale production still cannot be easily performed by the classical chemical procedures, which require long protection–deprotection steps and give low final yields.

Thus, the enzyme-catalyzed synthesis of oligosaccharides represents an interesting alternative to the classical chemical methods, allowing the control of both the regioselectivity and the stereochemistry of bond formation. The enzymatic approach involves mainly two class of enzymes: glycosyl transferases and glycosyl hydrolases. Glycosyl transferases have been used for oligosaccharide synthesis (Gijsen et al. 1996), but their low availability and the high cost of their substrates have limited their exploitation. Glycosyl hydrolases, which occur in all living organisms and allow the use of relatively inexpensive substrates, represent an alternative choice.

The two main approaches for the glycosidase-catalyzed synthesis of oligosaccharides are reverse hydrolysis and transglycosylation, in which the glycosyl–enzyme intermediate is transferred to an acceptor other than water. In both methodologies, which are described in excellent reviews (Sinnot 1990; Legler 1993; Watt et al. 1997; Zechel and Withers 2000), the product of the reaction is still a substrate for the enzyme and subject to hydrolysis. Therefore, the overall stereochemistry and final yields of the reaction depend on the relative activity of the enzyme on the substrate and the different products formed. Reaction conditions thus must be carefully controlled and reactions must be closely monitored for best performance.

The rise of interest in thermophilic enzymes, or thermozymes, as potential biotechnological tools is historically based on their intrinsic resistance to the harsh conditions used in several bioprocesses, such as high temperatures, high concentrations of substrate, and organic solvents. However, recent interest in thermozymes is also motivated by their biodiversity, which has revealed novel enzymatic activities (Hough and Danson 1999). Typical examples are thermophilic glycosyl hydrolases, which show peculiar enzymological properties, such as unique substrate specificities or reduced substrate/product inhibition (Trincone et al. 1991), and allow the synthesis of new products that are not produced by their mesophilic counterparts (Fischer et al. 1996).

This review highlights the most recent work on the use of thermophilic glycosyl hydrolases in carbohydrate synthesis. Two examples of glycosyl hydrolases from the hyperthermophilic archaeon *Sulfolobus solfataricus* are described: the preparation and development of thermophilic glycosynthases and the use of a novel α-xylosidase in transxylosidation reactions.

Reaction mechanisms and the catalytic machinery of glycosyl hydrolases

Glycosyl hydrolases follow two distinct mechanisms, which are termed *retaining* or *inverting*, depending on whether the enzymatic cleavage of the glycosidic bond liberates a sugar hemiacetal with the same or the opposite anomeric configuration compared with the glycosidic substrate, respectively. The two mechanisms described in Fig. 1A and Fig. 1B were proposed for the first time by Koshland (1953) and are still largely accepted. *Inverting* enzymes (Fig. 1A) use a direct displacement mechanism in which the two carboxylic acid residues in the active site are positioned so that one acts as a general acid and the other provides general base catalytic assistance to the attack of water. The catalytic mechanism of *retaining* enzymes (Fig. 1B) proceeds via a two-step double-displacement mechanism involving the formation of a covalent glycosyl intermediate. The two carboxylic residues in the active site play different roles in this case, one acting as a general acid or base catalyst and the other as the nucleophile of the reaction.

In the first step, commonly termed enzyme glycosylation, the concerted action of the general acid and of the nucleophile residues leads to glycosidic oxygen protonation and the departure of the aglycon group with the formation of a glycosyl–ester intermediate. In the second step (enzyme deglycosylation), a water molecule partially deprotonated by the conjugate base of the catalytic acid attacks the anomeric carbon and cleaves the glycosyl–ester intermediate, leading to the overall retention of the anomeric configuration of the substrate. When acceptors other than water intercept the reactive glycosyl–enzyme intermediate, retaining enzymes work in transglycosylation mode. This property makes the retaining glycosyl hydrolases interesting tools for the synthesis of carbohydrates.

Despite the differences, the two mechanisms show significant similarities: both classes of enzymes employ a pair of carboxylic acids at the active site and both mechanisms operate via transition states with substantial oxocarbenium ion character. However, strong evidence supports the hypothesis that a covalent intermediate is formed in retainers (Sinnot 1990; McCarter and Withers 1994). Moreover, X-ray and nuclear magnetic resonance (NMR) data on both classes of enzymes reveal that, despite the general structural similarity in the active sites of the two classes of enzymes, the distance between the catalytic residues in inverters is significantly greater than in retainers (9.5 Å versus 5.5 Å) allowing, in the former, the simultaneous binding of water and substrate to the active site (White and Rose 1997).

The reaction mechanism and the residues involved in catalysis of any glycosyl hydrolase can be identified by many techniques. Several excellent reviews of the different approaches are available (McCarter and Withers 1994; White and Rose 1997; Ly and Withers 1999). Briefly, kinetic analysis of site-specific mutants (see following) and the classification of glycosyl hydrolases into families and superfamilies on the basis of their amino acid sequence and three-dimensional (3-D) structure (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html) are commonly used to identify the active site residues and the reaction mechanism of any newly identified glycosyl hydrolase.

Recently, specific labeling with mechanism-based inhibitors such as activated 2-deoxy-2-fluoro-glycoside, in combination with mass spectrometry, amino acid sequence alignment, and crystal structure inspection, have also been shown to be successful approaches for the identification of active site residues (Zechel and Withers 2000). Among others, the use of mechanism-based inhibitors is nowadays the most powerful method for the direct identification in retainers of the carboxyl group acting as the nucleophile of the reaction, even in the absence of the amino acid sequence of the enzyme. In contrast, no reliable chemical methods for the identification of the acid/base catalyst are currently available. Hence, site-directed mutagenesis followed by kinetic analysis of the mutants remains the approach most used for the definition of the role played by the active site carboxylic groups. This approach is also the starting point for the preparation of new enzymes, termed glycosynthases, that have been recently described in the literature and which allow the synthesis of oligosaccharides at high yields (Mackenzie et al. 1998).

Reactivation of glycosyl hydrolases: glycosynthases

In retaining enzymes, replacement of the active site nucleophile with a nonnucleophilic residue completely inactivates the enzyme because the new amino acid side chain cannot form the glycosyl–enzyme intermediate. Nevertheless, the rest of the active site is intact and the small cavity created on mutation can accommodate a small anion. Under these conditions and in the presence of a substrate with good

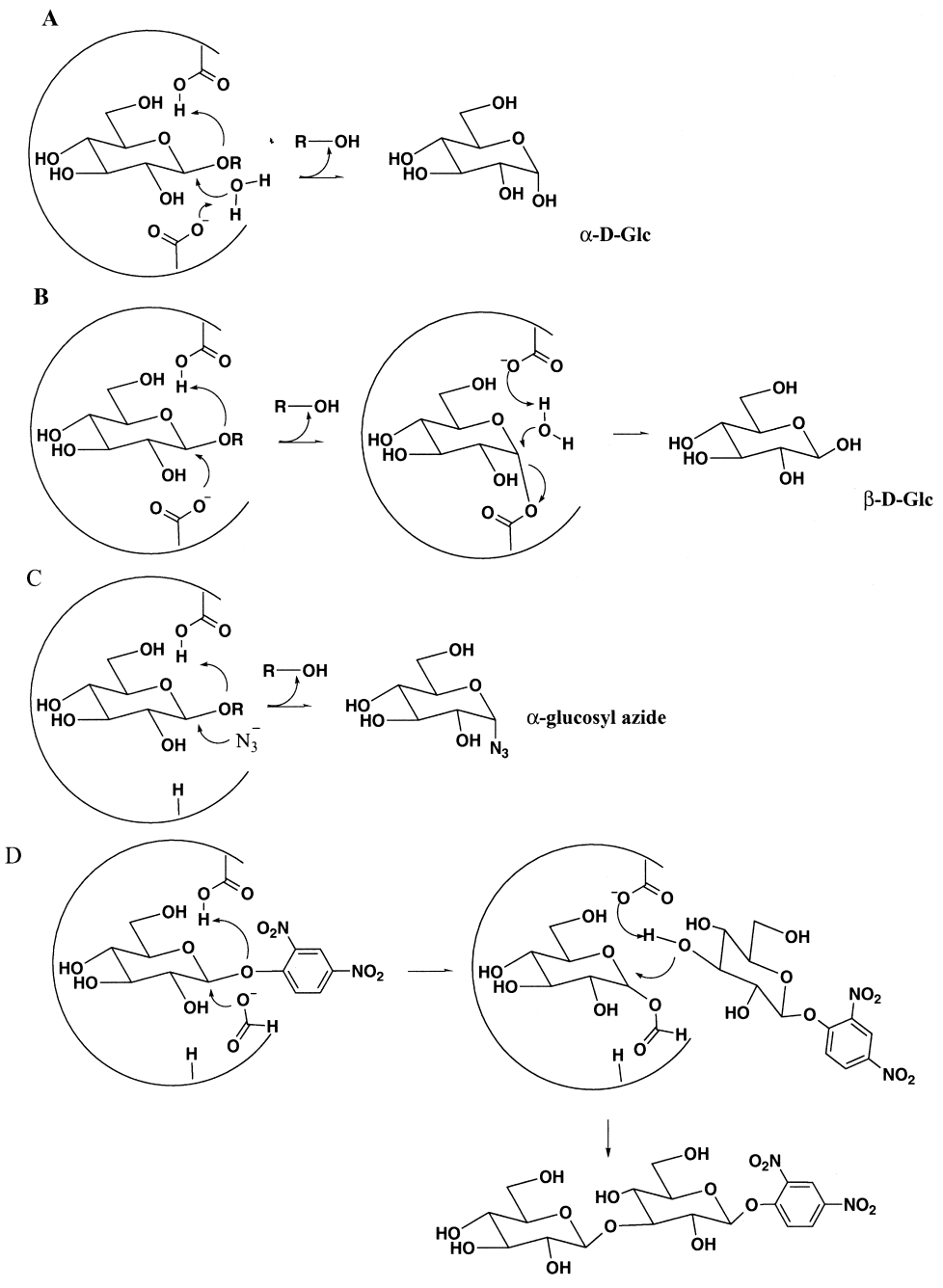

Fig. 1. Reaction mechanism of *inverting* (**A**) and *retaining* (**B**) β-glycosyl hydrolases. Proposed reaction mechanism of Ssβ-gly Glu387Gly mutant reactivated with sodium azide (**C**) and sodium formate (**D**)

leaving group ability, which assists the glycosylation step of the reaction, the activity of the mutant can be restored. This approach was developed for the first time by Steven Withers and collaborators on the β-glucosidase from *Agrobacterium faecalis* (Abg) (Wang et al. 1994). When the active site nucleophile of this *retaining* glycosyl hydrolase was changed into alanine (Glu358Ala), the activity of the mutant on 2,4-dinitrophenyl-glycosidase (2,4-DNP-Glc) was almost abolished as compared to the wild type.

However, the addition of high concentrations of sodium azide or sodium formate accelerated catalysis 10^5 fold. In the case of azide, the reaction proceeded through the direct attack of the exogenous nucleophile to the anomeric center of the substrate from the α side of the pyranose ring (Fig. 1C), leading to α-glucosyl azide as product; hence, the activity of the azide-restored mutant followed an *inverting* mechanism as compared to the wild type. With the same approach, three other enzymes, the exoglucanase/xylanase from *Cellulomonas fimi* (Cex), the 1,3-1,4-β-glucanase from *Bacillus licheniformis*, and the β-glycosidase from *Sulfolobus solfataricus* (Ssβ-gly) were reactivated and their reaction mechanism was changed from *retaining* to *inverting* (MacLeod et al. 1996; Viladot et al. 1998; Moracci et al. 1998). These results demonstrated that this methodology

Table 1. Kinetic parameters of wild-type and mutant glycosyl hydrolases reactivated with exogenous nucleophiles

Enzymes		Reaction conditions	k_{cat}/K_M (s^{-1}/mM^{-1})	Reactivation (%)
Abg (Wang et al. 1994; Zechel and Withers 2000)	Wild type	DNPG	2871	–
	E358A	DNPG	7.1×10^{-5}	–
		DNPG+2 M azide	0.289	0.010
		DNPG+4 M formate	2.727	0.095
		DNPG+5 M formate	26.933	0.938
	E358S	DNPG+2 M azide	0.160	0.006
		DNPG+5 M formate	15	0.522
Cex (MacLeod et al. 1996)	Wild type	DNPC	116.4	–
	E233A	DNPC	–	–
		DNPC+4 M azide	0.09	0.077
		DNPC+4 M formate	1.73	1.49
Bacillus β-glucanase (Viladot et al. 1998)	Wild type	DNPG4G3G	3.0×10^{6}	–
	E134A	DNPG4G3G	4.4	–
		DNPG4G3G+3.3 M azide	5×10^{1}	1.6×10^{-6}
		DNPG4G3G+4 M formate	6×10^{3}	0.002
Ssβ-gly (Moracci et al. 1998)	Wild type	DNPG	1617	–
	E387A	DNPG	–	–
		DNPG+2 M azide	8	0.49
	E387G	DNPG	–	–
		DNPG+2 M azide	611	37.8
		DNPG+2 M formate	323	19.97

DNPG, 2,4-dinitrophenyl-β-D-glucoside; DNPC, 2,4-dinitrophenyl-D-cellobioside; DNPG4G3G, 2, 4-dinitrophenyl-3-*O*-β-cellobiosyl-β-D-glucoside

could be of general applicability for β-glycosyl hydrolases, involving both *exo*- and *endo*-enzymes from diverse sources and with different functions and specificities.

The degree of reactivation obtained in the presence of sodium azide and formate as external nucleophiles varied significantly when compared to the wild type. In Table 1, the kinetic parameters for reactivated glycosyl hydrolases are reported. Kinetic constant values were obtained at the optimal conditions of pH and temperature that differed greatly among the enzymes described; however, for a simple comparison, only the values obtained on 2,4-DNP-glycoside substrates are reported. The comparison among the mutants in which the catalytic nucleophile was changed into alanine revealed that the highest restoration of activity was obtained from the Glu387Ala Ssβ-gly mutant, presumably because of the general increased resistance of the thermophilic enzyme to the high ionic strength observed at the nucleophile concentration used. This restoration was much more evident in the Ssβ-gly mutant Glu387Gly, suggesting that the increased size of the cavity created could play a relevant role. Remarkably, this mutant also showed a feature unique among reactivated glycosyl hydrolases, that of being active on 2-NP-Glc, a commercially available substrate of β-glucosidases, which shows an aglycon with poorer leaving ability (Moracci et al. 1998).

The analysis of the reaction products of modified Ssβ-gly demonstrated that the azide- and formate-restored activities worked differently at the molecular level. As already described, the isolation of α-glycosyl azide led to the consideration that an *inverting* enzyme had been generated by mutation. By contrast, the same Glu387Gly mutant in the presence of sodium formate catalyzed the formation of the 2,4-DNP-β-laminaribioside product (Fig. 1D) (Moracci et al. 1998). The β-form of the new glycosidic bond implies a two-step mechanism in which the external nucleophile assists the leaving of the phenolate group in the glycosylation step and then stabilizes the glucosyl unit until it is transferred to the acceptor. Similar conclusions were drawn for the *endo*-glucanase from *B. licheniformis* (Viladot et al. 1998), and the biomimetic role of formate has been described as an assistant nucleophile with formation of the intermediate, which has also been observed by ^{1}H[NMR].

The methodology described can be usefully applied to the synthesis of carbohydrates. In fact, these modified glycosyl hydrolases, lacking their catalytic nucleophile, can act only on activated substrates by synthesizing new glycosidic bonds but cannot hydrolyze the products formed. These compounds, in fact, show groups with poor leaving ability, which are resistant to the attack of the external nucleophiles and which accumulate in the reaction. For these reasons, these mutated enzymes were called glycosynthases; the Glu387Gly mutant of Ssβ-gly is the only thermophilic glycosynthase described so far.

Glycosynthases in oligosaccharide synthesis

Glycosynthases are novel biocatalysts that operate by the two mechanisms described in Fig. 2. In the first case, the donor substrate contains an α-bonded fluoride group that shows good leaving ability. In this case, space created by removal of the carboxylic nucleophile group in the enzyme can accommodate a substrate that mimics the glycosyl

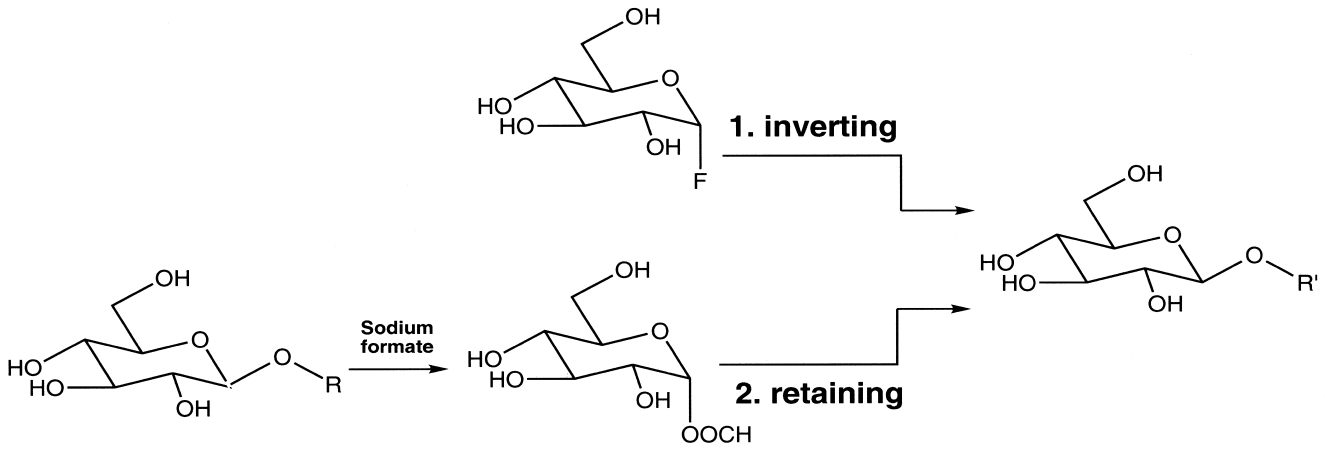

Fig. 2. Pathways followed by glycosynthases in oligosaccharide synthesis. The deglycosylation step is common to both the pathways, and the carbohydrate molecule is transferred to the acceptor (*R′*)

Fig. 3. Oligosaccharides formed by thermophilic glycosynthase

1. β-Glc-[1 -> 3]-β-Glc-O-oNp

2. β-Glc-[1 -> 6]-β-Glc-O-oNp

3. β-Glc-[1 -> 4]-β-Glc-O-oNp

4. β-Glc-[1 -> 6]
β-Glc-[1 -> 3]-β-Glc-O-oNp

5. β-Glc-[1 -> 6]
β-Glc-[1 -> 3]-β-Glc-O-oNp

6. β-Glc-[1 -> 3]-β-Glc-[1 -> 6]
β-Glc-[1 -> 3]-β-Glc-O-oNp

intermediate and which can be transferred to an acceptor with production of HF. The β-bond formed in the product led to the definition of an *inverting glycosynthase* mechanism. In the second pathway (Fig. 2), products are synthesized from activated β-donors in the presence of formate as an assistant nucleophile; this is called the retaining glycosynthase mechanism. Glycosynthases with both the described pathways have been used, but differ in the selectivity of the reaction catalyzed and in the use of different donors.

After the preliminary report, Withers and collaborators described the synthesis of different oligosaccharides by using the Abg mutant in the presence of α-F-Glc or -Gal and different aryl glycosides as donors and acceptors, respectively (Mackenzie et al. 1998). More recently, a different mutant of the same Abg enzyme (Glu358Ser) revealed improved glycosynthase activity (Mayer et al. 2000). Also, the mesophilic glycosynthase from *Bacillus licheniformis* performed the synthesis only by pathway 1 (see Fig. 2), by using as donor α-F-laminaribioside (Malet and Planas 1998). By contrast, the thermophilic Ssβ-gly glycosynthase is able to act on α-F-Glc by following pathway 1, but also on a wide range of donors of 2- and 2,4-DNP-glycosides (β-glucoside, β-fucoside, β-galactoside, and β-xyloside) through pathway 2 (Trincone et al. 2000).

Yields and selectivity are key features of these reactions: in the case of Abg glycosynthase, the yield ranged from 64% to 92%, and , with the exception of 4-NP-xyloside (β-1,3), β-1,4 glycosidic bonds were always formed. Differences in the composition of products were reported depending on the glucosyl and galactosyl fluorides used as donors. In the presence of α-F-Glc, tri- and tetrasaccharides were observed, whereas only disaccharide formation was observed from α-F-Gal, showing the inhibitory effect of the axial OH group of this substrate on sequential transferring reactions (Mackenzie et al. 1998).

Selectivity of formation versus the β-1,4 glycosidic link has also been observed with the glycosynthase enzyme from *B. licheniformis* (Malet and Planas 1998). In this case, α-laminaribiosyl fluoride is the donor and is used with different acceptors such as methylumbelliferyl mono- and disaccharides and 4-NP-galactoside and 4-NP-GlcNAc. The products formed were laminaribiosyl derivatives of the acceptors; the yield in the best case reported was 90%. It is worth noting that, in this case, the transfer directly involved a disaccharide unit. This finding demonstrates that an *endo*-glucanase can be transformed into an *endo*-glucansynthase able to transfer preformed sequences of carbohydrate units. More recently, another mutated *endo*-glucanase, from *Humicola insolens*, was used in the synthesis of natural or modified cellooligosaccharides and for the synthesis of different fluorescent oligosaccharides (Boyer et al. 1999).

The thermophilic Ssβ-gly *glycosynthase* is the only biocatalyst of this kind described so far possessing both

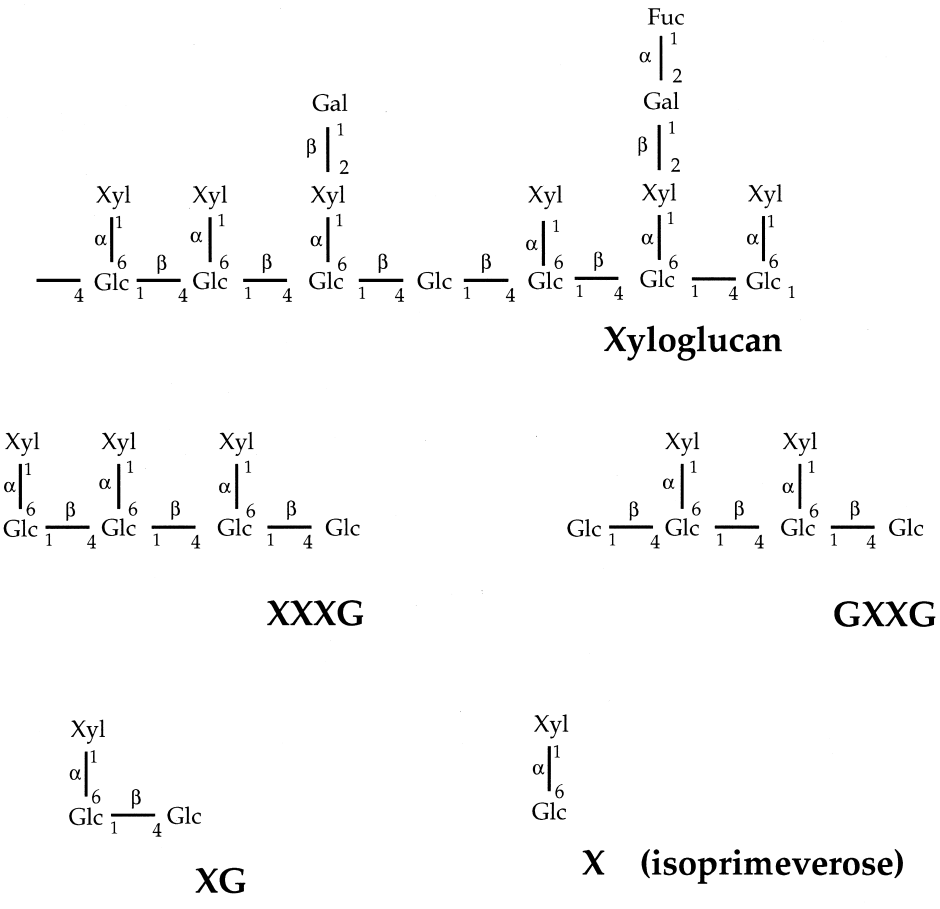

Fig. 4. Structure of xyloglucan and its building blocks. *Xyl*, *Glc*, *Gal*, and *Fuc* denote α-D-xyloside, β-D-glucoside, β-D-galactoside, and α-L-fucoside residues, respectively. *XXXG*, *GXXG*, *X*, and *XG* are the abbreviated names according to the nomenclature of Fry et al. (1993)

inverting and retaining glycosynthase activities (see Fig. 2). The enzyme can be used with DNP- and 2-NP-Glc in the presence of formate, leading to the synthesis of mainly β-1,3-disaccharides and tri- and tetrasaccharides containing both β-1,3- and β-1,6-bonds with high regioselectivity and about 85% yields (Fig. 3) (Trincone et al. 2000). The branching functionalization represents a unique characteristic of Ssβ-gly glycosynthase. However, by use of α-F-Glc as donor, this enzyme acts as an *inverting* glycosynthase, allowing the synthesis of single products with high selectivity and more than 80% yield (Fig. 3) (Trincone et al. 2000).

Interestingly, the wild-type Ssβ-gly used in transglycosylation mode never produced oligosaccharides, suggesting that these compounds were actively hydrolyzed and could not accumulate in the reaction, precluding their isolation. This idea was confirmed by the activity of the enzyme on both linear β-1,4 and branched β-1,3/β-1,6 oligosaccharides. Interestingly, this high specificity of wild-type Ssβ-gly for branched oligosaccharides could not be predicted even by detailed inspection of the 3-D structure of the enzyme (Aguilar et al. 1997). Instead, the separation of the synthetic from the hydrolytic activity obtained by the glycosynthase approach revealed this new substrate specificity of Ssβ-gly.

These studies demonstrated that it is possible, with this thermophilic glycosynthase, to widen the potentiality of these novel biocatalysts by synthesizing oligosaccharides of applicative interest. In fact, by using pathway 2 (Fig. 2), the diversity of the products obtained can be used in a wide screening for new substrates or inhibitors for glycosidases. Afterward, the specific compound of interest can be synthesized at high yields by using pathway 1 and by designing the appropriate donors and acceptors.

Preparation of xyloglucan oligosaccharides

Xyloglucan is widely distributed in plants, being the principal hemicellulose component in the primary cell wall (20% of the total cell wall) and one of the most abundant storage polysaccharides in seeds (>40% in weight in some species). This polymer is composed of a β-(1,4)-glucan backbone, with α-(1,6)-D-xylose groups linked to about 75% of the glucosyl residues (Fig. 4). Thus, the disaccharide isoprimeverose [α-D-xylopyranosyl-(1,6)-D-glucopyranose] represents the building block of xyloglucan. Additional ramifications of β-D-galactosyl-(1,2)-α-xylosyl and α-L-fucosyl-(1,2)-β-D-galactosyl-(1,2)-α-xylosyl chains are also α-(1,6) linked to the main backbone (Fig. 4). Xyloglucans from different plant tissues and species greatly vary in molecular mass and chemical composition: storage xyloglucans from seeds are fucosylated to a lesser extent if compared to the same polymer from primary cell walls (Crombie et al. 1998). Xyloglucans not only have structural functions but can also be involved in more complex

Table 2. Kinetic constants of XylS at 65°C

Substrates	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
Maltose[a,b]	17 ± 3.2	1.51 ± 0.07	0.09
Isoprimeverose[b]	28.92 ± 3.48	31.04 ± 1.54	1.07
4-NP-β-isoprimeveroside	1.72 ± 0.46	15.96 ± 1.62	9.28
4-NP-α-xyloside	17 ± 2.1	4.69 ± 0.27	0.28

[a]Kinetic values are calculated taking into account the fact that two glucose equivalents were produced for every glucosidic bond hydrolyzed
[b]Hydrolysis products were detected as glucose, and K_M values are shown as glucose equivalents

biological roles. For instance, some xyloglucan oligosaccharides can promote the elongation of stem segments (Lorences and Fry 1994) and therefore play a role in the regulation of plant growth. In plant seeds, the hydrolysis of xyloglucan occurs after germination, during the mobilization of this storage polysaccharide. For these reasons, there is clearly a need for purified xyloglucan oligosaccharides and isoprimeverose for enzymological and metabolic studies (Lorences and Fry 1994; Chaillou et al. 1998). Relatively little is known about the mechanism of xyloglucan degradation and the enzymatic systems involved in the metabolism of xyloglucan oligosaccharides and isoprimeverose. These compounds are not commercially available and can be prepared by several steps of hydrolysis of the natural polymer xyloglucan by different glycosyl hydrolases such as *endo*-β-1,4-glucanases, β-galactosidases, α-L-fucosidases, isoprimeverose-producing oligoxyloglucan hydrolases, α-xylosidases, and β-glucosidase (Kato and Matsuda. 1980).

This approach is limited by the huge number of different enzymatic activities required and by the production of mixtures of oligosaccharides difficult to purify and always contaminated by monosaccharides usually removed microbiologically. These drawbacks can be overcome by synthesizing isoprimeverose and specific xyloglucan oligosaccharides by using the enzymes involved in the degradation of xyloglucan in transglycosylating mode. In the course of our study on an archaeal α-xylosidase (XylS), the need for isoprimeverose-based substrates emerged, and we planned the enzymatic synthesis of isoprimeverose, its 4-NP-β-derivative, and the trisaccharidic unit of xyloglucan XG [abbreviated name according to the nomenclature of Fry et al. (1993)] (Fig. 4) by using the enzyme in transxylosidation mode (Moracci et al. 2000).

The α-xylosidase from *Sulfolobus solfataricus*

In an effort to determine the full set of glycosyl hydrolases produced by the hyperthermophilic archaeon *S. solfataricus*, we have identified a novel α-xylosidase that was classified in Family 31 of glycosyl hydrolases and showed high specificity for isoprimeverose and for its 4-nitrophenyl-β-derivative (Table 2) (Moracci et al. 2000). Moreover, the enzyme was active on the xyloglucan oligosaccharide XXXG, producing xylose and the oligosaccharide GXXG (Fig. 4). The latter compound, GXXG, was not a substrate of XylS and accumulated in the reaction. This experiment demonstrated that XylS hydrolyzes xyloglucan oligosaccharide substrates from their nonreducing end. GXXG, which shows at the nonreducing end a glucose residue that is β-1,4 bonded, was resistant to hydrolysis because XylS is specific for α-bonds (Trincone et al., in preparation).

We took advantage of the ability of XylS to synthesize glycosides containing α-bonds by transxylosylation reaction to produce the substrates used for the characterization of the enzyme itself. In fact, XylS, which follows the retaining reaction mechanism just like the other α-glycosyl hydrolases of Family 31, produced by transxylosylation reaction 4-NP-β-isoprimeveroside from 4-NP-α-Xyl and 4-NP-β-Glc as donor and acceptor, respectively (Moracci et al. 2000). The xylosyl–enzyme intermediate was transferred to the acceptor to form 4-NP-β-isoprimeveroside as the main product and, in trace amounts, different regioisomers in which xylose was transferred to different glucose positions (probably the OH in C3 and in C4). Free isoprimeverose was obtained by using glucose and α-xylosyl fluoride as acceptor and donor, respectively. Glycosyl fluorides, alternatives to aryl-glucosides, are very reactive substrates of glycosidases, whereas glucose is inexpensive and its determination permits estimation of the synthetic yields at the end of the reaction by calculating the amount of enzymatically xylosylated glucose.

For the synthesis of the trisaccharidic unit of xyloglucan XG (Fig. 4) and to test the selectivity of xylose transfer, the disaccharide 4-Np-β-cellobioside and α-xylosyl fluoride were used as acceptor and donor, respectively. Because of the seven free OH groups of 4-Np-β-cellobioside, the synthesis of this trisaccharide by XylS is difficult due to the possible formation of multiple products that are hard to purify. In our case, only three of seven compounds are formed at yields of 15%.

Spectroscopic analysis of the isolated products determined that in the most abundant compound, 4-NP-β-XG, xylose was always bound to the external glucose molecule. In the other regioisomers, present in trace amounts, xylose was transferred to the O3- and O4-positions of the external glucose unit of 4-NP-β-cellobioside. These findings demonstrate that the *exo*-acting characteristic of this enzyme is operative also in synthetic mode, as found in the hydrolytic reaction.

Conclusions

We have summarized here the results obtained in recent years on two glycosyl hydrolases from the hyperthermophilic archaeon *S. solfataricus*. We described how the β-glycosidase from this source, modified by site-directed mutagenesis, could be transformed in a glycosynthase-producing branched oligosaccharide. This approach allowed expanding our knowledge about the substrate specificity and the mechanism of transglycosylation of this enzyme.

Moreover, we described the transxylosidic activity of the α-xylosidase from *S. solfataricus*, which allowed the production of interesting compounds for the study of xyloglucan metabolism that are usually prepared by traditional methods. This study confirmed the importance of the biodiversity of hyperthermophilic enzymes for the isolation of new enzymatic activities and for increasing the development of oligosaccharides to be used for both applied and basic research.

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