Developing Promiscuous Glycosidases for Glycoside Synthesis: Residues W433 and E432 in *Sulfolobus solfataricus* β-Glycosidase are Important Glucoside- and Galactoside-Specificity Determinants

Susan M. Hancock,[a] Kevin Corbett,[b] Anthony P. Fordham-Skelton,[b] John A. Gatehouse,[b] and Benjamin G. Davis*[a]

Two residues that have been implicated in determining the substrate specificity of the thermophilic β-glycosidase from the archaeon Sulfolobus solfataricus *(SsβG), a member of the glycosyl hydrolase family 1, have been mutated by site-directed mutagenesis so as to create more versatile catalysts for carbohydrate chemistry. The wild-type and mutated sequences were expressed in* E. coli *with a His_7-tag to allow one-step chromatographic purification. The E432C and W433C mutations removed key interactions with the OH-4 and OH-3 of the sugar substrates, thus reducing the discrimination of glucose, galactose and fucose with respect to other glycosides. This resulted in two glycosidases with greatly broadened substrate specificities. Observed changes include a 24-fold increase in Man:Gal activity and an 18-fold increase in GalA:Gal activity. This promiscuous substrate tolerance was further illustrated by the parallel synthesis of a β-glycoside library of glucose, galactose, xylose and mannose in one pot at 50 °C, in organic solvent. The synthetic potential of the catalysts was further evaluated through alkyl glycoside transglycosylation yields, including the first examples of synthesis of β-mannosides and β-xylosides with SsβG.*

Introduction

The role of oligosaccharides is increasingly implicated in biological recognition and signalling mechanisms,[1] thus further elevating their potential as therapeutics. This prominence creates a strong need for efficient and versatile methods for stereo- and regioselective glycoside synthesis. The enzymatic synthesis of glycosidic linkages with carbohydrate-processing enzymes has been widely investigated to address this issue[2,3] and is attractive in its ability to circumvent often long-winded protection regimes. Glycosyltransferases have proven to be highly efficient in the synthesis of glycosidic linkages.[4] However, although the expense of the nucleotide sugar substrates has been overcome by the creation of complex recycling systems,[5] their tight substrate specificity, and low enzyme availability still limits their application. Glycosidases are attractive for large-scale application since they are more abundant, relatively inexpensive, commercially available, exhibit broader acceptor–substrate specificity and use simpler substrates.[2] However, stringent donor specificities can limit their use.

The family 1,[6] which retains *exo*-β-glycosidase from *Sulfolobus solfataricus* (SsβG), has previously been cloned[7] and expressed in *E. coli*.[8,9] Family 1 glycosyl hydrolases have been shown to operate by a double displacement mechanism, via a glycosyl–enzyme intermediate, effecting net retention at the anomeric centre.[10,11] The 2.6 Å-resolution crystal structure[12,13] of SsβG revealed a $(\alpha/\beta)_8$ triose phosphate isomerase (TIM) barrel structure, with the active site located in a radial channel in a kink in the fifth α/β repeat, and, together with mutagenesis studies,[9,14] the catalytic nucleophile and general acid/base residues were identified. The wild-type (WT) enzyme has a half life of 48 h at 85 °C.[9] This thermostability has been explained by an extended network of ionic bridges that hold the tertiary structure together upon heat-induced vibrations.[12] Advantageously, this attribute also confers tolerance to organic solvents; aliphatic alcohols have been found to stimulate SsβG activity,[15] with an activity enhancement of 30% being achieved in 80 mM butan-1-ol. This increased activity was observed without any concomitant changes in secondary structure by CD and FTIR analyses. These valuable properties highlight potential synthetic utility.

Although glycosidases naturally hydrolyse glycosides, the formation of glycosidic linkages can be catalysed by using two strategies: thermodynamically controlled reverse hydrolysis and kinetically controlled transglycosylation. Reverse hydrolysis involves the reaction between a monosaccharide aldose donor with an excess of an acceptor, such as an alcohol or sugar, to give a glycoside and water, through equilibrium displace-

[a] *S. M. Hancock, Dr. B. G. Davis*
Chemistry Research Laboratory, University of Oxford
Mansfield Road, Oxford OX1 3TA (UK)
Fax: (+44) 1865-275-674
E-mail: ben.davis@chem.ox.ac.uk

[b] *K. Corbett, Dr. A. P. Fordham-Skelton, Dr. J. A. Gatehouse*
Department of Biological Sciences, University of Durham
South Road, Durham DH1 3LE (UK)

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ment.[16] Transglycosylation uses reactive glycosyl donors, such as sugar fluorides and *p*-nitrophenyl (pNP) sugars, to maintain a steady-state concentration of glycosyl–enzyme intermediate, which can be intercepted by an acceptor, to synthesise glycosides.[16] In order to improve yields, the thermodynamic activity of water is often lowered by using high temperatures and organic cosolvents, therefore thermophilic enzymes with solvent tolerance have obvious advantages over their mesophilic counterparts for the synthesis of glycosides. The crude homogenate of *Sulfolobus solfataricus,* and purified native and recombinant WT β-glycosidase have previously been used to catalyse the formation of alkyl gluco- and galactosides with primary, secondary,[17] polyol[18] and chiral[19] alcohols by transglycosylation and 2-deoxy-β-glucosides from glucal.[20] Primary hydroxyls were favoured over secondary hydroxyls, and SsβG was found to tolerate high concentrations of the alcohol acceptors. However, as a result of the tight specificity of SsβG, these syntheses were limited to just β-glucosides and β-galactosides.

Clearly glycosidases have great potential as glycosylation catalysts; however, their often tight donor specificities and the resulting "one enzyme, one linkage" hypothesis[21] limit utility. In order to have access to a broad range of glycosides, many different glycosidases are currently required. As an alternative, "broadened" glycosidases—single catalysts capable of processing many different sugars—would be of high interest. Several groups have targeted glycosidase specificity by site-directed mutagenesis. For example, the β-glucosidase from *Pyrococcus furiosus* and the 6-phospho-β-galactosidase from *Lactococcus lactis* were both mutated on the basis of sequence conservation in order to produce the counterpart 6-phosphogalactosidase and galactosidase activities, respectively. Glucose-6-phosphate (Glc-6-P) activity decreased 200-fold with respect to Glc in the 6-phosphogalactosidase mutants,[22] whereas upon mutation the β-glucosidase showed no increase in the hydrolysis activity of 6-phosphorylated substrates.[23] In another example, the residues believed to interact with the 2-hydroxyl, thus effecting mannose specificity in the β-mannosidase from *Pyrococcus horikoshii*, were interchanged with the residues in the β-glucosidase from *Pyrococcus furiosus* and vice versa.[24] All these investigations have focused on transforming one substrate activity into another. However, attempts to broaden glycosidase specificity appear to have been neglected.

Donor sugar specificity in SsβG has been associated with residues in the −1 subsite (for nomenclature see ref. [25]). Interestingly, those family 1 hydrolases that differ at certain of these residues show altered specificities (vide infra). This implies that alteration of these donor-site residues in SsβG to remove the discriminating ability that they convey to the enzyme might result in the formation of a glycosidase with more promiscuous substrate specificity. To explore this concept, we have identified and mutated two putative residues that are believed to play a key role in substrate-specificity determination. A brief preliminary discussion of the kinetic parameters for these mutants has previously been described,[26] and in this paper we present the full kinetic and synthetic evaluation of their broadened specificity and discuss potential mechanistic reasons for its origin.

Results and Discussion

Identification of residues critical to substrate binding in SsβG

In order to identify which residues were responsible for the determination of substrate specificity in SsβG, we analysed the three-dimensional structure of SsβG (RCSB-PDB 1gow) and the close structural homologue *Bacillus polymyxa* β-glycosidase (BpβG), which has been crystallised with D-gluconate[27] (1bgg) and as the 2-deoxy-2-fluoro-α-D-glucosyl-enzyme intermediate (1e4i, Figure 1 a). By using these structures for homology modelling and docking analysis, a minimum energy structure of SsβG containing β-D-glucopyranose as a substrate mimic was

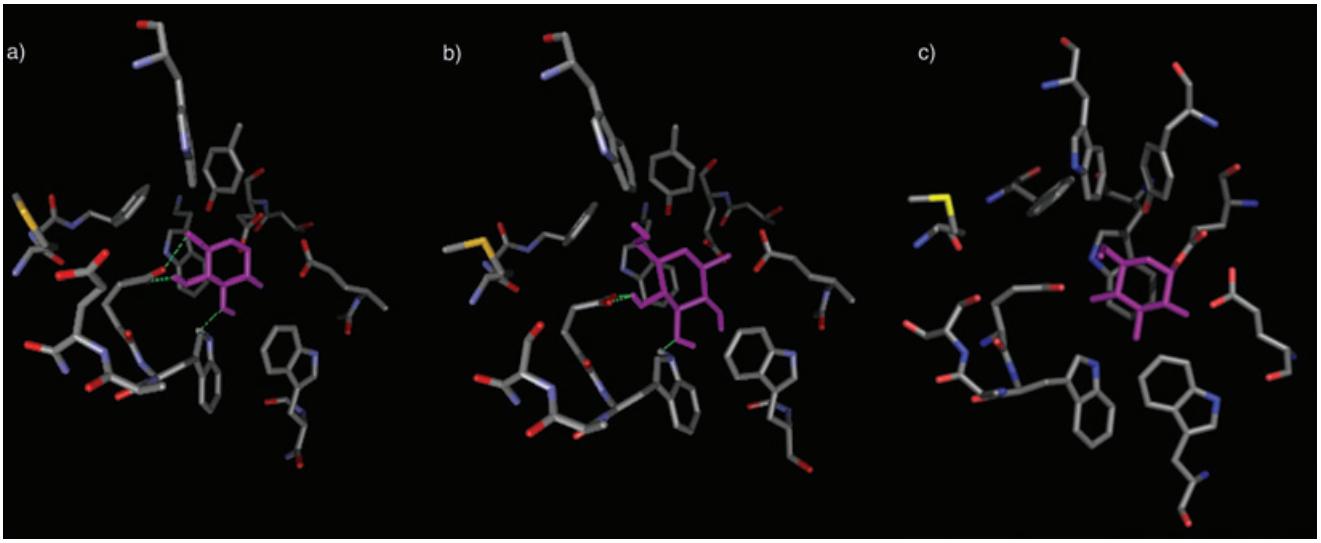


Figure 1. *A comparison of family 1 glycosyl hydrolase structures indicating the critical binding of substrates in a)* Bacillus polymyxa *β-glycosidase (BPβG)-2-deoxy-2-fluoro-α-D-glucopyranosyl enzyme intermediate (1e4i), b)* Sulfolobus solfataricus *β-glycosidase (SsβG, 1gow) containing docked and energy minimised β-D-glucopyranose, and c) (SsβG)-2-deoxy-2-fluoro-α-D-glucopyranosyl enzyme intermediate (1uws).*

created by molecular mechanics (Figure 1b). This illustrated that both BpβG and SsβG contain the conserved residues E432 and W433 (numbering corresponds to the original SsβG sequence), which create vital hydrogen bonds to the OH-4 and OH-3 of their substrates, respectively (Figure 2). Recently, the crystal structure of the 2-deoxy-2-fluoro-α-D-glucosyl enzyme intermediate of SsβG has been published (1uws, Figure 1c).[28] Reassuringly, comparison of this structure to the SsβG modelling, on which we based our hypothesis (Figure 1a), reveals that the structures are remarkably similar. The glycosyl–enzyme intermediate crystal structure reveals a bidentate interaction between E432 and OH-6 and OH-4, the only difference between gluco and galacto substrates being the interaction with Oε1 or Oε2, respectively. W433 was shown to hydrogen bond with OH-3. Sequence analysis also substantiates the role of these residues in specificity determination: E432 and W433 are largely conserved across family 1 glycosyl hydrolases (Figure 2). E432 has been implicated in the rejection of 6-phosphoglycosides and glycosides of galacturonic and glucuronic acid as substrates, due to repulsive electrostatic interactions.[12] For example, S432 (SsβG numbering) rather than E432 is found in the phosphogalactosidase (E.C. 3.2.1.85) from *Staphylococcus aureus*.[29] At position 433, G433 rather than W433 is found in the broad-specificity glycosidase/cerebrosidase human lactase phlorizin hydrolase (E.C. 3.2.1.62).[30]

For these reasons, residues E432 and W433 were chosen for mutagenesis, as potential determinants of substrate selectivity. Each amino acid was mutated to cysteine. Although cysteine is rarely used in such point mutagenesis studies, we consider it to be more suitable in certain cases than, for example, alanine. The side-chain-volume alteration of, for example, tryptophan or glutamate to cysteine is less dramatic than to alanine. Cysteine has been shown in many protein structures to function similarly to tryptophan, methionine[31] and serine, to which it is near isosteric, but eliminates or reduces any potential hydrogen-bonding interactions; thereby allowing the dissection of key interactions within the donor sugar-binding site. These cysteine residues could also be used as functional handles for further investigation of the critical contacts in the active site, for example by chemical modification.[32]

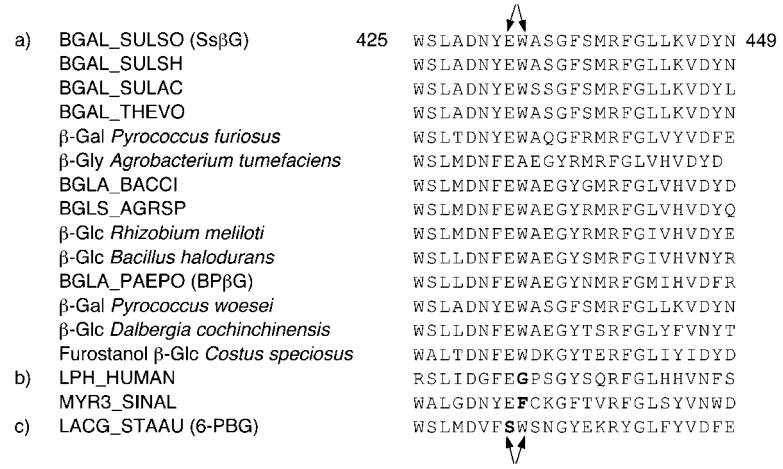


Figure 2. *Partial sequence alignment of the −1 binding pocket motif of* Sulfolobus solfataricus *β-glycosidase (SsβG) with family 1 glycosyl hydrolases of high sequence similarity; glycosidases with similar substrate specificity to SsβG (a) and glycosidases with different and/or broadened specificities in which W433 (b) and E432 (c) differ (bold). The residues analogous to E432 and W433 are indicated by arrows.*

Mutagenesis and kinetic characterisation of WT and mutant SsβG

WT SsβG, E432C and W433C were expressed in *E. coli* and purified by virtue of a His_7-tag (yields typically ~15 mgL^{-1}). In order to prevent interference with the putative C-terminal multimer-forming interactions[12,33] that have been implicated in functional multimerisation, the histidine tag was constructed at the N terminus. After nickel-affinity purification, the recombinant WT and mutated SsβG proteins were >95% pure by SDS-PAGE (~57 kDa), gel filtration and ESMS analysis.

The Michaelis–Menten parameters for the WT and mutant enzymes were determined with a broad range of 4-methylumbelliferyl (4-MU) substrates at the previously ascertained optimum pH of the enzyme (pH 6.5).[9,34] WT SsβG has been shown to have no α activity,[35] so only β substrates were investigated. The 4-MU kinetics were initially carried out at 80°C by using a discontinuous assay; this allowed activities to be determined at a high level of sensitivity at the optimum temperature of the enzyme (Table 1).

Under these conditions glucosides (Glc), galactosides (Gal) and fucosides (Fuc) were hydrolysed well by WT SsβG, but the xyloside (Xyl) substrate was hydrolysed relatively poorly, at approximately 3% of turnover relative to the k_{cat} for glucoside. Interestingly, low levels of β-mannoside and β-glucuronide activities were also detected, which had previously not been observed (~1% and 0.5% of turnover compared to β-D-glucoside, respectively). Intriguingly, the (pNPFuc) substrate, essentially 6-deoxy-pNPGal, had higher activity than even gluco or galacto substrates. This arises primarily from ground-state binding and through a four- to sixfold lower K_M, respectively, and a similar k_{cat}. One rationalisation of this is a potential hydrogen bond to the OH-6 position of Gal. Such an interaction in *Agrobacterium* β-glucosidase provides substantial stabilisation in the ground state and only equivalent stabilisation at the transition state, thus contributing to reducing catalytic efficiency.[36] Removing some of this stabilisation, by removing the interaction with OH-6, as in the fuco substrate, would increase catalytic efficiency as observed here. The preference for Fuc > Glc > Gal in β-glucosidase from *Streptomyces* sp.[37] has been explained by compensation of the less-favourable axial OH-4 interaction in

Table 1. *Kinetic parameters for SsβG enzymes with 4MU glycoside substrates at 80 °C, pH 6.5.*

SsβG	substrate	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$mM^{-1}s^{-1}$]
WT	4MUβGal	98 ± 7	0.066 ± 0.017	1490
	4MUβGlc	140 ± 20	0.046 ± 0.017	2900
	4MUβXyl	3.8 ± 0.3	0.13 ± 0.03	30
	4MUβFuc	80 ± 2	0.011 ± 0.002	7300
	4MUβMan	1.8 ± 0.2	0.036 ± 0.009	50
	4MUβGlcA	0.81 ± 0.18	1.3 ± 0.4	0.6
W433C	4MUβGal	14.6 ± 6	2.2 ± 1.2	6.3
	4MUβGlc	33 ± 5	1.61 ± 0.35	20
	4MUβXyl	1.5 ± 0.3	0.59 ± 0.19	2.5
	4MUβFuc	31 ± 3	0.41 ± 0.09	76
	4MUβMan	0.92 ± 0.05	0.18 ± 0.02	5.1
	4MUβGlcA	[a]	[a]	[a]
E432C	4MUβGal	5.4 ± 0.8	0.47 ± 0.14	11
	4MUβGlc	5.1 ± 0.5	0.34 ± 0.07	15
	4MUβXyl	2.8 ± 0.3	1.26 ± 0.21	2.2
	4MUβFuc	18 ± 1	0.34 ± 0.04	53
	4MUβMan	2.8 ± 0.7	0.90 ± 0.26	3.2
	4MUβGlcA	[a]	[a]	[a]

[a] No activity detected.

Gal/Fuc, by the increase in hydrophobicity in the C6-deoxy (Fuc), which interacts with the "hydrophobic platform" of residues that are ubiquitous in family 1 glycosyl hydrolase −1 subsites.[38] Interestingly, in SsβG, following the alteration of an active-site residue close to the region interacting with the OH-6, the preference for Fuc over Glc is removed.[26] This interesting observation is under investigation, and further details will be published in due course.

The E432C and W433C mutations have a striking effect upon activity, specifically towards 4MUGlc and 4MUGal substrates. The Glc k_{cat}/K_M is reduced 200-fold or 140-fold, and Gal k_{cat}/K_M is reduced 130-fold or 230-fold for E432C and W433C, respectively. Although the Man and Xyl activities were also reduced in the mutants, this was to a much smaller extent, E432C and W433C k_{cat}/K_M values were only 10- to 16-fold lower than for WT. In agreement with the crystal structure of the glycosyl-enzyme intermediate of SsβG and the molecular modelling shown in Figure 1, the E432C and W433C mutations appear to eliminate hydrogen bonds to OH-4 (E432C) and OH-3 (W433C); this results in a decrease in k_{cat}/K_M that corresponds to 4.5–10.5 kJ mol^{-1} destabilisation.[39] These reductions in k_{cat}/K_M were mainly a consequence of reduced ground-state binding, with K_M values generally increased up to 37-fold; the greatest K_M increases in both W433C and E432C were observed for Glc, Gal and Fuc. Variations in k_{cat} in the mutants E432C and W433C were less uniform; there were large overall reductions in Gal and Glc turnover (k_{cat} decreased by ca. five- to 30-fold), whereas k_{cat} for Fuc, Xyl and Man in E432C and W433C are essentially the same as for WT. This indicates that destabilisation of the transition state in the E432C and W433C mutations is exclusive to Gal and Glc.

These observations fit well with an emerging paradigm in glycosidase mechanism. The interactions with the OH-4 and OH-3 positions of the sugar substrate are believed to contribute predominantly to transition-state stabilisation.[36] It is proposed that the transition states for mannoside and glucoside hydrolysis, which are supported by both stereoelectronic,[40] inhibitor,[41] and X-ray structure analyses,[11,42,43] adopt $B_{2,5}$ and $^4H_3$ conformations, respectively. Although mannosides and glucosides differ in the configuration of C-2, the major difference between the $B_{2,5}$ mannoside and $^4H_3$ glucoside conformations is the orientation of the OH-3, which in $B_{2,5}$ is pseudoaxial and in $^4H_3$ is equatorial (Figure 3). Removal of the OH-3 interaction in

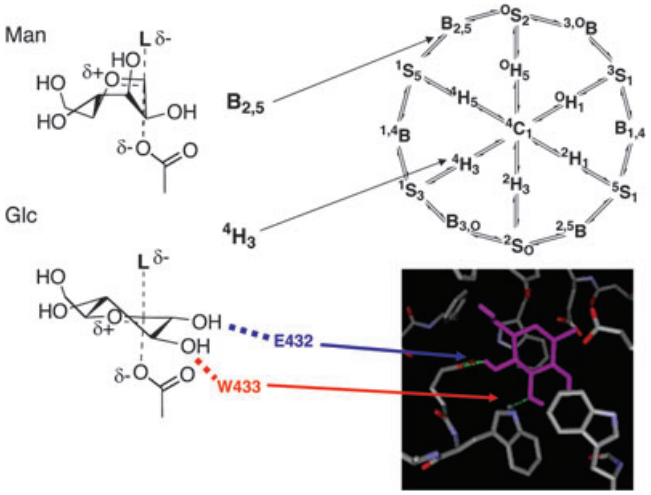


Figure 3. *Conformational pseudorotational itinerary[43,44] of pyranose conformations, highlighting the conformation of the transition states of manno and gluco substrates.*

the transition state by the W433C mutation would therefore account for the observed loss in distinction between Gal/Glc and Man substrates. As such we believe that these observations represent some of the first mutagenic experiments to support the conformational pseudorotational itinerary mechanism.

The E432C and W433C mutations altered k_{cat}/K_M values for different substrates to produce enzymes with remarkably broader specificity than WT. For example, the variation of k_{cat}/K_M for Glc:Gal:Xyl:Man moves from a restrictive 100-fold specificity range for WT to a broad eightfold range for W433C (WT, 100:52:1:2 → W433C, 8.1:2.5:1:2).

As a second phase of kinetic evaluation, we also explored pNP glycosides. The lower accessibility of fluorescent substrates reduces their usefulness as sugar donors in prospective biocatalysis experiments; this necessitates the evaluation of pNP glycosides as more suitable donor sugars. The pNP substrates were assayed at 45 °C with a continuous assay. Although this temperature is below the temperature optimum of the enzyme, the continuous-assay conditions are more reliable and precise than the higher-temperature discontinuous assays. Although absolute rates are reduced at 45 °C, relative donor processing remains representative; SsβG donor selectivity has been shown to remain constant with temperature in the range 50–80 °C.[45]

The ~10 mM solubility limit of pNP substrates prevented the determination of the full kinetic parameters for Xyl (W433C),

Man (W433/E432C), Fuc (E432C) and GalA (WT) due to their very high K_M values. An approximation of k_{cat}/K_M was therefore obtained from the rate of product formation (v), by using the limiting case of the Michaelis–Menten equation for low substrate concentration, where $v \approx (k_{cat}/K_M)[S][E]_o$ for $[S] \ll K_M$ and [S] and $[E]_o$ are substrate and enzyme concentrations, respectively.

In comparison to 4MU substrates, pNP substrate K_M values were approximately an order of magnitude higher than the corresponding 4MU substrates, and the k_{cat}/K_M values were 100-fold lower (Table 2). This is probably a consequence of the assay temperature, but the positioning and interaction with the two different aglycones in the +1 subsite may also contribute to this divergence. In addition, the different leaving-group abilities of the 4MU versus pNP will have a direct influence if the formation of the glycosyl intermediate is rate determining. As the pK_a values of 4MU and pNP are 9.16 and 7.22, respectively, the highest activities might be expected for the pNP substrates, if significant partial negative charge is developed on the anomeric O-1 during the transition state of the rate-determining step. However, this is not observed, thus suggesting that either the observed activity differences may be attributed to altered aglycone accommodation in the active site or, more likely, that, for the pNP substrates, the rate of glycosyl enzyme intermediate formation is so high that *deglycosylation* becomes rate limiting ($k_3 \ll k_2$). In the latter case, this would result in alterations in the K_M that reflect a change in the mechanistic regime. This has implications for donor choice in synthesis and justifies the need to conduct kinetics on appropriate substrates.

Table 2. *Kinetic parameters for SsβG enzymes with pNP glycoside substrates at 45 °C, pH 6.5.*

SsβG	substrate	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$M^{-1}s^{-1}$]
WT	pNPβGal	7.20 ± 1.06	0.57 ± 0.07	12736
	pNPβGlc	4.35 ± 0.53	0.15 ± 0.01	29796
	pNPβXyl	3.95 ± 0.75	5.10 ± 0.67	774
	pNPβFuc	5.16 ± 0.72	0.21 ± 0.02	42641
	pNPβMan	2.65 ± 0.90	24.2 ± 7.28	109
	pNPβGalA	[a]	[a]	60 ± 4.3[a]
W433C	pNPβGal	0.08 ± 0.01	5.28 ± 1.35	15
	pNPβGlc	2.72 ± 0.56	22.1 ± 4.51	123
	pNPβXyl	[a]	> 10[a]	3 ± 0.6[a]
	pNPβFuc	9.43 ± 1.94	18.6 ± 4.00	506
	pNPβMan	[a]	> 10[a]	5 ± 0.7[a]
	pNPβGalA	0.004 ± 0.0008	2.72 ± 0.80	1.29
E432C	pNPβGal	0.047 ± 0.005	0.81 ± 0.14	58
	pNPβGlc	0.018 ± 0.002	0.51 ± 0.09	36
	pNPβXyl	0.028 ± 0.007	13.7 ± 4.00	2
	pNPβFuc	[a]	> 10[a]	68 ± 23[a]
	pNPβMan	[a]	> 10[a]	1 ± 0.1[a]
	pNPβGalA	0.004 ± 0.001	3.46 ± 1.24	1

[a] High K_M prevented determination of full Michaelis–Menten parameters. k_{cat}/K_M was determined using the low substrate concentration approximation. An indication of errors is given.

Noticeable differences between the activities of the 4MU and the pNP substrates were observed for mannosides and xylosides. In particular, unusually high K_M values were observed for pNPXyl and pNPMan for WT SsβG and mutants. Regardless, the relative levels of activity, Fuc > Glc > Gal were identical to the 4MU substrates, thus confirming the value of these data in relative processing abilities. As a parameter, k_{cat} is often of the greatest value when applied to the synthetic utility of an enzyme; most reactions are conducted at substrate concentrations in excess of K_M and so permit useful transglycosylation activities with substrates in spite of poor K_M values. Interestingly, the changes in kinetic parameters with the pNP and 4MU substrates upon mutation result in W433C having a similar activity ratio to WT: Fuc > Glc > Gal. However, in E432C, the preference for Glc to Gal is changed, most notably with the pNP substrates, where the ratio Glc to Gal is reversed from 2:1 in WT to 1:1.6 in E432C. This turnaround has also been observed in *Spodoptera frugiperda* β-glycosidase upon mutagenesis of glutamate E451, which corresponds to E432.[46] E432 therefore appears to be a key residue in the determination of Gal/Glc preference. It has been proposed that hydrogen bonding with this residue stabilises the equatorial (Glc) and axial (Gal) OH-4 by 52.5 and 32.8 $kJmol^{-1}$, respectively, thus destabilising Glc transition states more than Gal transition states upon removal of this interaction.[46] Although no uronic acid GlcA activity was observed with the 4MU substrates with E432C and W433C, low-level activities towards epimeric uronic acid GalA were detected with pNP substrates (3–9% relative to galactoside k_{cat}/K_M) with the mutants; this corresponds to an 18-fold increase in GalA:Gal activity from WT SsβG. This observation is consistent with the proposal that the equivalent residue to E432 in other family 1 glycosidases is also responsible for the modulation of carbohydrate substrate *O*-6 substituent binding and, in particular, the rejection of negatively charged substituents.[12] W433C SsβG also showed a 11.5-fold increase in Man:Glc ratio; this highlights the potential of this mutation for the synthesis of β-mannosides.

A screen of cosolvent systems with pNPGal at 45 °C in 50 mM phosphate buffer (pH 6.5) was undertaken to establish which cosolvents were most suitable for use with SsβG, causing least denaturation at the highest concentration possible and thereby maximising transglycosylation yield (Table 3). The drop in dielectric constant upon addition of organic solvent causes pH to rise, but pH measured with a probe must also reflect the changes in proton-activity coefficient caused by the cosolvent.[47] pH quoted in this work therefore refers to that of the aqueous buffer before addition of the organic solvent.

As Figure 4 and Table 3 show, the activities of WT SsβG in 30% acetonitrile, butanone and acetone were higher than those in DMSO, DMF and 1,4-dioxane: acetonitrile, butanone and acetone give rise to lower K_M values at the same concentration than the other solvents. Consistent with previous suggestions of "active site loosening by organic solvents", all K_M values are higher in the presence of organic solvents than without. The low boiling point of acetone limited the application of this solvent in thermophilic enzyme reactions. The activity of WT SsβG in acetonitrile and butanone was therefore investigated further.

5–20% MeCN cosolvent concentrations increased WT activity with respect to that in phosphate buffer (Figure 4). This is con-

Table 3. *Kinetic parameters for a range of cosolvents with WT and mutant Ssβ-G using pNPGal substrate.*

SsβG	solvent system	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$M^{-1}s^{-1}$]
WT	50 mM phosphate, pH 6.5	7.20 ± 1.06	0.56 ± 0.07	12736
	50% MeCN	24.66 ± 3.84	5.06 ± 0.50	4873
	40% MeCN	19.73 ± 2.80	2.81 ± 0.27	7021
	30% MeCN	17.58 ± 2.41	2.90 ± 0.24	6061
	20% MeCN	12.24 ± 1.91	0.67 ± 0.16	18380
	10%MeCN	11.55 ± 1.42	0.58 ± 0.04	19872
	5% MeCN	11.38 ± 1.67	0.49 ± 0.06	23039
	30% DMF	8.32 ± 1.20	5.27 ± 0.45	1579
	30% DMSO	18.08 ± 2.91	6.33 ± 0.69	2857
	30% 1,4-dioxane	8.83 ± 1.09	5.12 ± 0.25	1724
	30% butanone	18.08 ± 3.38	2.20 ± 0.44	8220
	5% butanone	13.91 ± 1.88	0.49 ± 0.05	28331
	30% acetone	14.67 ± 2.10	1.84 ± 0.20	7973
	5% acetone	12.77 ± 1.70	0.73 ± 0.06	17545
W433C	30% MeCN	0.08 ± 0.03	18.3 ± 7.00	4
	30% butanone	0.19 ± 0.04	12.5 ± 3.15	15
E432C	30% MeCN	0.056 ± 0.006	1.30 ± 0.18	43
	30% butanone	0.067 ± 0.007	1.65 ± 0.25	40

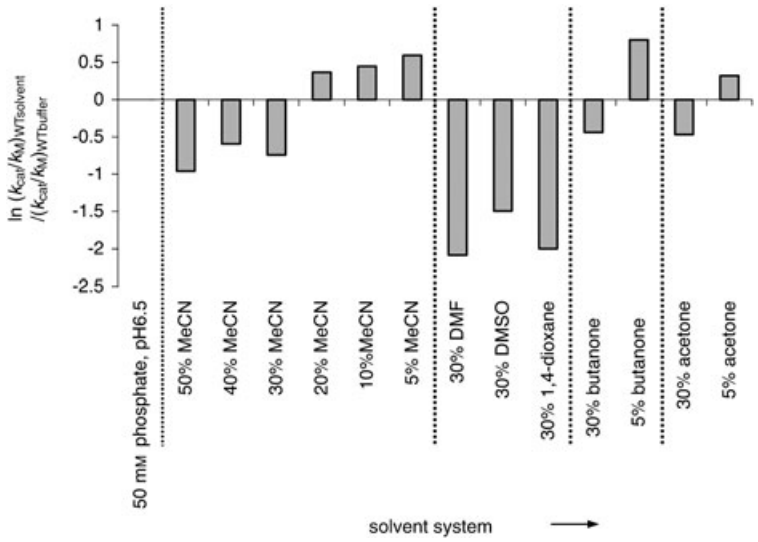


Figure 4. *Comparison of WT Ssβ-G activity in a range of cosolvent systems to that in phosphate buffer, with pNPGal as a substrate at 45 °C.*

sistent with the reports of thermophilic enzyme activation by classical denaturants, such as urea and guanidine hydrochloride,[48] and is in agreement with Ssβ-G activity increasing upon the addition of low concentrations of SDS.[49] This phenomenon may be explained by increased accessibility to the catalytic site and diminished steric hindrance upon dissociation of tetramers into dimers. However, increasing cosolvent concentration further, for example beyond 20% MeCN, caused dramatic loss of activity, perhaps through preferential protein–solvent binding and unpacking of the hydrophobic protein core, thus reducing binding and preventing orientation of the essential residues for catalysis. Fortunately, concomitant increases in K_M are less relevant to these solvent systems in biocatalysis experiments, as here substrate concentrations are high, typically 20–40 mM. As long as K_M is within the low millimolar range and accessible by solubility, it is k_{cat} that will primarily determine reactivity. k_{cat} in 50% MeCN was very high, 3.4-fold higher than in phosphate buffer alone, but the K_M became prohibitively high, increasing twofold upon going from 40% to 50% to a value of ~5 mM, some tenfold higher than phosphate buffer. Conversely, at 5–10% MeCN, little benefit from the organic solvent would be observed in transglycosylation reactions. 30% cosolvent was therefore chosen as a suitable compromise between enhanced k_{cat}, reduced binding affinity and the advantageous effects of organic solvent on transglycosylation activity. These optimised cosolvents were tested on the E432C and W433C mutants for which 30% acetonitrile and butanone induced tolerable increases in the K_M and did not affect k_{cat} considerably (Table 3).

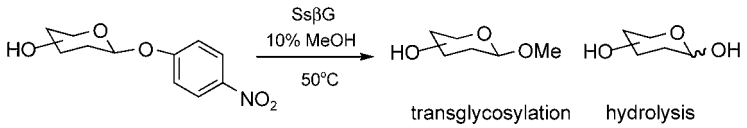
Synthesis of balanced libraries of β-glycosides

The high activity of WT Ssβ-G at 80 °C resulted in the formation of mutants that were still usefully active in spite of the large reductions in k_{cat}/K_M caused by the E432C and W433C mutations. In order to relate analyses of kinetic efficiency to those of synthetic utility, a competition experiment was conducted to directly compare the synthesis of methyl β-glycosides of Glc, Gal, Xyl and Man in one pot at 50 °C in 1:9 MeOH/buffer solutions (Scheme 1). For WT Ssβ-G, the strong bias towards Gal and Glc substrates resulted in the formation of only methyl glucoside **1** and galactoside **2** by transglycosylation after 45 min. After extended periods (4 h), small amounts of mannoside **3** and xyloside **4** were observed, but by this time all initially formed **1** and **2** had been hydrolysed. WT Ssβ-G is therefore incapable of creating balanced libraries of glycosides in this way. In comparison, both W433C and E432C yielded mixtures of methyl Glc, Gal, Xyl and Man glycosides **1**–**4**. The E432C mutant was exceptionally proficient at producing a balanced library, **1**–**4** being present in almost equal amounts. This highlights the potential of E432C and W433C in combinatorial biocatalysis.[50]

Synthesis of alkyl glycosides

Building on this success in broadening Ssβ-G towards parallel synthesis under kinetic, competitive control, we next investigated catalysis of glycoside synthesis under conditions in which thermodynamics play a greater role. Simple transglycosylation reactions to form alkyl glycosides in the presence of a large excess of acceptor alcohol were therefore conducted to probe the utility of WT and mutants (Tables 4 and 5).

The mutant Ssβ-G enzymes showed similarly good synthetic utility under these different conditions. E432C produced methyl β-galactoside and methyl β-xyloside in 100% and 50% yields, respectively, and W433C gave methyl-β-glucoside in



		Product distribution / %							
SsβG	Time	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
WT	45 min	40	33	2	3	14	8	-	-
WT	4 h	-	-	8	20	33	30	4	5
E432C	8 h	36	18	8	13	11	6	3	5
W433C	8 h	22	24	14	17	7	9	3	4

Scheme 1. *Parallel glycoside synthesis with WT and mutant SsβG as transglycosylation catalysts. The yields of hydrolysed and transglycosylation products are given in the table.*

87% yield, in agreement with the kinetic observations. WT SsβG also displayed good utility in the synthesis of glycosides of Xyl, Glc and Man. Alternative alkyl acceptors to methanol, such as benzyl alcohol and cyclohexanol at 30% *v/v*, were also explored by using WT enzyme. In these biphasic systems, benzyl glucoside and cyclohexyl glucoside were synthesised in 56 and 37%. Upon increasing the complexity of the acceptor moiety to other alcohols and sugars, transglycosylation yields dramatically decreased. Clearly, the identification of methods for reducing product hydrolysis is essential for the further development of glycosylation catalysts. Interestingly, methyl mannoside synthesis proved optimal with WT SsβG (53% yield); this is particularly promising given the low specificity of the enyzme towards this activity. In this case, the broadened specificity of the mutants was negated by an observed reduction in stability at the high temperatures and in organic solvents. In striking contrast to the mannoside-hydrolysis kinetics, the 13 and 17% yields for the synthesis of the β-mannoside linkage with W433C and E432C were disappointing. This might be due to competing product hydrolysis. Product concentration increases to a maximum at which the rates of synthesis and glycoside hydrolysis become equal. At this point, kinetic control is lost, and thermodynamic control ensues, thus leading to product hydrolysis. Therefore, unlike the kinetic competition experiments, reaction yield is greatly influenced by reaction conditions and enzyme properties. Nevertheless, these syntheses with SsβG demonstrated for the first time that β-xylosides and β-mannosides may be accessed, and these yields compare well to literature values for which the crude homogenate of *Sulfolobus solfataricus* was used to catalyse the formation of methyl glucoside in only up to 25% yield.[17]

Conclusion

Through mutagenesis of SsβG residues E432 and W433, we have shown that glycosidases can be tailored to have broadened substrate specificity. Kinetic analyses have revealed that these residues are important glucoside and galactoside specificity determinants, presumably by stabilising the $^4H_3$ transition state through hydrogen bonding. Removal of this interaction by mutagenesis produces mutant glycosidases with reduced discrimination between proposed glucosidase ($^4H_3$) and mannosidase ($B_{2,5}$) transition states; this results in the observed substrate promiscuity. These represent some of the first examples of mutagenesis to support the conformational pseudorotational itinerary mechanism.[11,43] WT and mutant SsβG have been shown to be useful synthetic catalysts, successfully generating balanced glycoside libraries by parallel synthesis and by the synthesis of alkyl glycosides, most notably methyl β-mannoside and methyl β-xyloside in one step in 53% and 92% yields, respectively. These represent the first examples of SsβG being used to form mannosides and xylosides by transglycosylation.

Experimental Section

Cloning, mutagenesis, expression and purification. The β-glycosidase from *Sulfolobus solfataricus* was cloned into pET24d (Novagen) with a heptahistidine tag. Mutations were introduced according to the QuikChange mutagenesis system (Stratagene). The WT and mutant glycosidases were expressed in *E. coli* BL21(DE3) and purified by nickel-chelation chromatography. The experimental detail has been previously reported.[26] Protein concentration was quantified by the method of Bradford[51](reagents from Biorad, Netherlands). Purified proteins were analysed by SDS-PAGE,[52] gel fitration chromatography and ESMS (Micromass LCT, with an error of ±8 Da).

Characterisation of kinetic properties. Parameters were determined by the initial-rate method. Activity was measured in time-course assays of the hydrolysis of 4-methylumbelliferyl β-D-glycosides (β-D-gluco, β-D-galacto, β-D-fuco, β-D-manno, β-D-xylo, β-D-glucurono) at 5–15 concentrations (0.001–1.5 mM) incubated at 80°C in 50 mM sodium phosphate buffer, pH 6.5. Reactions were terminated at 2, 5, 10, 15 min by the addition of ice-cold Na_2CO_3 (100 μL, 1 M, pH 10) and analyzed on a Labsystems Fluoroscan Ascent plate reader, excitation 355 nm, emission 460 nm. The hydrolyses of *p*-nitrophenyl glycosides were performed at 45°C in sodium phosphate buffer (50 mM, pH 6.5). Reactions were initiated by adding enzyme (0.9 or 11 μg WT, 18 or 36 μg E432C, 18 or 28 μg W433C, the higher enzyme concentration for pNPMan, pNPGalA, pNPGlcNAc, pNPXyl substrates) to substrate (190 μL, 10–0.0125 mM), and *p*-nitrophenol release was monitored at 405 nm on a Molecular Devices Spectra Max Plus plate reader ($\varepsilon_{405} = 3212\,M^{-1}cm^{-1}$, 50 mM phosphate buffer, pH 6.5). Extinction coefficients for pNP in the organic cosolvents were calculated from standard solutions of pNP.

The initial rates were calculated by using Microsoft Excel, and K_M and v_{max} were determined from curve fitting, nonlinear regression by using GraFit 4 (Erithacus Software Ltd, Staines, UK).

In cases in which the high K_M and substrate solubility prevented determination of the Michaelis–Menten parameters, approximate k_{cat}/K_M was determined by using the limiting case of the Michaelis–Menten equation at low substrate concentration. Substrate concentrations used were 0.25 (pNPMan–W433C and pNPGal–WT) and 0.1 mM (pNPXyl–W433 and pNPFuc and pNPMan–E432C), with

identical enzyme concentrations to the full kinetic analyses, except for pNPGalA determination with WT (4.3 μg).

Sequence analysis and molecular mechanics Sequence alignment was performed using ClustalW based on a BLOSUM42 matrix. The enzymes of interest were: BGAL_SULSO, *Sulfolobus solfataricus* β-glycosidase (SsβG);[7] PFBG, *Pyrococcus furiosus* β-glucosidase (*CelB*) (PFβG);[53] PFBM, *Pyrococcus furiosus* β-mannosidase;[54] BGLA_THEMA, *Thermotoga maritima* β-glucosidase;[33] BGLA_PAEPO (BPBG), *Bacillus polymyxa* β-glycosidase (BPβG);[27] LPH_HUMAN, human lactase phlorizin hydrolase;[30] LACG_STAAU (6-PBG), *Staphylococcus aureus* 6-phosphogalactosidase;[29] *Dalbergia cochinchinensis* β-glycosidase;[55] MY3_SINAL, myrosinase;[56] *Costus speciosus* furostanol β-glycoside hydrolase.[57]

The energy minimisation and docking analysis was conducted as previously reported.[26] The SsβG X-ray structure (RCSB-PDB entry 1gow) figures were prepared by using Cerius^2 or Pymol.[58]

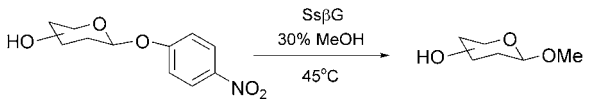
General synthetic methods. Silica gel (Merck, 400 mesh) was used for column chromatography. TLC was performed on Merck F254 silica gel coated, aluminium-backed sheets. Optical rotations were measured on a Perkin–Elmer 241 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded on Bruker 400/500 MHz spectrometers, peak assignments were made by COSY, HMQC and APT experiments and are quoted in ppm with respect to the residual solvent peak, with coupling constants quoted in Hz.

Alkyl glycoside synthesis. The methyl β-D-glycoside library was created as previously described.[26]

The synthesis of individual methyl glycosides was conducted as follows: *p*-Nitrophenyl donor was dissolved in 30% (*v*/*v*) MeOH (or benzyl alcohol or cyclohexanol) in phosphate buffer (50 mM, pH 6.5), to a final concentration of 10 mM. Enzyme was added and the reaction incubated at 45 °C with shaking at 200 rpm. Reactions were worked up by removing solvent in vacuo. Flash chromatography (4:1, EtOAc/MeOH) was used to purify the methyl glycoside products. Yields are given in Tables 4 and 5.

Methyl β-D-glucopyranoside. TLC R_f = 0.34 (chloroform/MeOH/water/acetic acid, 60:30:3:5); $[\alpha]_D^{30} = -8$ (c = 1.25 in H_2O) [lit.[59] $[\alpha]_D = -32$ (c = 0.3 in H_2O), lit.[60] $[\alpha]_D = -6.6$]; ^{1}H NMR (400 MHz, CD_3OD) δ = 3.21 (dd, J = 7.6, 9.0 Hz, 1H; H2), 3.27–3.34 (m, 2H; H4/5), 3.36 (dd, J = 9.0, 9.2 Hz, 1H; H3), 3.57 (s, 3H; CH_3), 3.71 (ddd, J = 4.0, 11.9 Hz, 1H; H6), 3.91 (dd, J = 1.6, 11.9 Hz, 1H; H6′), 4.21 (d, J = 7.6 Hz, 1H; H1) [lit.[61] ^{1}H NMR (400 MHz, CD_3OD) δ = 3.15 (dd, J = 8.0, 9.2 Hz, 1H), 3.28 (m, 1H), 3.38 (m, 2H), 3.47 (s, 3H), 3.61 (dd, J = 6.0, 12.4 Hz, 1H), 3.82 (dd, J = 2.4, 12.4 Hz, 1H), 4.27 (d, J = 8.4 Hz, 1H)]; ^{13}C NMR (100 MHz, CD_3OD) δ = 56.3 (q, CH_3), 61.7 (t, C6), 70.7 (d), 74.1 (d, C2), 77.0 (2d), 104.4 (d, C1); ESI$^-$: *m/z* (%): 229 (85) [M+Cl$^-$].

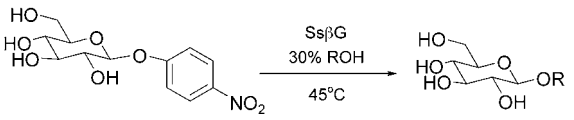
Table 4. *Transglycosylation yields of methyl glycosides with WT SsβG and mutants.*



SsβG	donor	enzyme [μg]	time [h]	yield MeGly [%]
WT	pNPGlc	15	16.5	93[a]
	pNPMan	150	62	53[b]
	pNPXyl	150	14	92[a]
E432C	pNPGal	2872	18	100[b]
	pNPXyl	2475	110	50[b]
	pNPMan	2245	110	17[b]
W433C	pNPGlc	2872	18	87[b]
	pNPMan	2245	110	13[b]

[a] Isolated yield. [b] Determined by NMR integration.

Table 5. *Transglycosylation yields of alkyl glycosides from biphasic reactions with WT SsβG.*



SsβG	acceptor	enzyme [μg]	time [h]	yield RGlc [%]	yield hydrolysis [%]
WT	benzyl alcohol	11	15	56[a]	8
	cyclohexanol	11	15	37[a]	0

[a] Determined by NMR integration

Methyl β-D-galactopyranoside. TLC R_f = 0.5 (chloroform/MeOH/water/acetic acid, 60:30:3:5); $[\alpha]_D^{30} = -0.2$ (c = 1.41 in H_2O) [lit.[62] $[\alpha]_D^{20} = 0$ (c = 0.7 in H_2O)]; ^{1}H NMR (400 MHz, CD_3OD) δ = 3.48 (dd, J = 3.3, 10.1 Hz, 1H; H3), 3.51 (dd, J = 7.2, 10.1 Hz, 1H; H2), 3.52 (ddd, J = 1.0, 5.5, 6.6 Hz, 1H; H5) 3.55 (s, 3H; CH_3), 3.74 (dd, J = 5.5, 11.4 Hz, 1H; H6), 3.78 (dd, J = 6.6, 11.4 Hz, 1H; H6′), 3.85 (dd, J = 1.0, 3.3 Hz, 1H; H4), 4.15 (d, J = 7.5 Hz, 1H; H1) [lit.[63] ^{1}H NMR (400 MHz, D_2O) δ = 3.51 (dd, J = 8.0, 9.9 Hz, 1H; H2), 3.59 (s, 3H; CH_3), 3.65 (dd, J = 3.3, 9.9 Hz, 1H; H3), 3.71 (ddd, J = 0.9, 4.4, 8.0 Hz, 1H; H5) 3.76 (dd, J = 4.4, 11.2 Hz, 1H; H6), 3.80 (dd, J = 8.0, 11.2 Hz, 1H; H6′), 3.93 (dd, J = 0.9, 3.3 Hz, 1H; H4), 4.32 (d, J = 8.0 Hz, 1H; H1)]; ^{13}C NMR (100 MHz, CD_3OD) δ = 56.3 (q, CH_3), 61.5 (t, C6), 69.3 (d, C4), 71.5 (d, C2), 74.0 (d, C3), 75.7 (d, C5), 105.1 (d, C1); ESI$^-$: *m/z* (%): 193 (100) [M−H$^-$].

Methyl β-D-xylopyranoside. TLC R_f = 0.8 (chloroform/MeOH/water/acetic acid, 60:30:3:5); $[\alpha]_D^{30} = -43$ (c = 1.53 in H_2O); ^{1}H NMR (400 MHz, CD_3OD) δ = 3.16 (dd, J = 7.5, 9.0 Hz, 1H, H2), 3.21(dd, J = 10.2, 11.4 Hz, 1H; H5α), 3.32 (dd, J = 8.9, 9.0 Hz, 1H; H3), 3.49 (ddd, J = 5.3, 8.9, 10.2 Hz, 1H; H4), 3.50 (s, 3H; CH_3), 3.88 (dd, J = 5.3, 11.4 Hz, 1H; H5β), 4.13 (d, J = 7.5 Hz, 1H; H1) [lit.[64] ^{1}H NMR (D_2O) δ = 3.16 (dd, J = 7.8, 7.8 Hz, 1H; H2), 3.23 (dd, J = 8.0, 11.3 Hz, 1H; H5α), 3.34 (dd, J = 7.8, 7.8 Hz, 1H; H3), 3.53 (dd, J = 5.3, 7.8, 8.0 Hz, 1H; H4), 3.87 (dd, J = 5.3, 11.3 Hz, 1H; H5β), 4.23 (d, J = 7.8 Hz, 1H; H1)]; ^{13}C NMR (100 MHz, CD_3OD) δ = 57.6 (q, CH_3) 67.3 (t, C5), 71.6 (d, C4), 75.3 (d, C2), 78.2 (d, C3), 106.5 (d, C1); ESI$^-$: *m/z* (%): 199 (18) [M+Cl$^-$], 223 (100) [M+AcO$^-$].

Benzyl β-D-glucopyranoside. TLC R_f = 0.6 (chloroform/MeOH/water/acetic acid, 60:30:3:5); $[\alpha]_D^{30} = -31.7$ (c = 0.625 in MeOH) [lit.[65] $[\alpha]_D^{25} = -35$ (c = 0.13 in H_2O)]; ^{1}H NMR (400 MHz, CD_3OD) δ = 3.27 (dd, J = 8.0, 9.1 Hz, 1H; H2), 3.30 (m, 1H; H5), 3.31–3.35 (m, 2H; H3/4), 3.71 (dd, J = 5.6, 11.9 Hz, 1H; H6), 3.91 (dd, J = 2.1, 12.0 Hz, 1H; H6′), 4.37 (d, J = 7.7 Hz, 1H; H1) 4.68 (d, J = 11.8 Hz, 1H; C*H*H), 4.96 (d, J = 11.8 Hz 1H; CH*H*′) 7.29 (m, 1H; Ph), 7.35 (m, 2H; Ph), 7.45 (d, J = 7.3 Hz, 2H; Ph) [lit.[65] ^{1}H NMR (400 MHz, CD_3OD) δ = 3.25 (dd, J = 8.1, 9.2 Hz, 1H; H2), 3.27 (m, 1H; H5), 3.30–3.37 (m, 2H; H3/4), 3.69 (dd, J = 5.5, 11.7 Hz, 1H; H6), 3.89 (dd, J = 1.8, 11.7 Hz, 1H; H6′), 4.35 (d, J = 7.7 Hz,1H; H1), 4.66 (d, J = 11.7 Hz,1H; CH_2), 4.93 (d, J = 11.7 Hz, 1H; CH_2′), 7.27 (d, J = 7.3 Hz,1H; Ph), 7.32 (dd, J = 5.1, 7.3 Hz, 2H; Ph), 7.42 (d, J = 8.0 Hz, 2H; Ph)]; ^{13}C NMR (100 MHz, CD_3OD) δ = 61.7 (t, C6), 70.4 (d), 70.7

(t, CH_2Ph), 74.0 (d, C2), 77.0 (2d), 102.6 (d, C1), 127.5 (d, Ph), 127.8 (2d, Ph), 128.0 (2d, Ph) 138.1 (s, Ph); ESI^-: *m/z* (%): 305 (100) $[M+Cl^-]$.

Cyclohexyl β-D-glucopyranoside. TLC $R_f = 0.4$ (EtOAc/MeOH, 4:1); ^{1}H NMR (400 MHz, CD_3OD) $\delta = 1.33$ (m, 5H; CH_2), 1.57 (m, 1H; C*H*H), 1.78 (m, 2H; CH_2), 1.96 (m, 2H; CH_2), 3.15 (dd, $J = 8.1$, 9.1 Hz, 1H; H2), 3.26–3.33 (m, 2H; H4/5), 3.37 (at, $J = 9.0$ Hz, 1H; H3), 3.67 (dd, $J = 5.6$, 12.0, 1H; H6), 3.70 (m, 1H; C*H*) 3.89 (dd, $J = 2.0$, 12.0, 1H; H6′), 4.41 (d, $J = 7.8$ Hz, 1H; H1) [lit.[66] ^{1}H NMR (400 MHz, CD_3OD) $\delta = 4.52$ (d, $J = 8.1$ Hz, 1H; H1)]; ^{13}C NMR (125 MHz, CD_3OD) $\delta = 25.5$ (2t, CH_2), 27.2 (t, CH_2), 33.1 (t, CH_2), 35.1 (t, CH_2), 61.7 (t, C6), 70.7 (d, C4), 77.2 (d, CH) 74.1 (d, C2), 76.8 (d, C5), 77.1 (d, C3), 101.3 (d, C1); ESI^-: *m/z* (%): 297 (10) $[M+Cl^-]$, 217 (40)-$[(M-C_6H_{11})+Cl^-]$.

Abbreviations

BpβG = *Bacillus polymyxa* β-glycosidase, Fuc = fucose, Gal = galactose, GalA = galacturonic acid, Glc = glucose, Glc-6-P = glucose-6-phosphate, GlcNAc = *N*-acetyl glucosamine, Man = mannose, 4MUGly = 4-methylumbelliferyl β-D-glycoside, pNPGly = *para*-nitrophenyl β-D-glycoside, SsβG = *Sulfolobus solfataricus* β-glycosidase, Xyl = xylose

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Keywords: carbohydrates · enzymes · glycosidases · glycosides · substrate specificity

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