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Enzymatic Thioxyloside Synthesis: Characterization of Thioglycoligase Variants Identified from A Site-Saturation Mutagenesis Library of *Bacillus Circulans* Xylanase

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Thioglycoligases are engineered enzymes for the synthesis of thioglycosides that are derived from retaining glycosidases by replacing the acid/base catalyst. The optimal choice of substitution for the acid/base mutant is currently unknown, so to investigate this question a complete acid/base library of the model glycosidase *Bacillus circulans* xylanase (Bcx) was generated by using site-saturation mutagenesis. A novel screening approach combining active site titration with semiquantitative product analysis by thin layer chromatography was established and used to evaluate specific activities of each mutant enzyme within crude cell lysates. The six most active Bcx variants were analyzed in more detail, a pH optimum of 8.5 was established and the identity of reaction products was confirmed. Optimal choices for substitution were small, preferably polar amino acids such as threonine, cysteine, and serine. We discuss the resultant data in the context of previously published studies on thioglycoligases.

Introduction

Oligosaccharides play important roles in many biological processes,[1–3] thus considerable potential exists for their use as therapeutics in blocking undesirable processes.[4–6] However, natural oligosaccharides and glycoconjugates are prone to degradation by endogenous glycosidases and thus would have limited utility in such a role. A solution to this problem is the use of thioglycosides in which the glycosidic oxygen is replaced by a sulfur, because such thioglycosides are good mimics, but are resistant to glycosidase digestion. In recent years several reports have been published in which mutant glycosidases, wherein the acid/base residue has been replaced by a neutral residue (Scheme 1), have been successfully turned into thioglycoligases, which catalyze the synthesis of thioglycans.[7–14] Incubation of the variant with a donor sugar bearing a good leaving group such as dinitrophenolate or fluoride results in the relatively rapid formation of a covalent glycosyl–enzyme intermediate without need for the acid catalyst. Because the mutant enzymes also lack the general base catalyst, the rates of transglycosylation to water or hydroxyl-containing sugars are extremely low. However, efficient transfer occurs to acceptor sugars bearing a suitably positioned thiol group, because the thiolate anion is more nucleophilic and does not require general base assistance.

While considerable effort has been expended in identifying optimal replacements for the catalytic nucleophile in glycosynthases by saturation mutagenesis at that position, only one such study to optimize thioglycoligases has been reported.[12]

Consequently, information on which residues provide optimal substitutions is limited. A well-studied model glycosidase on which to perform such a study is the *Bacillus circulans* xylanase Bcx. This has the added advantage that we are interested in the generation of selective sulfur-linked xylo-oligosaccharides as enzymatically inert analogues for protein structural studies on that same system.

Here we report the generation and characterization of a wide series of acid/base variants of Bcx that function as thioglycoligases. Site-saturation mutagenesis was performed to generate a complete acid/base catalyst mutant library of Bcx. A novel screening approach in crude cell lysates that combines the active site titration method[15,16] to quantify native enzyme with semiquantitative thin layer chromatography (TLC) to quantify products was used to pick active variants. The best six of these were characterized in more detail.

Results and Discussion

Generation of acid/base catalyst mutant library and screening

The enzymatic synthesis of *S*-glycosidic linkages employing thioglycoligases, a new class of engineered enzymes, is a concept that has recently emerged. So far four β-glycosidases[7,10–14] and two α-glycosidases[8] have been successfully turned into thioglycoligases by replacing the acid/base catalyst. Activated

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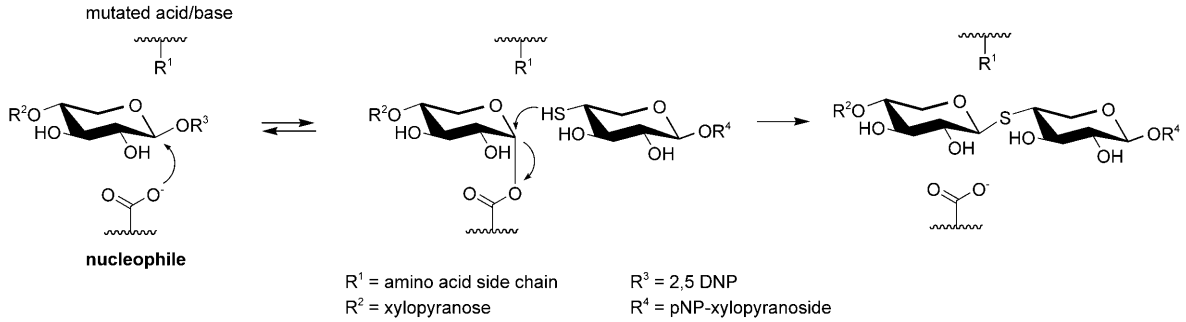


Scheme 1. Mechanism of transglycosylation by a thioglycoligase.

donor sugars are employed to facilitate the formation of the glycosyl enzyme intermediate in the absence of the acid catalyst. Thiosugar acceptor moieties then accomplish the nucleophilic attack without the need for base catalysis, yielding an *S*-glycosidic product. By using PCR and the appropriate degenerate primers a Bcx gene library containing all 20 possible variants modified at the acid/base position (Glu172) was generated and cloned into the pET-27b expression system (Novagen). The mutant library was expressed in *Escherichia coli* BL21 cells and the quantities of each active enzyme in the crude cell lysate and in pure protein preparations were determined by active site titration employing the mechanism-based inhibitor 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside.[16,17] This reagent rapidly forms a covalent glycosyl-enzyme intermediate with stoicheometric release of 2,4-dinitrophenol. Turnover of this intermediate through hydrolysis is slower than its formation—leading to a burst phase followed by a steady state. Examples of such active site titrations for three of the Bcx acid/base variants are shown in Figure 1. From these curves, the *y*-intercept of the back extrapolation of the linear region yielded the amount of 2,4-dinitrophenol released, thus, through its extinction coefficient, the enzyme concentration. In the initial screen the active site titration method affords a very precise and rapid quantitation of active variants from crude cell lysates without the need for purification.

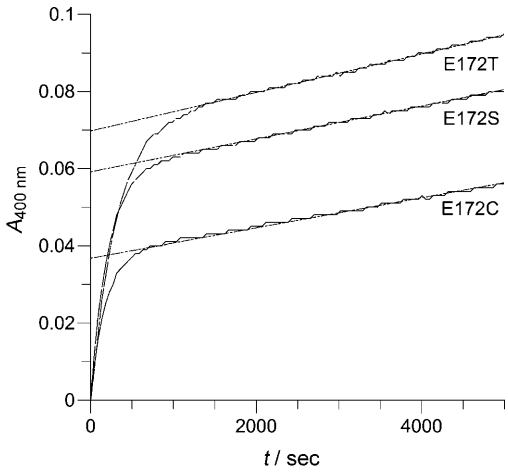


Figure 1. Determination of enzyme concentration by active site titration. The active site titration curves for Bcx E172T, E172S, and E172C are shown with a linear fit (-----) for each. The y-intercept and the extinction coefficient of 2,4-dinitrophenol were used to calculate the active site concentration.

To test the relative abilities of each acid/base variant to catalyze the transglycosylation of xylose moieties onto a thio sugar, mutant enzymes were mixed with donor sugar 2,5-dinitrophenyl β-xylobioside (DNP-X2) and acceptor sugar 4-nitrophenyl 4-thio β-xylobioside (4S-*p*NP-X2). Equal amounts of each enzyme, based upon the active site titration data, were used and product yield was estimated by semiquantitative TLC using a digital camera and the software ImageJ (NIH).[19] Mutants in which glutamate 172 was replaced by threonine, serine, cysteine, valine, alanine, glycine, leucine, isoleucine, methionine, asparagine, glutamine, proline or phenylalanine were found to produce the expected thioglycoside whereas the other amino acid replacements, including the wild type enzyme, did not form detectable amounts of product (Figure S1 in the Supporting Information). At that stage only the six most active acid/base variants (threonine, serine, cysteine, valine, alanine and isoleucine) were chosen for purification and more detailed analysis by HPLC.

pH profile of Bcx thioglycoligase reaction

Preliminary screening of the alanine variant at pH 6.0, 7.0, and 8.0 indicated that pH 8.0 gave the best yields and tests with the other variants concurred, with roughly comparable yields in each case. Consequently, the best of these, the E172T variant, was chosen as a representative to determine the pH-dependence of the thioglycoligase reaction catalyzed by Bcx acid/base mutant enzymes. As shown in Figure 2 the largest amount of product was formed at pH 8.5, with only very little product formation at pH 6.0, the pH optimum of the native enzyme for xylan hydrolysis.[18] This corresponds well with previous studies on another thioglycoligase; these studies showed that the pH dependence apparently reflects formation of the thiolate anion as the reactive species.[12] The pK_a measured in this case (~8) is somewhat lower than would be expected for an alkanethiol, but is likely perturbed downwards due to its binding in an active site cavity that formerly contained an anion (Glu172). Interestingly, we observed a decrease in product formation at pH values above 8.5. Possible reasons for this drop in yield at higher pH values include instability of the

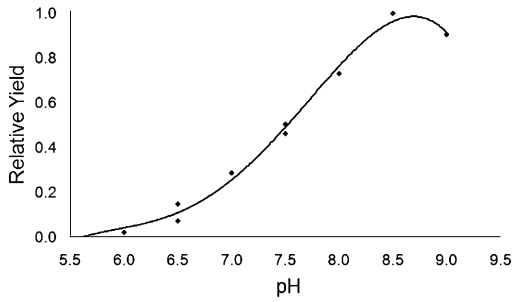


Figure 2. The pH profile for thioglycoside synthesis by Bcx E172T. Relative yields of product formed from the reaction of DNP-X2 (1 mM) with 4S-*p*NP-X2 (1 mM) at a series of pH values. Product was quantitated by HPLC (UV/Vis detection) relative to an internal standard.

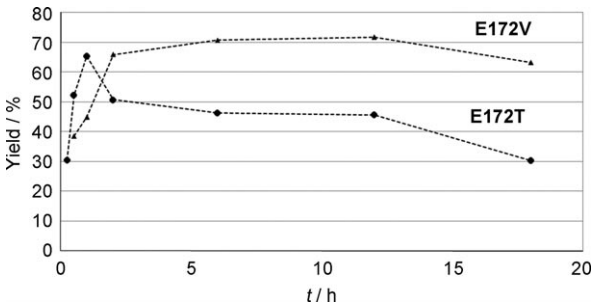


Figure 3. Time course for reaction of the E172T and E172V Bcx variants. Percentage yield of product formed from reaction of DNP-X2 (1 mM) with 4S-*p*NP-X2 (1 mM) at pH 8.5 at several time points for E172T and E172V. Product was quantitated by HPLC (UV/Vis detection) relative to an internal standard.

enzyme, greater hydrolysis or possibly a need for a protonated residue of higher pKa to assist in carboxylic acid departure.

Characterization of acid/base mutants by HPLC

The efficacies of the six most active variants as thioglycoligases were analyzed in more detail and the results are summarized in Table 1. Reactions were performed at the pH optimum (pH 8.5) and aryl xylotetraoside formation was analyzed by reversed-phase HPLC with UV/Vis detection. Product was quantified at two time points by monitoring the absorbance of *p*-nitrophenol (*p*NP) at 300 nm and quantifying the peak area. At 1 h the most active acid/base mutant enzymes were the threonine and cysteine variants with yields of 65 or 62%, respectively. Interestingly, after 18 h the yields for the variants possessing polar amino acids (threonine, serine, and cysteine) in place of the acid/base residue were lower than after 1 h, suggesting product hydrolysis, while for the nonpolar variants (valine, alanine, and isoleucine) reactions were initially slower, but yields after 18 h were higher.

Table 1. Reaction yields for selected Bcx acid/base variants.

Mutant	Yield [%] after 1 h	Yield [%] after 18 h
E172T	65	30
E172C	62	47
E172S	56	37
E172V	45	63
E172A	43	58
E172I	35	47

A more in-depth analysis of time-dependent yield was performed for the E172T and E172V mutant enzymes as representatives of each class, Figure 3. The threonine variant reacts faster than the valine variant over the first hour; however, this is followed by a substantial drop in yield. By contrast the valine acid/base mutant enzyme starts out more slowly, but once at its peak the yield decreases only very little, if at all. The most likely explanation for this behaviour of the threonine variant, thus likely of the other polar substitutions, was that these variants are capable of hydrolyzing the nitrophenyl moiety from the substrate and/or product–rendering products "invisible" by UV/Vis. Indeed, TLC analysis of such reaction mixtures using an ammonium molybdate/sulfuric acid stain revealed not only the expected *p*NP-tetrasaccharide product, but also the free tetrasaccharide and disaccharide. This hydrolytic reactivity was therefore further probed kinetically.

Kinetic studies of the hydrolysis of 4-nitrophenyl xylobioside

The kinetic parameters for enzymatic hydrolysis of 4-nitrophenyl xylobioside (*p*NP-X2) catalyzed by the E172S, E172T, E172C, E172V, E172A and E172I variants in the absence of thiosugar acceptor are shown in Table 2. The K_M value for E172I was substantially higher than the concentration of *p*NP-X2 that could be achieved, thus substrate depletion analysis was performed to determine the k_{cat}/K_M value. In general, mutant enzymes with polar substitutions at the acid/base position exhibit higher rates of hydrolysis than do those with nonpolar residues. This corresponds well with the results observed in Table 1 and Figure 3 where the total amount of *p*NP-tetrasaccharide product detected for polar variants is decreased after longer incubation times.

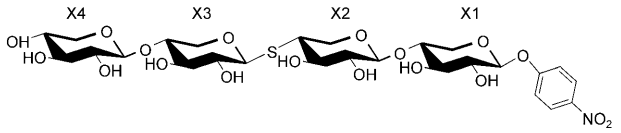
Table 2. Kinetic parameters for the hydrolysis of 4-nitrophenyl xylobioside by Bcx variants.

Enzyme	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [s^{-1} mM^{-1}]
Bcx[a]	24	49	0.48
E172S	0.036	2.6	0.014
E172T	0.028	3.6	0.0076
E172C	0.015	7.0	0.0021
E172A	0.0079	1.5	0.0052
E172V	0.0050	6.2	0.00082
E172I	n.d.[b]	n.d.[b]	0.00034

All values are ± ca. 10%. [a] Parameters published previously at a pH of 6.0.[22] [b] n.d., not determined.

The only previous such comparison of activities of thioglycoligase variants is that for mutant enzymes developed from *Agrobacterium sp.* β-glucosidase (Abg).[12] Interestingly, in that case substitution of the acid/base catalyst of Abg by a glutamine residue resulted in the highest activity, with the next highest activity variants being those in which asparagine, glycine, alanine, threonine, and serine were present. Preference for Gln was particularly great when less activated donor sugars were employed. A number of other glycosidases (*Cellulomonas fimi* β-mannosidase,[10] *Escherichia coli* α-xylosidase,[8] *Sulfolobus solfataricus* α-glucosidase,[8] *Xanthomonas manihotis* β-galactosidase,[7,13] and *Thermotoga maritima* β-glucuronidase[11]) have been successfully converted into thioglycoligases by replacement of the acid/base catalyst with alanine: presumably a more polar substituent would be a better choice for future developments. Those results compare well with the findings of this study. Substitution of the acid/base catalyst with residues having small, polar or amide side chains appears to work best to create a thioglycoligase, though care must be taken with reaction timing to minimize hydrolysis of aryl glycoside bonds in the products—or noncleavable thioglycosides should be employed.

Table 3. ^{1}H and ^{13}C NMR chemical shifts and coupling constants of the thioglycoside product.



δ Ring	H-1	H-2	H-3	H-4	H-5a	H-5b
X1	5.15	3.61	3.55	3.79	4.08	3.52
X2	4.47	3.21	3.36	2.95	4.01	3.29
X3	4.39	3.26	3.45	3.69	4.03	3.39
X4	4.35	3.14	3.31	3.51	3.86	3.19

J Ring	$J_{1,2}$ [Hz]	$J_{2,3}$ [Hz]	$J_{3,4}$ [Hz]	$J_{4,5a}$ [Hz]	$J_{4,5b}$ [Hz]	$J_{5a,5b}$ [Hz]
X1	7.8	9.0	9.6	8.4	10.8	12.0
X2	9.6	9.0	9.6	4.8	10.8	11.4
X3	8.4	9.0	9.0	4.8	12.0	12.0
X4	7.8	9.0	9.0	5.4	12.0	11.4

δ Ring	C-1	C-2	C-3	C-4	C-5
X1	100.2	73.8	73.0	76.4	63.5
X2	102.2	74.7	72.9	45.6	66.9
X3	84.5	72.4	75.4	76.5	66.5
X4	102.2	73.1	76.0	69.6	65.6

Product characterization by mass spectrometry and nuclear magnetic resonance spectroscopy

Initial confirmation of the formation of the thioglycoside, rather than an O-glycoside, which would leave a free thiol in the product, was provided by TLC tests in the presence and absence of Ellman's reagent (5, 5′-dithiobis-(2-nitrobenzoic acid), DTNB). No shift in TLC behaviour was observed. The structure of the trisaccharide product was fully confirmed by mass spectrometry and NMR analysis. Indeed the mass spectrum (Figure S2) displayed the expected peak at 706 *m/z*, corresponding to the sodium adduct of the product ($C_{26}H_{37}NO_{18}S+Na^{+}$). Assignments of the ^{13}C- and ^{1}H NMR spectra (see Table 3 and Figure S3) were carried out by using the assignments for *p*NP xylotetraoside as a guide.[19,20] X1 refers to the xylose residue closest to the reducing end, and X2–X4 are numbered towards the nonreducing end. The anomeric carbon on X3 as well as C4 on X2 show substantially lower chemical shifts than would be expected for an O-linked tetrasaccharide ($\delta = 84.5$ for C1X3 and $\delta = 45.6$ for C4X2 compared to 102.885 and 77.601 for the O-analogue), thus indicating that they are attached to a sulfur atom. Spin–spin coupling constants show that all four anomeric carbons, including the anomeric centre involved in the sulfur linkage, have the β-configuration ($J_{1,2} = 7.8$–9.6). These data therefore fully support the formation of the expected thioglycoside product. Such thiol-directed regioselectivity of transglycosylation reactions catalyzed by thioglycoligases is consistent with findings on other thioglycoligases.[10,11,21]

Conclusions

Semiquantitative TLC screening combined with an active site titration method to quantify enzyme provided a novel and rapid method for the evaluation of the library of candidate thioglycoligases in crude cell extracts. This approach could be applied much more broadly to speed the analysis of mutant enzyme libraries. In conjunction with data from previous studies, we conclude that replacing the acid/base catalyst of a retaining glycosidase with a residue having either a small or a polar side chain or an amino acid with an amide on the side chain generated the most active thioglycoligases. As discussed previously[10,12] the inactivity of the wild type enzyme in thioglycoside formation is probably a result of deleterious electrostatic interactions rather than steric repulsion, as evidenced by the activity of the Gln mutants.

Experimental Section

Site-saturation mutagenesis and cloning: A PCR approach was applied to replace the acid/base catalyst E172 with all other possible amino acid residues, employing the N-terminal primer Bcx_NdeI and a degenerate primer Rev_AA that binds at the C terminus (Table S1). 25 PCR cycles were performed. The denaturation step lasted 30 s at 95 °C, and the annealing step was performed at 55 °C for 40 s. Elongation was performed for 40 s at 72 °C. The library was then cloned into pET-27b vector (Novagen) using NdeI and HindIII restriction enzyme sites and subsequently transformed into *E. coli* BL21 DE3 cells. 63 colonies were grown overnight at 37 °C in deep-well plates containing LB medium (600 μL) and kanamycin (50 $\mu g\,mL^{-1}$). Sequence analysis revealed that all possible amino acid substitutions were produced except for E172Q, E172H, and E172I. In order to complete the acid/base mutant library, the three missing variants were generated by PCR using individually designed primers (Table S1) and cloned identically into the expression vector.

Protein expression and purification: Individual clones of *E. coli* BL21 DE3 cells, each harbouring a coding sequence of one of the 19 acid/base mutants or the wild type, were grown in deep-well plates (96 wells) overnight as described above. The next day the overnight culture (100 μL) was used to inoculate LB medium (500 μL) containing kanamycin (50 $\mu g\,mL^{-1}$) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.7 mM). The cultures were grown under vigorous shaking at 30 °C for 4 h, then centrifuged at 3200 *g* for 15 min (Eppendorf Centrifuge 8510R). Cell pellets were resuspended in MES buffer (20 mM, pH 6.0), containing sodium chloride (50 mM) and polymyxin B sulfate (1 $mg\,mL^{-1}$) and incubated at 37 °C for 30 min under gentle shaking. Crude cell extracts obtained after centrifugation (16000 *g* for 15 min) were used to screen for thioglycoligase activity by thin layer chromatography (TLC).

Pure enzyme preparations for analysis were obtained from 0.5 L cultures. Cells were induced with IPTG at an $OD_{600\,nm}$ ~0.6 (final IPTG concentration = 0.2 mM) and grown at 30 °C overnight. After centrifugation cell pellets were resuspended in MES buffer (20 mM, 30 mL, pH 6.0) and treated with lysozyme (25 mg, Sigma Aldrich) and Benzonase® Nuclease (125 units, Novagen) at 37 °C under gentle shaking for 30 min. After centrifugation (27000 *g* for 15 min) cell debris was discarded and the supernatant was further diluted with two volumes of MES buffer and loaded onto SP-Sepharose ion-exchange columns (HiTrap SP FF, 5 mL, GE Healthcare) and eluted with a 0–1 M NaCl gradient. Fractions containing Bcx variants were pooled and concentrated (Amicon Ultra Centrifugal Filters Ultracel 10000 NMWL, Millipore) and further purified by size-exclusion chromatography (HiPrep 16/60 Sephacryl S-100, GE Healthcare) in HEPES buffer (20 mM, pH 8.0), containing NaCl (50 mM).

Active site titration: To determine the enzyme concentration crude or purified enzyme preparations (20 μL) were inactivated at 40 °C in MES buffer (20 mM, 200 μL, pH 6.0), containing NaCl (50 mM) and the inactivator 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (2F-DNP-X2, 0.25 mM).[22] The reaction mixture was placed into a UV/Vis spectrophotometer (UNICAM UV4) equipped with Vision 3.0 software and the release of 2,4-dinitrophenol was monitored at a wavelength of 400 nm until a linear rate of hydrolysis of the inactivator was observed. The y-intercept of the linear region was determined by linear regression using the program GraFit 5.0.[23] The obtained values and the extinction coefficient of 2,4-dinitrophenol (11.40 $mM^{-1}\,cm^{-1}$) were then used to calculate the enzyme concentration.

Thioglycoligase screening: To identify acid/base variants that catalyze the thioglycoligase reaction, samples of each Bcx variant (0.1 μM) were assayed in reaction mixtures containing HEPES (20 mM), NaCl (50 mM), DNP-X2 (donor, 1.6 mM), 4S-*p*NP-X2 (acceptor, 1.6 mM), and DTT (2.5 mM) at pH 8.0 at 40 °C. Aliquots taken after 10, 15, 20, 25, 30 and 60 min were frozen at −70 °C then analyzed by TLC using propan-2-ol/ethyl acetate/water (3:1:1) as solvent. TLC-plates were visualized under UV-illumination and the formed product was quantified on digital blots with ImageJ.[24]

Thioglycoside products of the six most active acid/base variants (threonine, serine, cysteine, valine, alanine and isoleucine) were also tested for the presence of free thiols by adding an equal amount (*v*/*v*) of a saturated solution of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) or water to the reaction mixture. Solutions were incubated at room temperature for 5 min and products were analyzed by TLC. The R_f values of the products did not shift when pre-incubated with DNTB indicating that the sulfur, in each case, was involved in an *S*-glycosidic linkage, thus not present as a free thiol. Control experiments with the thiol-containing acceptor sugar confirmed that this assay protocol was effective.

Mass spectrometry and NMR spectroscopy: For detailed characterization of the thioglycoside product a reaction mixture containing the E172V variant (8.5 μM) and DNP-X2 and 4S-*p*NP-X2 (2.5 mg each) in HEPES buffer (20 mM, pH 8.0), NaCl (50 mM), and DTT (2.5 mM) was incubated overnight at ambient temperature. The reaction mixture was then filtered (Millex GP 0.22 μm, Millipore) and loaded onto a reverse-phase cartridge (Sep-Pak® Light tC18, sorbent weight 145 mg, Waters). The column was washed with water (4 mL) and the product then eluted in 90% methanol. Eluted fractions were further purified by HPLC on a C18 column (particle size of 5 μm, pore size of 300 Å, Jupiter) using a 0–100% gradient of acetonitrile in water. Fractions containing the formed thioglycoside (~2 mg in total) were pooled, dried on a rotary evaporator, then subsequently dissolved in deuterium oxide (1 mL) for NMR analysis. The purified product was also analyzed by mass spectrometry (Waters LC-MS system, ZQ2000).

Determination of thioglycoligation reaction yields by HPLC: Quantification of yields was achieved by analysis of the peak area corresponding to the eluted thiosugar by using reversed-phase HPLC (Waters Delta 600E multisolvent delivery system). Reaction mixtures (200 μL) containing purified enzyme (0.89 μM), NaCl (50 mM), DNP-X2 (1 mM), 4S-*p*NP-X2 (1 mM), DTT (2.0 mM), and Tris (pH 8.5, 20 mM) were incubated at ambient temperature for either 1 or 18 h. A more detailed time course was collected for the E172V and E172T variants, sampling at several times between 30 min and 18 h. The reactions were terminated by incubation at 85 °C for 5 min then stored at −70 °C. The reaction mixture was filtered (Millex GP 0.22 μm, Millipore) then loaded onto a C18 column (particle size 5 μm, pore size 300 Å, Jupiter). A gradient of 0–100% acetonitrile in water was used to elute the product and the absorbance at 300 nm was monitored and quantified with the software Empower (Waters). *Para*-nitrophenyl cellobioside was employed as internal standard in all HPLC runs.

pH profile for Bcx E172T: Yields of the thioglycoligase reaction of Bcx E172T were determined at pH values ranging from 5.5–9.0. Reactions were performed as described above in a series of buffers (20 mM): MES (pH 5.5–6.5), PIPES (pH 6.5–7.5), and Tris (pH 7.5–9.0). Overlapping data points from different buffers indicated that no significant change in activity results from changing buffer identities. HPLC analysis was achieved as described above.

Kinetic studies of the hydrolysis of 4-nitrophenyl xylobioside: The rates of enzymatic hydrolysis of *p*NP-X2 were determined using a continuous assay. To an appropriate concentration of *p*NP-X2 in Tris pH 7.0 (20 mM) and NaCl (50 mM), warmed to 40 °C, an aliquot of enzyme was added. Substrate hydrolysis was monitored by measuring the rate of 4-nitrophenolate release at 400 nm. The millimolar extinction coefficient of $\varepsilon = 9.42$ was used to convert from absorbance units to millimolar values. The k_{cat}/K_M value for E172I was determined by substrate depletion.[25] For this analysis *p*NP-X2 (0.0114 mM) in Tris buffer (20 mM), pH 7.0, and NaCl (50 mM) was incubated at 40 °C, then an aliquot of enzyme was added (final concentration of 0.11 mM). The time course for release of *p*NP was monitored as above and fit to the equation: $A(t) = A_{inf}(1 - e^{kt}) + k$ using GraFit 5.0 software.[23] Division of this rate constant by the enzyme concentration yielded the k_{cat}/K_M value.

Glossary: Bcx, *Bacillus circulans* xylanase; DNP-X2, 2,5-dinitrophenyl β-xylobioside; 4S-*p*NP-X2, 4-nitrophenyl 4′-thio β-xylobioside; *p*NP-X2, 4-nitrophenyl xylobioside; 2F-DNP-X2, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside; DTT, Dithiothreitol; DNP, dinitrophen-

yl group; *p*NP, *para*-nitrophenyl group; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Tris, tris(hydroxymethyl)amino-methane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTNB, 5, 5′-dithiobis-(2-nitrobenzoic acid);

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Keywords: acid/base catalysts · enzyme catalysis · hydrolases · synthesis · thioglycoligases

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