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Catalytic properties of a mutant β-galactosidase from *Xanthomonas manihotis* engineered to synthesize galactosyl-thio-β-1,3 and -β-1,4-glycosides

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Abstract The identity of the acid/base catalyst of the Family 35 β-galactosidases from *Xanthomonas manihotis* (BgaX) has been confirmed as Glu184 by kinetic analysis of mutants modified at that position. The Glu184Ala mutant of BgaX is shown to function as an efficient thioglycoligase, which synthesises thiogalactosides with linkages to the 3 and 4 positions of glucosides and galactosides in high (>80%) yields. Kinetic analysis of the thioglycoligase reveals glycosyl donor K_m values of 1.5–21 μM and glycosyl acceptor K_m values from 180 to 500 μM. This mutant should be a valuable catalyst for the synthesis of metabolically stable analogues of this important glycosidic linkage.

Keywords: Glycoside hydrolase Family 35; *Xanthomonas manihotis* β-galactosidase; Acid/base catalyst; Thioglycoligase; Thioglycosides

1. Introduction

Due to the important roles of oligosaccharides and glycans in many biological processes in cells [1], the naturally occurring oligosaccharides and their analogues are of particular interest as carbohydrate-based therapeutics [2]. However, the application of carbohydrate-based therapeutics may be limited, in some cases, as a consequence of their susceptibility to enzymatic hydrolysis by endogenous glycosidases [3]. One strategy towards circumventing this problem is to replace the interglycosidic oxygen atom with a sulfur atom to give sulfur-linked oligosaccharides [4,5]. Glycosidase-resistant thioglycoside analogues of the original oligosaccharides are therefore attractive candidates as potential therapeutics, as stable antigens, and as biochemical probes due to their much lower rates of chemical and enzymatic hydrolysis and their similar conformations to those of their natural *O*-linked counterparts [6]. A number of successful chemical syntheses of thio-oligosaccharides have been reported [7–9], but control of stereochemistry remains challenging. Recently, enzymatic strategies have been reported using mutant forms of glycosidases: thioglycoligases [10] and thioglycosynthases [11] as well as, but less efficiently to date, glycosyltransferases [12].

Thioglycoligases are mutant enzymes derived from retaining glycosidases in which the acid/base carboxylic acid residue has been replaced by an amino acid that has no negative charge (Fig. 1). When these mutants are incubated with substrates bearing a good leaving group, such as dinitrophenol (DNP) or fluoride, they relatively rapidly form a covalent glycosyl-enzyme intermediate because they do not need assistance from the acid catalyst. Since the mutants have no general base catalyst, the rates of transglycosylation to normal acceptors such as water and hydroxyl-containing sugars are extremely low. However, transfer occurs efficiently to acceptors bearing a suitably-positioned thiol since the thiol group is much more nucleophilic and requires no general base catalytic assistance. The thioglycoligase strategy has been applied recently in the synthesis of thio-linked inhibitors for use as structural probes [13] and in the modification of glycans on the surface of a model glycoprotein [14].

A class of glycosidic linkages that is of particular interest, but which has not yet addressed by glycosynthase [15] or thioglycoligase strategies, is that of the Gal-β-1,3-GlcNAc and Gal-β-1,3-GalNAc linkages found in gangliosides and glycoproteins. While β-galactosidases are found in glycoside hydrolase (GH) Families 1, 2, 35, and 42, only those in Family 35 have specificities directed toward Gal-β-1,3-linkages. This family contains the human lysosomal β-galactosidases responsible for catabolism of such glycoconjugates and deficiencies in this enzyme arising from mutations lead to the neurological disorders GM1-gangliosidosis and Morquio B. syndrome [16]. Through the use of mechanism-based inactivators that result in trapping of a glycosyl-enzyme intermediate, the catalytic nucleophile of this enzyme was identified as Glu268 [17]. Labeling of the equivalent residue, Glu260, in the homologous bacterial enzyme from *Xanthomonas manihotis* confirmed the nucleophilic role of this residue [18]. No information was available on the identity of the acid/base catalyst until the relatively recent solution of the three-dimensional structure of the Family 35 β-galactosidase from *Penicillium* sp. by X-ray crystallography [19]. On the basis of a product complex with galactose,

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Abbreviations: DNP, 2,4-dinitrophenyl; PNP, 4-nitrophenyl; BgaX, β-galactosidase from *Xanthomonas manihotis*; Abg, β-glucosidase from *Agrobacterium* sp.; GH, glycoside hydrolase; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; ESI, electrospray ionization; DNPGal, 3,4-dinitrophenyl β-D-galactopyranoside; PNPGal, 4-nitrophenyl β-D-galactopyranoside; PNP4SGlc, 4-nitrophenyl 4-deoxy-4-thio-β-D-glucopyranoside; PNP3SGlc, 4-nitrophenyl 3-deoxy-3-thio-β-D-glucopyranoside; PNP6SGlc, 4-nitrophenyl 6-deoxy-6-thio-β-D-glucopyranoside; PNP4SGal, 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside; PNP3SGal, 4-nitrophenyl 3-deoxy-3-thio-β-D-galactopyranoside

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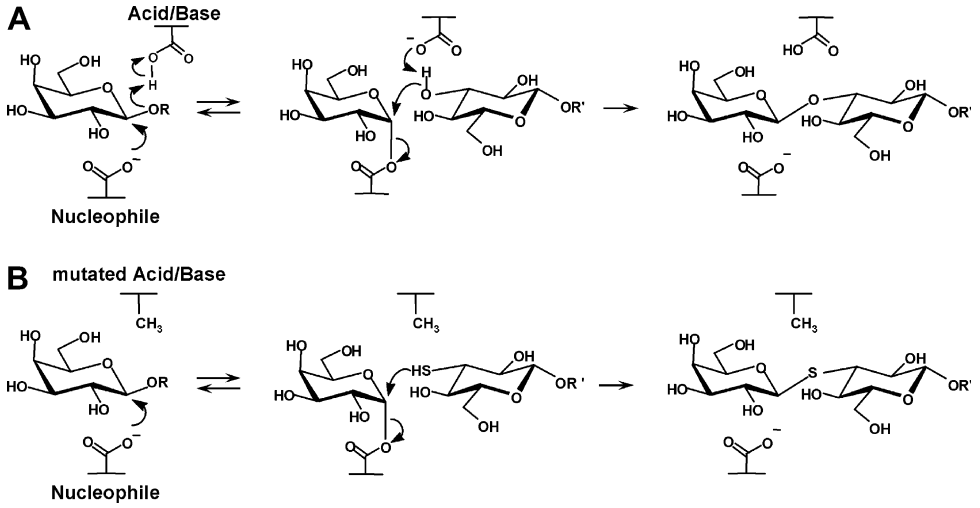


Fig. 1. Mechanisms of transglycosylation by retaining galactosidases (A) and thiogalactoligases (B).

Glu200 was identified as the putative acid/base catalyst. However, there has been no confirmation of this proposed role by kinetic analysis of mutants. The β-galactosidase from *X. manihotis* (BgaX) is a good test system with which to confirm this assignment and to assess the potential for generation of thioglycoligases of the desired specificity. The wild type enzyme has been shown previously to synthesise Gal-β-1,3-GlcNAc linkages via transglycosylation, albeit in rather low yields [20,21], though attempts to generate a glycosynthase from BgaX have been unsuccessful to date (Blanchard, J. and Withers, S.G., unpublished data).

Herein, we describe the confirmation of Glu184 as the acid/base catalyst in BgaX, analogous to Glu200 in *Penicillium* sp. β-galactosidase, by azide rescue analysis with the Glu184Ala mutant. We demonstrate that this mutant functions as an efficient thioglycoligase capable of synthesizing both β-1,3- and β-1,4-thioglycosidic linkages, depending on the position of the thiol-group in the acceptor. Detailed kinetic analyses revealed low K_m values for both glycosyl donors and thio-sugar acceptors, and substantial preference for transfer to the 3-position of gluco-configured acceptors.

2. Materials and methods

2.1. Materials and general analysis

All chemicals were obtained from the Sigma Chemical Co. unless otherwise specified. *Pwo* polymerase was purchased from Roche and restriction enzymes from Fermentas (Germany). 4-Nitrophenyl 4-deoxy-4-thio-β-D-glucopyranoside (PNP4SGlc), and 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside (PNP4SGal) were synthesized according to literature procedures [10]. The synthetic details of the preparation of 4-nitrophenyl 6-deoxy-6-thio-β-D-glucopyranoside (PNP6SGlc), 4-nitrophenyl 3-deoxy-3-thio-β-D-glucopyranoside (PNP3SGlc), and 4-nitrophenyl 3-deoxy-3-thio-β-D-galactopyranoside (PNP3SGal) will be published elsewhere. All ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz using a Bruker AV-400 spectrometer. Mass spectra of small molecules were recorded using a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an electrospray ionization (ESI) ion source. Thin layer chromatography (TLC) was performed on aluminum-backed sheets of silica gel $60F_{254}$ (Merck) of thickness 0.2 mm. The plates were visualized using UV light (254 nm) and/or by exposure to 10% sulfuric acid in methanol followed by charring.

2.2. Construction of BgaX-E184A and E184Q mutants

The mutations of the acid/base residue of BgaX were introduced using a mega primer PCR method. The gene encoding the 6x histidine tagged BgaX obtained by digestion of pTUG10N18/β-Gal [18] with *Nco*I and *Hin*dIII was subcloned into a pET28a vector (Novagen). The resulting plasmid, namely pET28BgaX$(His)_6$, was used as the template for the mutation of BgaX. The front ends of the genes for BgaX-E184A and BgaX-E184Q were first amplified using a T7 promoter primer and the following BgaX-E184A-rev primer (5′-GTCGGCGTAGGAGCCGTAGGCGTTCTCGAC-3′) and BgaX-E184Q-rev primer (5′-GTCGGCGTAGGAGCCGTACTGGTTCTCGAC-3′). The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and then used as megaprimers to obtain the full length genes with the T7 terminator primer. The final PCR products were digested with *Nco*I and *Hin*dIII, then extracted from the agarose gel using a QIAquick Gel Extraction Kit and ligated with pET28a that had been digested with the corresponding restriction enzymes. The resulting plasmids were designated pET28BgaX-E184A$(His)_6$ and pET28BgaX-E184Q$(His)_6$, respectively.

2.3. Expression and purification of BgaX-E184A mutant

The plasmids containing genes for BgaX mutants were transformed into *Escherichia coli*, and the recombinant *E. coli* was cultured for 6 h at 37 °C in Luria–Bertani medium containing 20 μg/mL of kanamycin, followed by overnight IPTG induction (0.2 mM) at 30 °C. The crude enzyme solutions were prepared by lysis of the harvested cells using Bugbuster™ solution (Novagen). The crude enzyme solutions were centrifuged at $10000 \times g$ for 30 min and the supernatants were loaded onto a HisTrap™ FF column (Amersham Biosciences). BgaX mutants were eluted using a step-gradient of 10 mM → 20 mM → 250 mM imidazole in 20 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl. For further purification, ammonium sulfate was added directly to the fractions containing BgaX mutants until the final concentration of ammonium sulfate was 2 M, then the enzyme solutions were subjected to hydrophobic interaction chromatography using a HiTrap™ Phenyl HP column (Amersham Biosciences). BgaX mutants were eluted with a linear gradient of 2 M → 0 M ammonium sulfate in 20 mM Tris–HCl buffer (pH 8.0). The pure enzyme fractions were combined, dialyzed, and then concentrated using an Amicon Ultra-4 filter unit (10000 Da, cut-off, Millipore). Concentrations of enzyme solution were quantified by the Bradford method using bovine serum albumin as a standard [22].

2.4. Hydrolysis and thioglycosylation kinetics

All kinetic studies were performed at 30 °C in pH 7.0, 100 mM phosphate buffer. Twenty microliters of BgaX-E184A was added to 100 μL of buffer containing either 3,4-dinitrophenyl β-D-galactopyranoside (DNPGal) or 4-nitrophenyl β-D-galactopyranoside (PNPGal) as donors and thio-sugar acceptors for transglycosylation. Hydrolysis of

the nitrophenyl galactosides was observed without thio-sugar acceptors. Rescue of the glycosidic bond cleaving activity of BgaX-E184A was performed in the presence of various concentrations of azide as a nucleophile at a fixed concentration of DNPGal (0.1 mM). The release of the nitrophenols was monitored at 400 nm using a microplate reader (SPECTRAMax plus, Molecular Devices Corporation). The values of K_m and k_{cat}, which are strictly apparent K_m and k_{cat} since they are determined at a fixed, non-saturating co-substrate concentration, were determined by fitting the initial velocity curves to the Michaelis–Menten equation using non-linear regression with the program GraFit [23].

2.5. Preparative thioglycoligase reactions and isolation of products

All thioglycoligase reactions were carried out at room temperature in 3–5 mL of 100 mM sodium phosphate buffer, pH 7.0. Thiosugar acceptors (~9 mg) were dissolved in 200 μL of DMF, and then an equal amount of DNPGal was added as donor. BgaX-E184A (0.3 mg) was added and the mixture then incubated at 25 °C. Reactions were monitored by TLC. Upon completion, the reaction mixtures were subjected to a C18 SEP PAK cartridge (Waters) to remove free sugars, enzyme, and salts, and then the solvent was evaporated under reduced pressure. Transfer products were purified by flash chromatography (EtOAc/MeOH/H_2O = 17:2:1 → 7:2:1) using Silica Gel 60 (230 ± 400 mesh) and reaction yields were determined by weighing the isolated products. The purified compounds were acetylated in pyridine and Ac_2O. The acetylated thio-disaccharides, purified using flash chromatography (EtOAc/hexanes = 1:1), were subjected to electrospray ionization mass spectrometry and ^1H and ^{13}C NMR spectroscopy.

3. Results and discussion

In order to confirm Glu184 as the acid/base catalyst in BgaX a mutant was made in which an alanine, a standard substitution to remove the charge and acid/base properties, was introduced at the position of Glu184 in BgaX. Michaelis–Menten parameters for hydrolysis of DNPGal and PNPGal (Table 1) were completely consistent with a role of Glu184 as acid/base catalyst, as follows. The observation of very similar k_{cat} values for two substrates of very different reactivity, DNPGal and PNPGal, with BgaX-E184A (2.1 and 2.6 min^{-1}) strongly suggested that the deglycosylation step (which would be independent of substrate leaving group ability) is rate-limiting in each case. In support of this conclusion are the very low K_m values, particularly that of around 100 nM for DNPGal with BgaX-E184A. Such a low value suggests accumulation of the intermediate, as would be expected if deglycosylation is rate-limiting. Importantly, k_{cat}/K_m values, which reflect the first irreversible step (glycosylation), differ by ~25-fold for the two substrates, as expected based upon their different reactivity. A second test of the role of Glu184 as acid/base catalyst is rescue of the activity of BgaX-E184A by an added nucleophile, such as azide anion. Generally, when reacted with activated substrates, the glycosyl-enzyme intermediate of retaining glycosidase mutants modified at the acid/base catalyst accumulates. However, upon introduction of azide or equivalent nucleophiles rapid turnover of the intermediate occurs via nucleophilic attack of the added external nucleophiles. Rescue of activity of the mutants thereby occurs with formation of a glycosyl azide product [24–26]. As expected, increasing concentrations of azide resulted in enhanced reaction rates of cleavage of DNPGal by BgaX-E184A (Fig. 2). These results, therefore, suggest that Glu184 of BgaX plays the role of the acid/base catalyst.

Table 1
Kinetic parameters for hydrolysis by BgaX wild type and mutant

Enzyme	Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})
WT[a]	PNPGal	2140	50	42 800
E184A	PNPGal	2.1	2.0	1050
E184A	DNPGal	2.6	0.08[b]	32 500[b]

[a]Data from reference [17]. Errors in kinetic parameters are less than 10% for K_m and 5% for k_{cat}.
[b]Errors on these parameters are ±50% due to the low enzyme concentration needed for the K_m determination in order to ensure Michaelian behaviour.

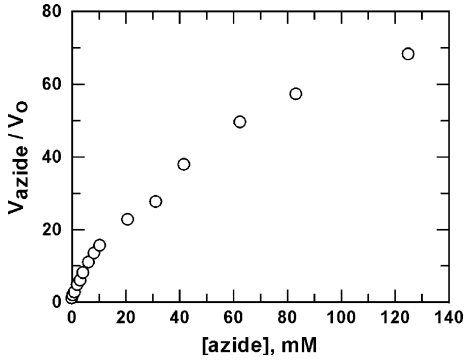


Fig. 2. Effect of added azide nucleophile on cleavage of DNPGal by BgaX-E184A. Enzyme activity was measured at increasing concentrations of azide (0–130 mM). V_{azide} and V_0 represent the initial velocities in the presence and absence of azide, respectively.

Based upon previous experience with *Agrobacterium* sp. β-glucosidase (Abg) thioglycoligases [27], a second acid/base mutant was also prepared: Glu184Gln. As a fast test of potential thioglycoligase activity of BgaX-E184A and BgaX-E184Q a series of small scale reactions was set up using DNPGal as donor and various PNP glycosides with thiol groups at positions 3, 4 and 6 as acceptors. After 12 h incubation, no useful amounts of thio-disaccharide product were seen with BgaX-E184Q, whereas a single product was clearly formed by BgaX-E184A in each reaction with 4-nitrophenyl 3-deoxy-3-thio-β-D-galactopyranoside (PNP3SGal), PNP4SGlc, and PNP3SGlc. No transfer was seen, however, to the axial thiol-group in PNP4SGal or the 6-thiol-group in PNP6SGlc, nor was any transfer observed to the hydroxyl group of DNPGal. While the fact that BgaX-E184Q does not function as a thioglycoligase differentiates it from Abg, in fact the same situation has been observed in the α-xylosidase from *E. coli* and the β-mannosidase from *Cellulomonas fimi* (Kim, Y. W., Jahn, M., Mullegger, J. and Withers, S. G., unpublished data).

To further investigate the catalytic properties of BgaX-E184A as a thioglycoligase, apparent kinetic parameters were determined for both DNPGal and PNPGal as donors in the presence of various thio-sugar acceptors (Table 2). These studies were considerably simplified by the fact that the products of thioglycoligase action are not acceptors for further transfer since they contain no free thiols, and by the finding that simple hydrolysis is much slower than transglycosylation to the thiosugars. Studies were first carried out at a fixed concentration of a thio-sugar acceptor (PNP3SGlc, 0.8 mM) using PNPGal or DNPGal as donors at a range of concentrations. The apparent k_{cat} values for DNPGal and PNPGal were enhanced in the presence of the thiosugar acceptor by 43.3-fold and 8.5-fold, respectively, compared to those for simple hydrolysis of each

Table 2
Comparison of kinetic parameters for transglycosylation of various substrates by BgaX-E184A

Variable substrate	Fixed substrate	k_{cat}[a] (min^{-1})	K_m[a] (μM)	k_{cat}/K_m[a] ($min^{-1}\,mM^{-1}$)
PNPGal	PNP3SGlc[b]	17.7	21	843
DNPGal	PNP3SGlc[b]	115	5.9	19491
DNPGal	PNP3SGal[b]	25.2	2.6	9692
DNPGal	PNP4SGlc[b]	35.8	1.5	23866
PNP3SGlc	DNPGal[c]	221	490	451
PNP3SGal	DNPGal[c]	22.5	180	125
PNP4SGlc	DNPGal[c]	34.5	320	108

Errors in kinetic parameters in this table are 10–14% for K_m and 2–5% for k_{cat}.
[a]Values are apparent parameters at the co-substrate concentration indicated.
[b]Concentrations of thio-sugars were fixed at 0.8 mM.
[c]Concentrations of DNPGal was fixed at 0.1 mM.

substrate. Observation of leaving group-dependent k_{cat} values for the thioglycoligase reactions means that, in contrast to what was seen for the hydrolytic reaction, the rate-limiting step has now shifted to the glycosylation step, at least for PNPGal. This is, of course, consistent with the dramatic elevation of the rates of deglycosylation by the thio-sugar acceptors. The higher glycosyl donor K_m values (apparent) for the thioglycoligase reactions compared to those seen for hydrolysis also support this interpretation.

Acceptor specificities of BgaX-E184A were then investigated by determining kinetic parameters for DNPGal at a fixed concentration (0.8 mM) of each thio-sugar acceptor (PNP3SGal, PNP3SGlc, and PNP4SGlc). Given the catalytic properties of the wild type enzyme we expected that β-1,3 thioglycosidic linkages would be formed more efficiently than β-1,4 linkages by BgaX-E184A and indeed PNP3SGlc was the best acceptor tested. Perhaps surprisingly, however, the next best acceptor was PNP4SGlc rather than PNP3SGal (Fig. 3A, Table 2). This outcome is possibly the result of either disfavored binding of a galactoside in the +1 subsite of this enzyme or inhibition of the enzyme by PNP3SGal. Hence, we investigated the reactions with varying concentrations of the thiosugar acceptors at a fixed concentration of DNPGal (0.1 mM). BgaX-E184A showed standard saturation kinetic behaviour for all acceptors (Fig. 3B) and the apparent values of k_{cat} for these thiosugar acceptors showed the same tendencies as those seen in the experiments above (Table 2). These kinetic data for BgaX-E184A with various acceptors suggest that glucosides are substantially preferred as acceptors over galactosides with this

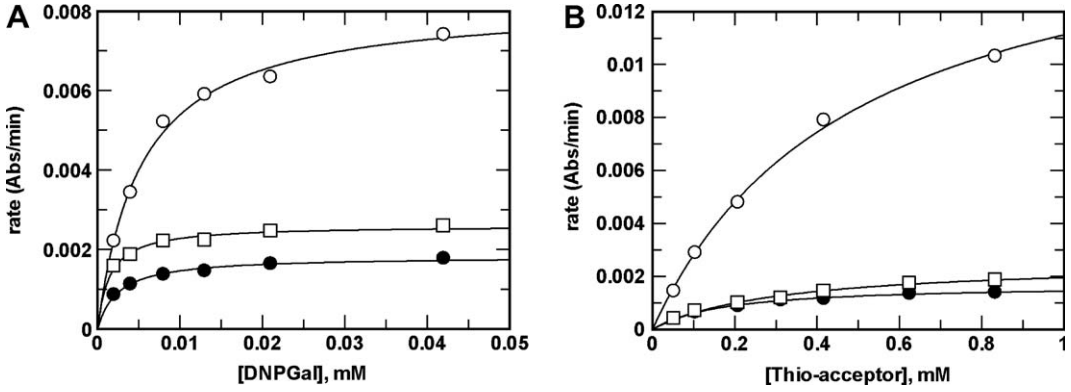
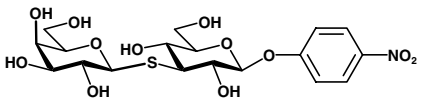
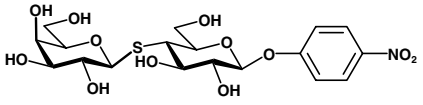
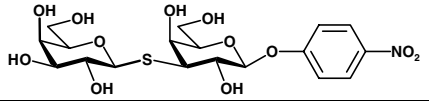


Fig. 3. Kinetics of transglycosylation catalysed by BgaX-E184A. (A) Dependence of rate on DNPGal concentration at fixed (0.8 mM) concentration of PNP3SGlc (□), PNP3SGal (●), and PNP4SGlc (□). (B) Dependence of rate on concentrations of PNP3SGlc (○), PNP3SGal (●), and PNP4SGlc (□) at a fixed (0.1 mM) concentration of DNPGal. The error ranges for data in this figure are less than 5%.

Table 3
Thioglycoligase reactions catalyzed by BgaX-E184A

Donor	Acceptor	Product	Yield (%)
DNPGal	PNP3SGlc		80
DNPGal	PNP4SGlc		80
DNPGal	PNP3SGal		83

enzyme. Indeed, this observation is consistent with our previous investigations of the acceptor specificity of the wild type BgaX wherein galactosides were shown not to function as reactivators of the 2-deoxy-2-fluorogalactosyl enzyme via transglycosylation [28].

Preparative scale reactions were carried out using DNPGal as a donor to various thio-sugar acceptors. An equimolar ratio of donor to acceptor was enough to obtain the thio-disaccharides in more than 80% yields (Table 3). As expected based on measured kinetic parameters for the thioglycoligase reactions, the reactions with PNP3SGlc were accomplished very efficiently (1 h incubation, 80% yield), while reactions with either PNP4SGlc or PNP3SGal needed longer incubation times (6 h and 10h incubation, respectively) to complete the consumption of the thio-sugar acceptors. ESI-mass spectrometry and NMR analyses of the acetylated products confirmed the structures shown in Table 3.

In conclusion, the catalytic acid/base of GH35 enzyme has been confirmed, for the first time, by kinetic analysis with a bacterial β-galactosidase (BgaX) mutant modified at the residue corresponding to the previously proposed acid/base catalyst based on the three-dimensional structure of β-galactosidase from *Penicillium* sp. The substitution of the acid/base catalyst with alanine allows synthesis of both β-1,3 and β-1,4-linked thiodisaccharides through the thioglycoligase mechanism. The BgaX thioglycoligase is therefore a valuable tool for the synthesis of thio-linked analogues of oligosaccharides found in many surface antigens.

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