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Research Paper

Rapid screening of the aglycone specificity of glycosidases: applications to enzymatic synthesis of oligosaccharides

Jan E. Blanchard, Stephen G. Withers*

Protein Engineering Network of Centres of Excellence of Canada and the Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Z1

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Abstract

Background: Retaining glycosidases can catalyse glycosidic bond formation through transglycosylation from a donor sugar to an acceptor bound in the aglycone site. The aglycone specificity of a glycosidase is not easily determined, thereby complicating the choice of the most appropriate glycosidase for use as a catalyst for transglycosylation. We have developed a strategy to rapidly screen the aglycone specificity of a glycosidase and thereby determine which enzymes are best suited to catalyse specific transglycosylation reactions.

Results: The reactivation, or turnover, of a glycosidase trapped as a fluoroglycosyl-enzyme species is accelerated in the presence of a compound that productively binds to the aglycone site. This methodology was used to rapidly screen six glycosidases with 44 potential acceptor sugars. Validation of the screening strategy was demonstrated by the identification of products formed from a transglycosylation reaction with positively screened acceptors for four of the enzymes studied.

Conclusions: The aglycone specificity of a glycosidase can be rapidly evaluated and requires only an appropriate fluorosugar inactivator, a substrate for assay of activity and a library of compounds for screening.

Keywords: Aglycone specificity; Screen; Glycosidase

1. Introduction

The enzymatic cleavage of glycosidic bonds in oligosaccharides is achieved by glycosidases, enzymes which are often highly specific with regard not only to the identities of their glycone (non-reducing end sugar), but also to their aglycone (reducing end sugar). The specificity of a glycosidase for the sugar on the non-reducing side of the scissile bond (the glycone) is readily determined, typically through the use of activated substrates such as nitrophenyl glycosides: this forms the basis for the most common means of classification of glycosidases. By contrast, determination of the specificity of a glycosidase for its aglycone site is much more difficult, requiring access to large numbers of oligosaccharides of known structure. However, such information is an important component of the characterisation of a glycosidase and is crucial for the selection of appropriate enzymes for use in the analysis of oligosaccharide structure. Such knowledge is also particularly important when selecting glycosidases for use in the synthesis of oligosaccharides via transglycosylation [1–3]. This paper describes a simple screen that can be used to elucidate the

Abbreviations: Abg, *Agrobacterium* sp. β-glucosidase; BgaC, *Bacillus circulans* β-galactosidase; BgaX, *Xanthomonas manihotis* β-galactosidase; CelB, *Streptomyces lividans* endoglucanase; Cex, *Cellulomonas fimi* xylanase/glucanase; Man2A, *Cellulomonas fimi* β-mannosidase; 2-deoxy-Gal, 2-deoxy-D-galactopyranose; 2-deoxy-Glc, 2-deoxy-D-glucopyranose; DNPCell, 2,4-dinitrophenyl β-cellobioside; 2FDNPCell, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside; 2FDNPGal, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside; 2FDNPGlc, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; 2FManF, 2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride; Gal-β-(1-3)-Glc-β-SPh, phenyl β-D-galactopyranosyl-(1-3)-1-thio-β-D-glucopyranoside; Gal-β-(1-4)-Glc-β-SPh, phenyl β-D-galactopyranosyl-(1-4)-1-thio-β-D-glucopyranoside; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-β-NH_2, 2-acetamido-2-deoxy-β-D-glucosyl amine; Me α-Cell, methyl α-cellobioside; Me β-Cell, methyl β-cellobioside; MeXyl, methyl β-D-xylopyranoside; *N*-Ac-D-glucalamine, 2-acetamido-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol; *o*NPXyl, *o*-nitrophenyl β-D-xylopyranoside; *p*CO_2MePhGlc, *p*-carboxymethylphenyl β-D-glucopyranoside; PhGlc, phenyl β-D-glucopyranoside; *p*NPCell, *p*-nitrophenyl β-cellobioside; *p*NPGal, *p*-nitrophenyl β-D-galactopyranoside; *p*NPGlc, *p*-nitrophenyl β-D-glucopyranoside; *p*NPMan, *p*-nitrophenyl β-D-mannopyranoside; *p*NP-α-Xyl, *p*-nitrophenyl α-D-xylopyranoside; *p*NPXyl, *p*-nitrophenyl β-D-xylopyranoside

* Correspondence: Stephen G. Withers;
E-mail: withers@chem.ubc.ca

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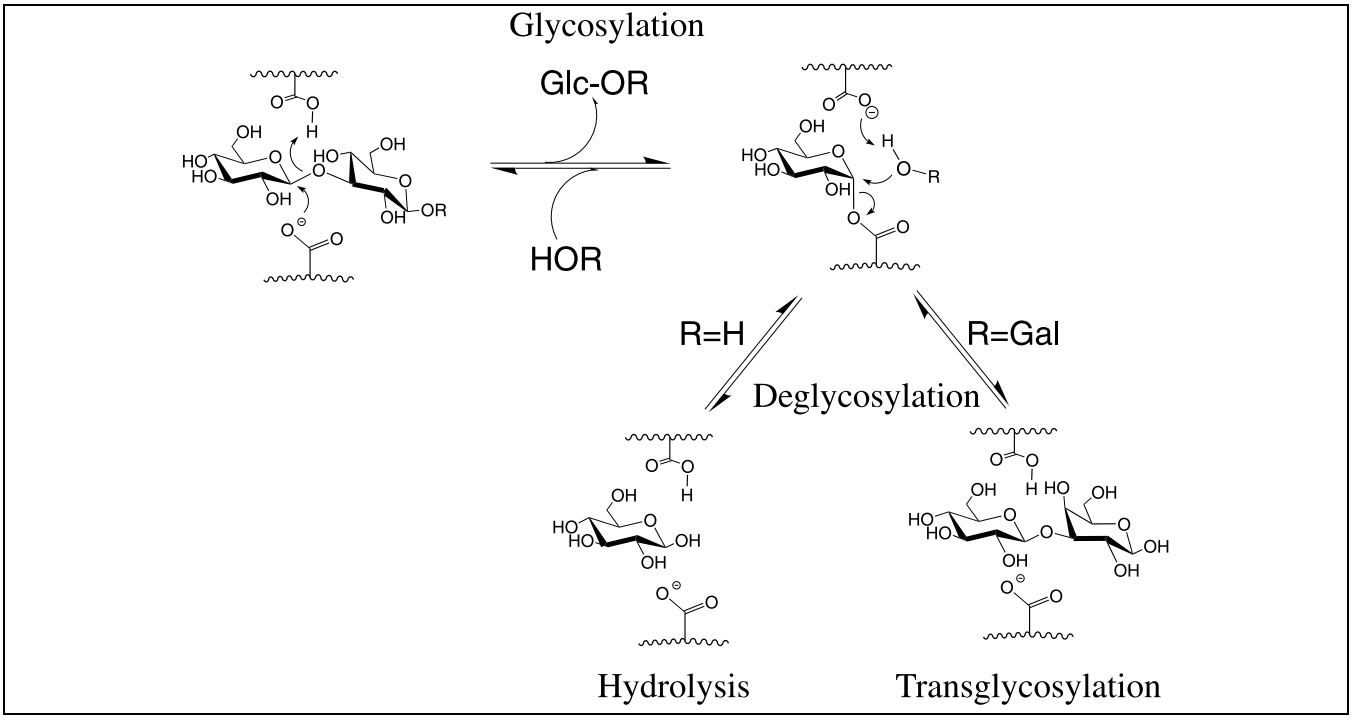

Fig. 1. The general mechanism of catalysis by retaining glycosidases. The first step (glycosylation of the enzyme) results in the formation of a covalent glycosyl-enzyme intermediate that subsequently undergoes either hydrolysis or transglycosylation.

aglycone specificity of glycosidases and thereby identify suitable enzymes for use in the synthesis of oligosaccharides.

Retaining glycosidases hydrolyse their substrates through a two-step, double-displacement mechanism involving a covalent glycosyl-enzyme intermediate [4–6] (Fig. 1). General base-catalysed attack of water on this intermediate leads to hydrolysis. However, if an appropriate sugar, capable of binding productively in the aglycone site, is present at sufficient concentration then transglycosylation occurs with the formation of a glycosidic bond. This process forms the basis for the use of glycosidases in the enzymatic synthesis of oligosaccharides [1–3].

A relatively recently developed class of mechanism-based glycosidase inactivators are activated glycosides in which a fluorine is substituted close (at C-2 or C-5) to the anomeric centre. Such reagents form a glycosyl-enzyme intermediate that is hydrolysed only extremely slowly but is nonetheless catalytically competent since it undergoes a transglycosylation reaction in the presence of a suitable acceptor sugar, resulting in reactivation of the enzyme [4,5] (Fig. 2). This reactivation process forms the basis of a new screen for aglycone site specificity. Once a glycosidase is inactivated by a fluoroglycoside, it is mixed with a variety of potential 'reactivating' sugars. After an incubation period, the amount of enzyme that has been reactivated, either through transglycosylation or hydrolysis, is quantified and compared to the amount of reactivation seen for enzyme in buffer alone. Those sugars that induce a higher degree of reactivation are those which are best accommodated in the enzyme's aglycone site. This analysis was performed with six retaining glycosidases and a library of 44 different potential reactivating sugars.

2. Results and discussion

An overview of the large scale screening strategy, using the *Agrobacterium* sp. β-glucosidase (Abg) as an illustrative example, is outlined in Fig. 3. (1) The enzyme is first trapped as the 2-deoxy-2-fluoroglucosyl-enzyme by reaction with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyr-

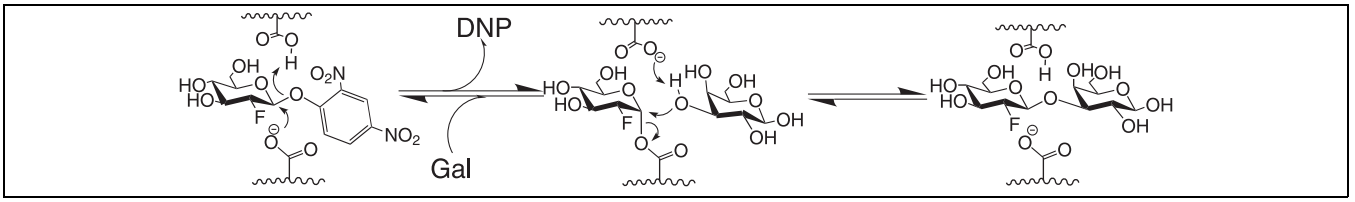

Fig. 2. The attempted hydrolysis of a C-2- or C-5-fluoroglycoside by a retaining glycosidase traps the covalent glycosyl-enzyme intermediate. Reactivation of the enzyme occurs slowly, but can be accelerated in the presence of a suitable acceptor.

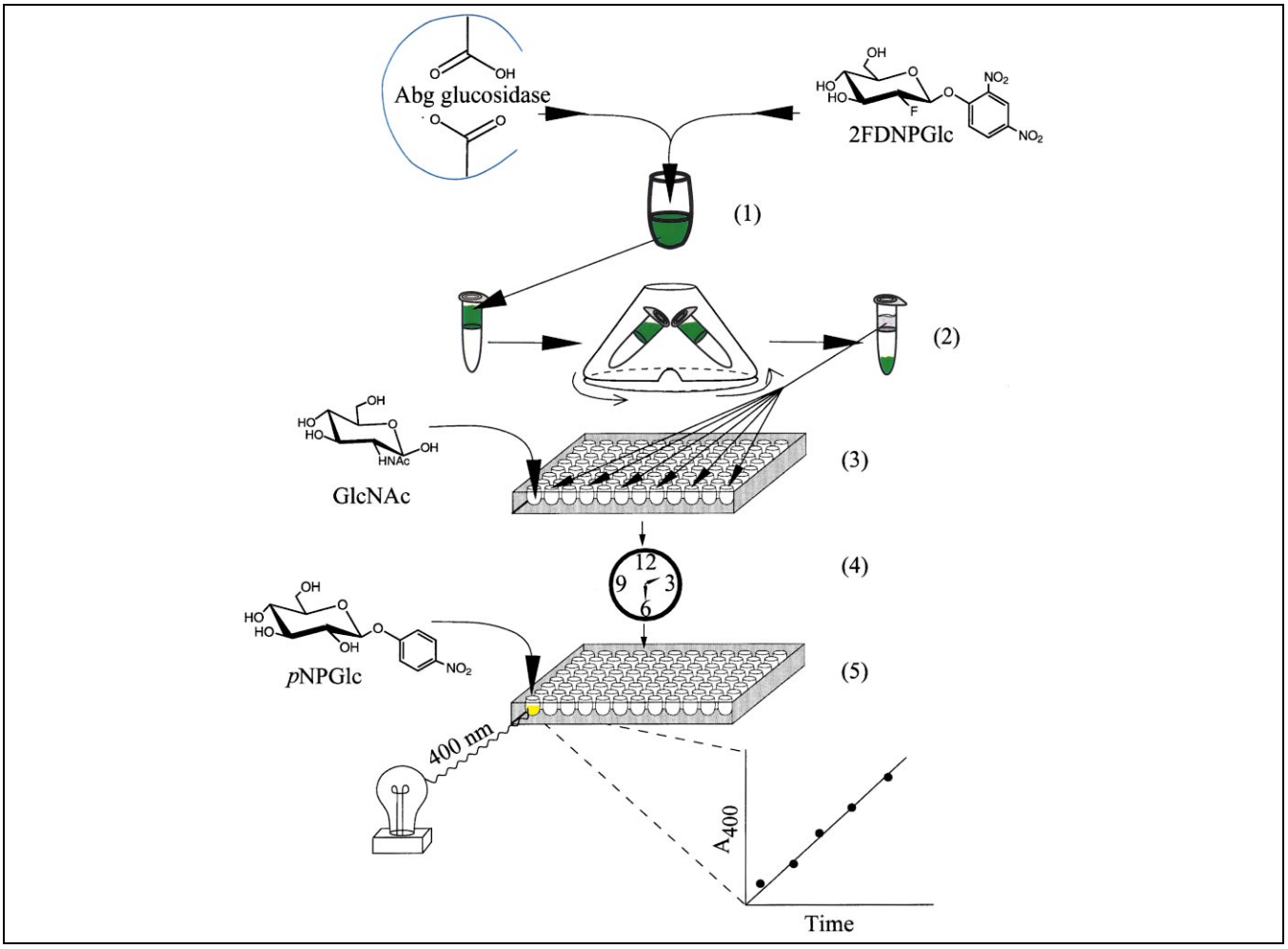

Fig. 3. Overview of the aglycone screening strategy: (1) inactivation; (2) removal of excess inactivator; (3) mixing of inactivated enzyme and potential reactivator; (4) incubation; (5) quantification of reactivated enzyme.

anoside (2FDNPGlc), and (2) dialysed via centrifugal ultrafiltration to remove excess inactivator. (3) Aliquots of the inactivated enzyme species are placed in individual wells of a 96-well plate; to each well are then added aliquots of different sugar 'reactivators' (e.g. *N*-acetyl-D-glucosamine (GlcNAc)) and (4) the plate incubated at room temperature for 2.5 h to allow transglycosylation (or hydrolysis), hence reactivation to occur. (5) Substrate is then added to each well and the activity of each enzyme sample determined using a 96-well plate reader. Activity in a well greater than that in the control sample incubated in buffer alone indicates that reactivation via transglycosylation has occurred, thus that the reactivator sugar binds productively in the aglycone site.

Those sugars included in the reactivator library were selected for several reasons: they are representative of carbohydrates that comprise many common synthetic targets; they are interrelated by simple substitutions or epimerisations, thereby illustrating the effect of subtle structural changes in the reactivator on aglycone binding; and they were readily available in the laboratory.

Positive 'hits' thus identified were then subjected to a second round of screening to more accurately determine relative reactivation rates and thereby to more precisely delineate the aglycone specificity. This involved incubating samples of inactivated enzyme with each candidate reactivator, removing aliquots at time intervals and assaying for return of activity in a conventional UV/Vis spectrometer. The initial rates of reactivation in the presence of each ligand (at the chosen concentration) were determined by applying a linear regression to the data of activity versus time. The results of this analysis for the six enzymes studied are shown in the form of histograms in Fig. 4.

The total number of positive hits for each glycosidase was relatively low, 4–16 out of a possible 44. Several general trends were seen with these positively screened carbohydrates: (i) Of the potential acceptors screened, aryl glycosides were generally better reactivators than their parent carbohydrates. Thus, for example, *p*-nitrophenyl β-D-galactopyranoside (*p*NPGal) was identified as a reactivating ligand for *Bacillus circulans* β-galactosidase (BgaC) and Abg, yet galactose was not. Similar trends were seen for aryl glucosides and aryl xylosides; these carbohydrates were positively screened with *Xanthomonas manihotis* β-ga-

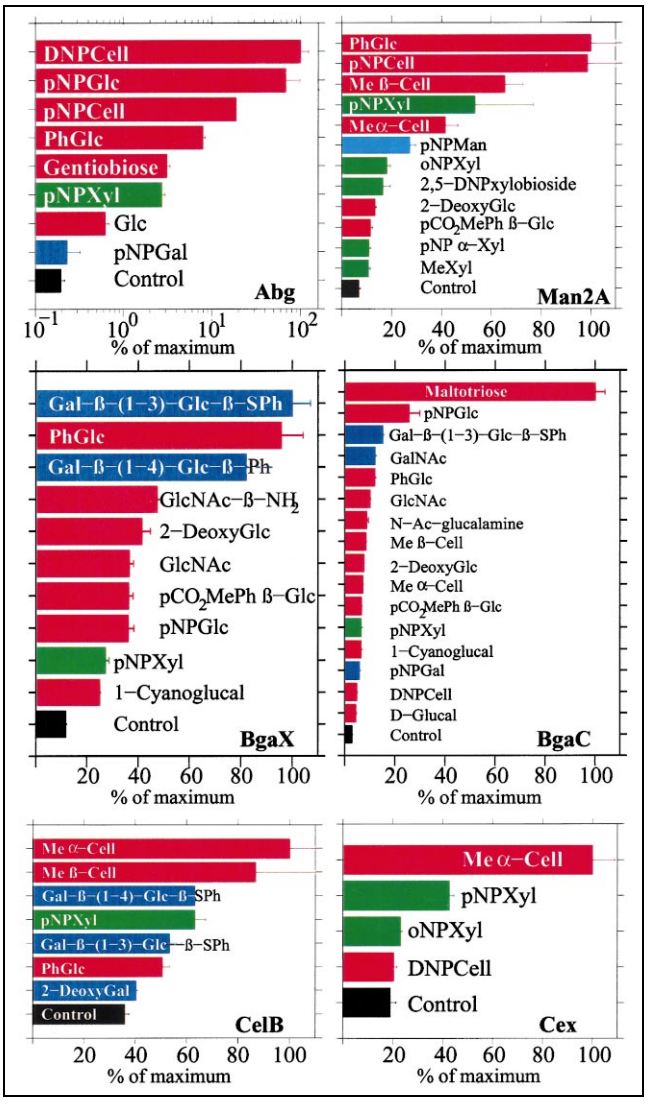

Fig. 4. Histograms depicting the relative initial rate of reactivation of the six enzymes studied in the presence of those compounds that were identified as positive hits through the screening protocol. BgaX, *Xanthomonas manihotis* β-galactosidase; BgaC, *Bacillus circulans* β-galactosidase; Man2A, *Cellulomonas fimi* β-mannosidase; Cex, *C. fimi* glycanase/xylanase; CelB, *Streptomyces lividans* cellulase. Note, for clarity data for Abg have been plotted on a logarithmic scale. Colours indicate the relative stereochemistry of the non-reducing sugar of each compound: red = glucose; dark blue = galactose; green = xylose; light blue = mannose. As well as those compounds listed in each plot, the following were also included in the sugar library: arabinose, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl α-L-arabinopyranoside, L-fucose, mannose, 2,4-dinitrophenyl β-D-fucopyranoside, galactose, *p*-nitrophenyl α-D-mannopyranoside, *N*-acetyl-D-mannosamine, *o*-nitrophenyl β-D-galactopyranoside, D-galactal, D-galacturonic acid, xylose, D-glucal, 1-cyano-D-glucal, 2-carboxymethyl β-D-glucoside, fructose.

lactosidase (BgaX), BgaC, and Abg, whereas negative results were obtained for all three enzymes with the parent compounds glucose and xylose. This general trend was seen previously in this laboratory for the reactivation of Abg and *Cellulomonas fimi* β-mannosidase (Man2A). The turnover of the 2-fluoro-glucosyl-enzyme species of Abg increased 2700-fold in the presence of *p*-nitrophenyl β-D-glucopyranoside (*p*NPGlc), yet no significant increase in reactivation rate was seen with glucose [7]. Similarly, the rate of reactivation of Man2A was significantly increased in the presence of *p*-nitrophenyl β-D-mannopyranoside (*p*NPMan) relative to a control, but not with the parent compound mannose [8]. This behaviour is most likely the result of favourable interactions between the aryl substituent and the enzymes' aglycone site. (ii) Although aryl glycosides were generally preferred as acceptors by comparison with their parent carbohydrates, the reactivation of some enzymes was sensitive to the position of the phenyl substituent. It appears that *para*-nitro-substituted phenyl glycosides are generally better reactivators relative to their *ortho*-derivatives: *p*NPGal and *p*-nitrophenyl β-D-xylopyranoside (*p*NPXyl) were positive hits for BgaC and Abg, yet *o*NPGal and *o*NPXyl were not. Similarly, BgaX accepted the *para*-substituted nitrophenyl *β*-xyloside, but not the *ortho*-substituted derivative. (iii) In general, disaccharide acceptors are more effective at reactivation than monosaccharides. This presumably reflects improved interactions with the aglycone sites of these enzymes. These examples illustrate the significant effects that small changes in acceptor structure have on productive binding to the aglycone site of the glycosidases studied.

One small drawback to this screening strategy is that it may not reliably identify a glycoside as a reactivator if it is a substrate for the enzyme, since it will be progressively hydrolysed during the incubation process. However, this is not necessarily a problem since even though BgaC, Abg and Man2A are known to hydrolyse *p*NPGal [9], *p*NPXyl [10] and *p*NPMan [11] respectively, these sugars were nonetheless identified as positive hits. This suggests a higher rate of reactivation versus hydrolysis at high concentrations of these particular substrates.

Following the completion of the screening procedure, confirmation was then sought that the specificities so determined correctly predict the synthetic potential of the enzyme. Thus four of the enzymes studied were incubated with its preferred nitrophenyl glycoside donor sugar in the presence of one of the best reactivator sugars identified through the screen, and the reaction followed by thin layer chromatography. After completion of the reaction, the mixtures were lyophilised and products subjected to acetylation, in two cases using 1-^{13}C-acetyl chloride in pyridine. Analysis of the ^{1}H-NMR spectrum readily revealed the site of linkage as being that at a carbon attached to a proton that was not *J*-coupled to ^{13}C. Substrates used and products obtained for each enzyme were: Abg with *p*NPGlc as donor and gentiobiose as acceptor: 21% β-D-glucopyranosyl-(1-4)-β-D-glucopyranosyl-(1-6)-β-D-glucopyranose; Man2A with *p*NPman as donor and phenyl β-glucoside as acceptor: 12% phenyl β-D-mannopyranosyl-(1-4)-β-D-glucopyranoside; BgaX with *p*NPGal as donor and *p*NPXyl as acceptor: 6% *p*-nitrophenyl β-D-galactopyranosyl-(1-3)-β-D-xylopyranoside; BgaC with *p*NPGal as donor and maltotriose as acceptor: 30% β-D-galacto-

pyranosyl-(1-3)-α-D-glucopyranosyl-(1-4)-α-D-glucopyranosyl-(1-4)-β-D-glucopyranose.

This approach provides a rapid means of screening the aglycone specificity of a retaining glycosidase without any need for oligosaccharide substrates. It requires only the relevant fluorosugar and a series of commercially available sugars and derivatives thereof. Non-saccharidic acceptors could also be screened, allowing testing of recent theoretical predictions of their relative efficacies [12]. While it does not provide direct information on the linkage specificity of the enzyme (1,2 versus 1,3 etc.) this information can be obtained by identification of the transglycosylation products formed once the identity of the preferred sugar has been determined using the screen. The approach should prove extremely valuable in the selection of the appropriate glycosidases for the synthesis of specific oligosaccharides as well as in determining aglycone specificities for use in enzyme analysis.

3. Materials and methods

3.1. General

All buffers, substrates, and reagents were purchased from Sigma-Aldrich Co. or Aldrich Chemical Company unless noted otherwise.

All enzymes were expressed and purified by either members of this laboratory, or were generously donated by collaborators; all such protocols have been published previously (BgaX [9], BgaC [9], Abg [13], Man2A [11], *C. fimi* xylanase/glucanase (Cex) [14], *Streptomyces lividans* endoglucanase (CelB) [15]). The buffer used for all enzyme studies was 50 mM sodium phosphate (NaPi), pH 7.0, except for BgaX (pH 6.0) and CelB (pH 6.5). Substrates used to measure enzyme activity were as follows: BgaX: 1.2 mM *p*NPGal; BgaC: 0.94 mM *p*NPGal; Abg: 0.50 mM *p*NPGlc; Man2A: 0.35 mM *p*NPMan; Cex: 1.2 mM *p*-nitrophenyl β-cellobioside (*p*NPCell); CelB: 0.50 mM 2,4-dinitrophenyl β-cellobioside (DNPCell).

Enzyme inactivators were generously donated by members of this laboratory: 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside (2FDNPGal), 2FDNPGlc, and 2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride (2FManF) (L. Mackenzie), 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside (2FDNPCell) (D. Zechel). Potential acceptors were purchased from Sigma-Aldrich Co. or Aldrich Chemical Company or were gifts from members of this laboratory: DNPFuc (J. McCarter), *p*CO_2MePh β-Glc (Q. Wang), D-glucal (L. Mackenzie), 1-cyano-D-glucal (E. Lai), DNPCell (H. Prade), methyl α-cellobioside (Me α-Cell), methyl β-cellobioside (Me β-Cell) (V. Ferro), phenyl β-D-galactopyranosyl-(1-3)-1-thio-β-D-glucopyranoside (Gal-β-(1-3)-Glc-β-SPh), phenyl β-D-galactopyranosyl-(1-4)-1-thio-β-D-glucopyranoside (Gal-β-(1-4)-Glc-β-SPh) (H. Prade), 2,5-DNPX_2 (D. Zechel). Stock solutions of potential acceptors were made in water to a final concentration, where possible, of ca. 50 mM; some of these solutions were saturated.

3.2. Large scale screening

Each enzyme (60–470 μg) was incubated with its respective inactivator (dictated by glycone specificity) (60–260 μmol) in buffer (total volume of 0.5–1.0 ml) until $< 5\%$ of the original activity remained. The inactivated enzyme was concentrated using a 30 kDa molecular weight cut-off Millipore Ultrafree 0.5 centrifugal filter to a volume of 10–20 μl, and fresh buffer was added to a final volume of 200 μl. This process of concentration and dilution was repeated until all excess inactivator was removed. The final concentration of inactivated enzyme was determined by absorption at 280 nm and used to calculate the maximal activity possible (V_o) if the enzyme were completely active.

An aliquot of each inactivated enzyme species plus buffer and each compound to be screened were added to the wells of a 96-well plate so that the final concentration of the potential acceptors was either 30 mM or 60% saturation (final volume = 50–100 μl). Controls of (i) non-inactivated enzyme and (ii) inactivated enzyme, each in buffer alone, were included. The plates were incubated at room temperature for 2.5–19 h. After incubation, 100 μl of the respective assay substrate was added to each well, and the release of the *p*NP or DNP phenolate ion was measured with a 96-well plate reader either by: (i) taking single absorbance measurements of each well at 400 nm over 0.5–6 h (using a Bio-Rad Model 2550 EIA Reader), or (ii) measuring the continuous change in absorbance of each well at 400 nm over 10–20 min (using a Molecular Devices Spectra Max 190 Reader), the latter procedure being preferred. Tested compounds were classified as a positive hit if they induced greater enzyme reactivation compared to the control of reactivating enzyme in buffer alone.

3.3. Further analysis of enzyme reactivation

Each enzyme was inactivated and the excess inhibitor removed as described; samples of these species (0.25–5.70 μg) were added to buffer containing those compounds identified as positive hits to a final concentration of each tested compound of 30 mM or 60% saturation (volume = 50–100 μl). All such mixtures were incubated in a water bath at 37°C, excluding BgaX (incubation at 20°C). Controls of non-inactivated enzyme were included to account for enzyme death over time. Aliquots (5 μl) of each mixture were removed over time and assayed for activity as described below. The resultant gain in fractional activity was fit to a linear expression, and the initial rate of enzyme reactivation determined from the slope of the plot.

3.4. Measurement of enzyme activity

Enzyme activity assays were performed with a Unicam UV4 ultraviolet/visible spectrometer; the spectrometer and cuvettes were pre-equilibrated to 37°C by the use of a circulating water bath. The rates of hydrolysis of nitrophenyl glycoside substrates were determined by the continuous spectroscopic monitoring of the release of *p*NP or DNP at 400 nm over 1–2 min. Spectrometer cuvettes contained 0.1% BSA (w/v), and the respective buffer and

assay substrate listed above for each enzyme. Assays were initiated by the addition of a 5 μl enzyme aliquot.

3.5. Preparative scale transglycosylation reactions

Enzyme (0.05–0.10 mg/ml) in buffer was incubated with acceptor (A) (55–220 mg) and a portion of donor (D) (D:A = 1:5) at room temperature so that the final concentration of A was 40–50 mM (volume = 5–8 ml). Reactions were monitored by TLC (solvent: 7:2:1 ethyl acetate/methanol/water). The rest of D was successively added over the course of the reaction. After total depletion of D (4–14 h), reactions were filtered through a 30 kDa membrane to remove the enzyme, lyophilised and then acetylated.

3.6. Acetylation of transglycosylation products

Transglycosylation products were acetylated with either (i) acetic anhydride (Ac_2O) or (ii) 1-^{13}C-acetyl chloride (1-^{13}C-AcCl) according to the following methods: (i) Ac_2O (3 ml) was added under N_2 to the mixture of transglycosylation products in dry pyridine (6 ml) at room temperature. Once the reaction was complete by TLC (solvent: 1:1 ethyl acetate/hexanes), the solvent was removed by evaporation in vacuo. (ii) 1-^{13}C-AcCl (150–200 μl) was added under N_2 to the mixture of transglycosylation products in dry pyridine (2 ml) at room temperature. Once the reaction was complete by TLC (solvent: 3:1 ethyl acetate/hexanes), the solvent was removed by evaporation in vacuo. Water and ethyl acetate were added to the residues of (i) and (ii) (ca. ∼10 ml of each) and successively washed with 1 M HCl, 1 M $NaHCO_3$ and saturated NaCl. The organic layer was recovered and evaporated in vacuo.

3.7. Separation of transglycosylation products

Acetylated transglycosylation products were loaded onto a silica gel column for chromatography in the minimum amount of CH_2Cl_2 and eluted with a solvent system of 1:1 or 2:1 petroleum ether/ethyl acetate, or a combination thereof. Fractions of each product were pooled and the solvent evaporated in vacuo. The isolated products were dried in vacuo for ∼48 h prior to NMR analysis. Product yields are based on isolated products and are referenced to the total amount of acceptor used in each reaction.

3.8. NMR data of transglycosylation products

3.8.1. 2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl-(1-3)-2,4,6-tri-O-acetyl-α-D-glucopyranosyl-(1-4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl-(1-4)-1-2,3,6-tetra-O-acetyl-β-D-glucopyranose

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.71 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 5.48 (dd, 1 H, $J_{2,3}$ 10.1 Hz, $J_{3,4}$ 9.0 Hz, H-3), 5.38 (dd, 1 H, $J_{3',4'}$ 9.7 Hz, H-3′), 5.33 (d, 1 H, $J_{3''',4'''}$ 3.3 Hz, H-4‴), 5.28 (d, 1 H, $J_{1'',2''}$ 4.1 Hz, H-1″), 5.22 (d, 1 H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.04 (dd, 1 H, $J_{2''',3'''}$ 10.4 Hz, H-2‴), 4.97–4.89 (m, 3 H, H-2, H-4″, H-3‴), 4.78 (dd, 1 H, $J_{2'',3''}$ 10.2 Hz, H-2″), 4.69 (dd, 1 H, $J_{2',3'}$ 10.3 Hz, H-2′), 4.61 (d, 1 H, $J_{1''',2'''}$ 8.0 Hz, H-1‴), 4.47–4.38 (m, 3 H, H-6a, H-4′, H-6a′), 4.24–4.12 (m, 5 H, H-6b, H-6b′, H-6b″, H-6a‴, H-6b‴), 4.09–3.99 (m, 3 H, H-4, H-5, H-3″), 3.95–3.81 (m, 4 H, H-4′, H-5′, H-6b″, H-5‴), 2.22, 2.14, 2.13, 2.11, 2.07, 2.03, 2.02, 1.99, 1.98, 1.97, 1.96, 1.93 (12 s, 42 H, Ac).

3.8.2. Para-nitrophenyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1-3)-2,4-di-O-acetyl-β-D-xylopyranoside

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, 2 H, $J_{a,b}$ 9.3 Hz, *p*NP), 7.06 (d, 2 H, $J_{c,d}$ 9.0 Hz, *p*NP), 5.37 (dd, 1 H, $J_{4',3'}$ 3.2 Hz, H-4′), 5.17 (dd, 1 H, $J_{2,3}$ 8.1 Hz, H-2), 5.16 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, H-2′), 4.99 (dd, 1 H, $J_{3',4'}$ 3.3 Hz, H-3′), 4.96 (ddd, 1 H, $J_{4,5eq}$ 4.3 Hz, H-4), 4.56 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.19 (dd, 1 H, $J_{5ax,5eq}$ 12.1 Hz, H-5eq), 4.00–4.14 (m, 2 H, H-6a′, H-6b′), 3.94 (dd, 1 H, $J_{3,4}$ 5.6 Hz, H-3), 3.89 (dd, 1 H, $J_{5',6a'}$ 6.2 Hz, $J_{5',6b'}$ 7.3 Hz, H-5′), 3.59 (dd, 1 H, $J_{5ax,4}$ 7.0 Hz, H-5ax), 2.14, 2.10, 2.09, 2.05, 2.03, 1.97 (6 s, 18 H, Ac).

3.8.3. Phenyl 2,3,4,6-tetra-O-(1-^{13}C-acetyl)-β-D-mannopyranosyl-(1-4)-2,3,6-tri-O-(1-^{13}C-acetyl)-β-D-glucopyranoside

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (m, 2 H, Ph), 7.20 (m, 3 H, Ph), 5.47 (ddd, 1 H, $J_{2',Ac}$ 3.2 Hz, $J_{2',3'}$ 3.2 Hz, H-2′), 5.32 (m, 1 H, $J_{4',Ac}$ 1.9 Hz, H-4′), 5.20 (ddd, 1 H, $J_{3,Ac}$ 3.18 Hz, $J_{3,4}$ 9.1 Hz, H-3), 5.13 (ddd, 1 H, $J_{2,Ac}$ 3.41 Hz, $J_{2,3}$ 10.0 Hz, H-2), 5.02 (ddd, 1 H, $J_{3',Ac}$ 3.2 Hz, $J_{3',4'}$ 6.4 Hz, H-3′), 4.66 (d, 1 H, $J_{1',2'}$ 0.5 Hz, H-1′), 4.30 (ddd, 1 H, $J_{6b,Ac}$ 3.1 Hz, $J_{6b,6a}$ 12.4 Hz, H-6b), 4.26 (m, 1 H, H-6a), 4.10 (d, 1 H, $J_{1,2}$ 7.1 Hz, H-1), 4.05–4.17 (m, 2 H, H-6a′, H-6b′), 3.88 (dd, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 3.79 (ddd, 1 H, $J_{5,6a}$ 2.44 Hz, $J_{5,6b}$ 5.2 Hz, H-5), 3.62 (ddd, 1 H, $J_{5',4'}$ 8.5 Hz, $J_{5',6a'}$ 2.2 Hz, $J_{5',6b'}$ 5.0 Hz, H-5′), 2.17, 2.15, 2.14, 2.09, 2.05, 2.04, 1.98 (7 s, 21 H, Ac).

3.8.4. 2,3,4,6-Tetra-O-(1-^{13}C-acetyl)-β-D-glucopyranosyl-(1-4)-2,3,6-tri-O-(1-^{13}C-acetyl)-β-D-glucopyranosyl-(1-6)-1,2,3,4-tetra-O-(1-^{13}C-acetyl)-β-D-glucopyranoside

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.68 (dd, 1 H, $J_{1,Ac}$ 3.3 Hz, $J_{1,2}$ 8.2 Hz, H-1), 5.32 (ddd, 1 H, $J_{3,Ac}$ 3.4 Hz, $J_{3,4}$ 9.1 Hz, H-3), 5.16 (ddd, 1 H, $J_{2'',Ac}$ 3.4 Hz, $J_{2'',3''}$ 11.2 Hz, H-2″), 5.14 (ddd, 1 H, $J_{3'',Ac}$ 3.3 Hz, H-3″), 5.14–5.03 (m, 2 H, H-3′, H-4″), 5.05 (ddd, 1 H, $J_{2,Ac}$ 1.5 Hz, $J_{2,3}$ 10.8 Hz, H-2), 5.04–4.89 (m, 1 H, H-4), 4.98 (ddd, 1 H, $J_{2',Ac}$ 0.1 Hz, $J_{2',3'}$ 10.8 Hz, H-2′), 4.51 (ddd, 1 H, $J_{6a',5'}$ 3.7 Hz, $J_{6a',Ac}$ 3.0 Hz, $J_{6a',6b'}$ 12.4 Hz, H-6a′), 4.47 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.33–4.20 (m, 3 H, H-6b′, H-6a″, H-6b″), 4.02 (d, 1 H, $J_{1'',2''}$ 8.3 Hz, H-1″), 3.93–3.80 (m, 2 H, H-6a, H-5′), 3.81 (ddd, 1 H, $J_{5,4}$ 8.8 Hz, $J_{5,6a}$ 2.5 Hz, $J_{5,6b}$ 3.7 Hz, H-5), 3.61 (m, 1 H, H-6b), 3.57 (dd, 1 H, $J_{3',4'}$ 9.0 Hz, H-4′), 2.13, 2.11, 2.10, 2.08, 2.07, 2.06, 2.02, 2.00, 1.98, 1.97, 1.77 (11 s, 33 H, Ac).

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