On expanding the repertoire of glycosynthases: Mutant β-galactosidases forming β-(1,6)-linkages

David L. Jakeman and Stephen G. Withers

Abstract: Oligosaccharide synthesis by enzymatic processes offers the potential for thrusting oligosaccharides to the forefront of pharmaceutical research, in part, due to expedient and scalable reaction protocols. Glycosynthases are an emerging class of mutant enzymes capable of synthesizing glycosidic linkages in high yield. We report a new glycosynthase enzyme generated by a point mutation of *E. coli* β-galactosidase that condenses α-galactosyl fluoride with aryl glucosides forming a β-(1,6) glycosidic linkage. A further point mutation within the enzyme, proximal to the active site, increases the yields significantly.

Key words: glycosyl transfer, glycosynthase, enzymatic oligosaccharide synthesis.

Résumé : La synthèse d'oligosaccharides par des procédés enzymatiques offre la possibilité de projeter les oligosaccharides à l'avant-plan de la recherche pharmaceutique en raison, en partie, au fait que les protocoles de réaction sont relativement simples et qu'il est possible de les réaliser à grande échelle. Les glycosynthases font partie d'une classe d'enzymes mutants en émergence capable de réaliser la synthèse de liens glycosidiques avec des rendements élevés. Dans ce travail, on la préparation d'une nouvelle glycosyntase, obtenue par mutation ponctuelle de la β-galactosidase du *E. coli*, qui permet de condenser le fluorure d'α-galactosyle avec des glucosides d'aryles pour former une liaison β-(1,6) glycosidique. Une autre mutation ponctuelle à l'intérieur de l'enzyme, à proximité du site actif, permet d'augmenter le rendement de façon significative.

Mots clés : transfert d'un glycosyle, glycosynthase, synthèse enzymatique d'un oligosaccharide.

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Introduction

Oligosaccharides are ubiquitious throughout nature and play a multitude of important roles in biological systems ranging from leukocyte trafficking by glycosaminoglycans (1–3) to the localization of glycoproteins via their glycan moieties (4–9). The diverse roles played by oligosaccharides therefore provide possibilities for the generation of new therapeutics for numerous disease states. Inspired by the possibilities for disease treatment and by the challenges posed by the complex structural architecture of oligosaccharides, chemists have devised and implemented impressive synthetic routes toward these challenging molecules. The very complexity of these compounds, however, hinders development of the efficient and economical syntheses required to produce the quantities of oligosaccharides needed for biological testing. Particularly demanding are the many protecting group manipulations that are needed to ensure control of regio- and stereochemistry. Enzymatic syntheses offer the promise of inherently scalable and shorter routes towards quantities of oligosaccharides because the vital regio- and stereochemical control is governed by the enzyme, thus avoiding laborious protecting group manipulation (10–15).

Glycosynthases (16) are a new class of mutant enzymes for the synthesis of oligosaccharides derived from retaining β-glycoside hydrolases, which cleave glycosides through a double displacement mechanism that proceeds via a covalent glycosyl–enzyme intermediate (Fig. 1*a*). Wild-type β-glycoside hydrolases have been used successfully in the synthesis of glycosides through the coupling of an activated donor sugar to the desired acceptor moiety via a transglycosylation mechanism (Fig. 1*b*). The yields are almost always modest because the product from the transglycosylation reaction is a substrate for hydrolysis (17). These low product yields have been circumvented by using glycosynthases: mutant glycosidases in which the nucleophilic carboxylic acid residue is engineered into a non-nucleophilic residue. The single-point mutation within the enzyme is sufficient to remove all hydrolytic activity yet maintain an active-site architecture appropriate for the condensation of an activated donor sugar (glycosyl fluoride) of the opposite anomeric configuration to that of the natural substrate, with an acceptor sugar. α-Glycosyl fluorides are therefore used with β-glycosynthases, binding into the enlarged active-site pocket generated by the mutation and mimicking the reactive α-glycosyl-enzyme intermediate formed during catalysis by the wild-type enzymes (Fig. 2). Since the report of the initial glycosynthase (AbgGlu358Ala) several glycosynthases have been reported in the literature. These condense various α-hexosyl fluorides onto acceptors with formation of β-(1,3)- or β-(1,4)- glycosidic linkages (18–23). Of particular importance has been the discovery that replacement of the nucleophilic amino

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Dedicated to the memory of Professor Raymond U. Lemieux.

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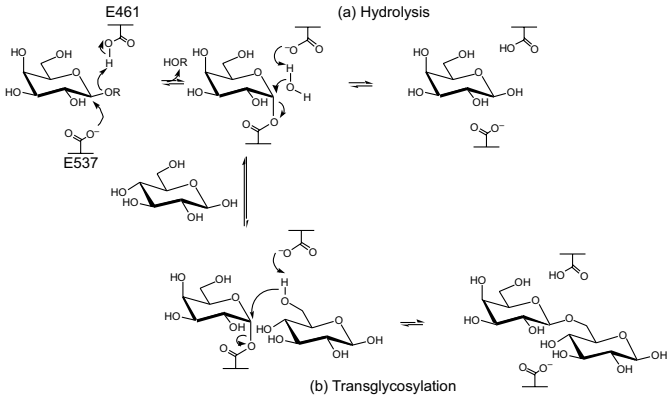
Fig. 1. Mechanisms of the hydrolysis and transglycosylation reactions catalyzed by wild-type LacZ α-galactosidase.



acid with serine rather than alanine leads to more active glycosynthase mutants (19, 23).

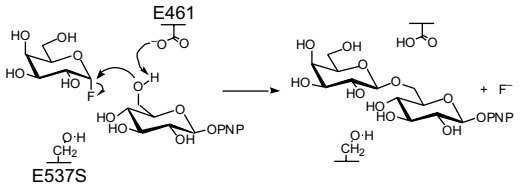
In this paper we describe the development of a glycosynthase from glycosyl hydrolase family 2 *Escherichia coli* β-galactosidase (LacZ), which synthesises Gal-β-(1,6)-linkages, a glycosidic linkage hitherto unavailable using known glycosynthases (Fig. 2).

Results and discussion

E. coli LacZ-galactosidase (EC 3.2.1.23) is one of the most commonly used enzymes within molecular biology and was instrumental in determining the operon model of gene regulation. It is responsible for the hydrolysis of lactose (Gal-β-(1,4)-Glc) to provide glucose as a starting point for glycolysis. It also performs transglycosylation reactions between the galactose and glucose moieties from lactose, to form allolactose (Gal-β-(1,6)-Glc), the natural lac-operon inducer. This enzyme is functional as a tetramer of 4 × 116 kDa, thus solution of its 3-D structure represented a considerable "tour de force" of X-ray crystallography (24). Previous work had identified the nucleophilic residue within the active site of LacZ galactosidase as Glu537 by trapping the covalent intermediate through the use of the mechanism-based inactivator, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside, coupled with electrospray ionization mass spectrometric analysis of HPLC-separated proteolytic digests of the 2-fluorogalactosyl-enzyme intermediate (25).

The stage was therefore set for generation of a glycosynthase from LacZ. To simplify the purification of the enzyme and also allow separation of the mutant from any chromosomally encoded wild-type contaminant, a construct was used that coded a N-terminal His_6-tag fusion. Upon mutation of the nucleophile to serine (Glu537Ser), the mutant protein was over-expressed in *E. coli* strain BL21 λDE3 and isolated by affinity chromatography on a nickel column. Unfortunately, the protein retained significant wild-type activity (1/200) as demonstrated by removal of all activity after incubation of the recombinant protein with sub-stoichiometric amounts of the mechanism-based inactivator. We surmized that this low wild-type activity arose from monomers of the wild-type LacZ galactosidase expressed constitutively from the lac operon associating with the mutant to form active tetrameric units within the cell. These tetrameric units presumably remain associated throughout the affinity purification and, therefore, a small but significant quantity of wild-type LacZ is co-purified with the mutant, despite its lack of an affinity tag. To circumvent wild-type contamination we obtained *E. coli* strain W2244 that has one third of the LacZ gene deleted, and consequently fails to grow on lactose as the sole carbon source (26). The strain was lysogenized with λDE3 phage to introduce the T7 RNA polymerase gene into the W2244 genome as is necessary for expression of genes under control of a T7 promoter. Purification of the gene product from *E. coli* strain W2244 λDE3 resulted in a lower yield of protein than from BL21 λDE3 stocks but, more significantly, wild-type activity was not observed.

Fig. 2. Transglycosylation catalyzed by LacZ glycosynthase mutants.

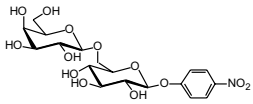
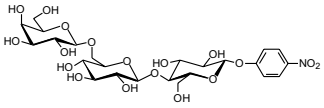
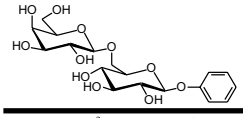


The results of transglycosylation reactions catalyzed by LacZ (Glu537Ser) glycosynthase are summarized in Table 1. Reaction between α-D-galactosyl fluoride (α-GalF) and *para*-nitrophenyl β-D-glucopyranoside (*p*NPGlc) catalyzed by the Glu358Ser mutant resulted in a modest 40% yield of a disaccharide product. To determine the regiochemistry of the linkage, the disaccharide product was acetylated and characterized by NMR. The relative downfield chemical shift of the glucopyranoside ring C-6 in comparison to the other centers, which are acetylated, indicated a β-(1,6)-linkage. The glycosidic linkage formed by the glycosynthase reaction is therefore the same as the linkage formed in the wild-type transglycosylation reaction, consistent with a preferred binding mode for galactose in the aglycone (+1) site in which the 6-hydroxyl of glucose is located close to the anomeric center of the galactosyl enzyme. Comparable yields of product were obtained using a transglycosylation reaction catalyzed by wild-type enzyme to synthesise 3-*O*-methyl allolactose. As with all transglycosylation reactions, product hydrolysis occurred over longer reaction times (27). The glycosynthase reaction yield was significantly greater than that obtained with the wild-type enzyme (40% vs. 10% total of allolactose, lactose and Gal-β-(1,3)-Glc) (28).

The reaction of α-GalF and *p*NP β-cellobioside was also catalyzed by the Glu537Ser mutant enzyme, affording a 63% isolated yield of Gal-β-(1,6)-Glc-β-(1,4)-Glc-*p*NP. While the reaction of α-GalF and phenyl β-D-glucoside afforded a 61% yield of Gal-β-(1,6)-Glc-Ph. Further improvements in yield were therefore sought through additional protein engineering.

Huber and co-workers (29) had reported earlier a single-point mutation (Gly794Asp) that increased the rate of glycosidic-bond cleavage 25-fold relative to the wild-type enzyme, using lactose as substrate. It therefore seemed likely that inclusion of this point mutation within the glycosynthase could improve the performance of this catalyst. The double mutant Glu537Ser/Gly794Asp was therefore generated and the protein was isolated. Two parallel reactions with *p*NP β-cellobioside and α-GalF as the reactants were monitored, one catalyzed by Glu537Ser and the other

Table 1. The reaction of α-D-galactosyl fluoride with various acceptors catalyzed by LacZ Glu537Ser and LacZ Glu537Ser/Gly794Asp.

Product	Isolated product yield %	
	Glu537Ser	Gly794Asp/ Glu537Ser
	40[a]	70
	51 (63[a])	80
	61	85

[a]Reaction performed with an enzyme concentration of 34 μM

catalyzed by Glu537Ser/Gly794Asp. TLC analysis revealed that the double mutant was considerably more active, and upon isolation of the disaccharide product the double mutant gave a higher product yield (80% vs. 51%). With phenyl β-D-glucoside as the acceptor improvements in yield were also observed for the double mutant (85% vs. 61%). The importance of a 794–804 amino-acid residue loop has been shown through recent X-ray crystallographic studies of the structures of LacZ and mutants bound to various substrate, transition-state, and intermediate analogues. Upon the binding of transition-state analogues, this loop was found to move up to 10 Å closer to the active site (30). Figure 3 shows the position of Gly794Asp modeled into the Glu537Gln LacZ-allolactose crystal structure (1jz8.pdb) to minimize steric interactions. This structure confirms that the introduced aspartic acid lies well within the active site and may well interact with the acceptor sugar to facilitate transglycosylation. In addition, since Gly794 resides at one end of this mobile loop, which seems to play a role in catalysis, it is quite possible that the mutation may well affect the preferred conformation of this loop in a way that optimizes turnover.

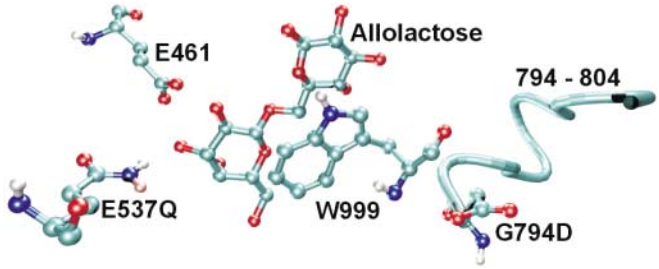
In conclusion, the existing glycosynthase methodology has been extended to the synthesis of Gal-β-(1,6)-linkages using a serine-nucleophile mutant of LacZ. Improvements in product yield have been obtained by an additional point mutation within the enzyme, which is close to the enzyme active site. Further improvements in the reaction rate should be possible using a combination of random mutagenesis and an appropriate screening protocol similar to that used for selecting improved glycosynthases from *Agrobacterium sp.* glucosidase (18).

Experimental

Enzyme mutagenesis: expression and purification

The plasmid pRSETlacZ was obtained from Invitrogen and codes for a N-terminal His-tagged LacZ gene under the control of a T7 promotor. It was mutated using the Promega GeneEditor in vitro Site-Directed Mutagenesis System as described in the manual with the following oligonucleotide to generate the Glu537Ser mutation: 5′-(P)GATCCTTGCAGTTACGCCCACG-3′ (the mutated codon is underlined). The Gly794Asp mutation was introduced into the wild-type gene using the Promega kit with the following oligonucleotide 5′-(P)AACGACATTGACGTCAGTGAAGCGACC-3′. Both mutants were digested with SacI and AccI restriction endonucleases and run on a 2% agarose gel. The 5000 bp and 800 bp fragments were cut out and purified using a Qiaex II gel extraction kit. The 5000 bp vector backbone from mutant Glu537Ser was ligated with the 800 bp insert encoding Gly794Asp and transformed into XL1-Blue cells to afford the double mutant Glu537Ser/Gly794Asp. Mutations were confirmed by sequence analysis.

Fig. 3. The crystal structure of E537Q LacZ (1jz8.pdb) with allolactose bound in the +1/+2 (shallow) binding site showing the acid–base catalyst (E461), the mutated nucleophile (E537Q), and allolactose bound over W999. The mutation G794D was generated using swisspdbviewer. The loop residues that move during catalysis in the wild-type enzyme are represented as a tube (794–804).



The plasmid coding for Glu537Ser was transformed by electroporation into *E. coli* BL21 λDE3 and induced at OD_{600} = 0.6 with IPTG (0.4 mM final concentration) and allowed to grow for a further four hours. Cells were harvested by centrifugation and passed twice through a French press at 4°C. The mutant, Glu537Ser, was isolated from the supernatant by Ni^{2+} chelation chromatography (His-bind resin, Novagen); yields of 50 mg L^{-1} were obtained. Protein was concentrated using Centricon Plus 20 kDa molecular-weight cut-off membranes.

Strain W2244 (*E. coli* genetic stock centre) was lysogenized with phage λDE3 using the Novagen λDE3 lysogenization protocol. Plasmids coding for Glu537Ser and Glu537Ser/Gly794Asp mutants were transformed into electrocompetent W2244 λDE3 cells. Protein expression and purification in W224 λDE3 was performed exactly as described for strain BL21 λDE3 except that final yields were 5 mg L^{-1}. Protein concentrations were determined by absorbance at 280 nm using the extinction coefficient $E_{280}^{0.1\%}$ = 2.1 cm^{-1}, determined from the amino acid sequence.

Oligosaccharide synthesis and characterization

Enzyme reactions were performed in 200 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$. Reaction mixtures were monitored by TLC (Kieselgel 60 F_{254} (Merck), ethyl acetate–methanol–water, 7:2:1) and visualized under UV light and by exposure to 10% ammonium molybdate in 2 M H_2SO_4. Reaction mixtures were passed through a 30-kDa ultrafiltration membrane to remove protein, applied to a TosaHaas Amide 80 HPLC column and separated by gradient elution of 80% acetonitrile to 60% acetonitrile. After analysis

and identification by mass spectrometry (ESI), products were acetylated with acetic anhydride–pyridine (1:2) and purified by flash chromatography (ethyl acetate–hexanes) on silica gel (230–400 mesh). ^{1}H and ^{13}C NMR (Bruker Avance, 400 MHz) spectra were internally referenced to the solvent.

4-Nitrophenyl* β-D-*galactopyranosyl-(1,6)*-β-D-*glucopyranoside

4-Nitrophenyl β-D-glucopyranoside (8.7 mg, 29.1 μM) and α-D-galactosyl fluoride (10.6 mg, 58 μM) were dissolved in phosphate buffer (1 mL) containing LacZ E537S (34 μM). After incubation at room temperature for 48 h, with TLC monitoring, the reaction mixture was purified to yield the disaccharide (5.4 mg, 40%).

An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 μM) was used as catalyst to yield the disaccharide (9.4 mg, 70%). ESI-MS *m*/*z*: 486 $[M + Na]^+$.

4-Nitrophenyl hepta-O-*acetyl* β-D-*galactopyranosyl-(1,6)*-β-D-*glucopyranoside*

^{1}H NMR δ: 1.82, 1.98, 2.02, 2.05, 2.06, 2.08, 2.18 (s, 21H, CH_3), 3.65 (dd, *J* = 8.1 Hz, 1H, H6), 3.02–3.91 (m, 2H, H5′, H6), 3.96 (ddd, *J* = 8.2, 1.8 Hz, 1H, H5), 4.09 (dd, *J* = 6.4, 11.3 Hz, 1H, H6′), 4.19 (dd, *J* = 6.7, 11.3 Hz, 1H, H6′), 4.48 (d, *J* = 8.0 Hz, 1H, H1′), 4.91–5.00 (m, 2H, H3′, H4), 5.12 (d, *J* = 7.2 Hz, 1H, H2), 5.21 (dd, *J* = 10.5, 8.0 Hz, 1H, H2′), 5.26 (dd, *J* = 9.3, 8.2 Hz, 1H, H3), 5.28 (d, *J* = 7.2 Hz, 1H, H1), 5.39 (d, *J* = 2.6 Hz, 1H, H4′), 7.10 (d, *J* = 7.2 Hz, 2H, Ph), 8.25 (d, *J* = 7.2 Hz, 2H, Ph). ^{13}C NMR δ: 20.5, 20.6, 20.6, 20.6, 61.3, 67.0, 68.3, 68.5, 68.7, 70.7, 70.9, 71.1, 72.5, 73.8, 98.2, 101.2, 116.6, 128.1, 143.2, 161.4, 169.2, 169.6, 170.0, 170.1, 170.3. HRMS (DCI+) *m*/*z*: calcd. for $C_{32}H_{43}N_2O_{20}$ $[M + NH_4]^+$: 775.2409; found: 775.2412.

4-Nitrophenyl* β-D-*galactopyranosyl-(1,6)*-β-D-*glucopyranosyl-(1,4)*-β-D-*glucopyranoside

4-Nitrophenyl β-cellobioside (10.6 mg, 22 μM) and α-D-galactosyl fluoride (18.4 mg, 100 μM) was dissolved in phosphate buffer (1 mL) containing LacZ E537S (17 μM). After incubation at room temperature for 48 h, with TLC monitoring, the reaction mixture was purified to yield the trisaccharide (7.0 mg, 51%).

An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 μM) was used as catalyst, yielding the trisaccharide (11.0 mg, 80%). ESI-MS *m*/*z*: 648 $[M + Na]^+$.

4-Nitrophenyl deca-O-*acetyl* β-D-*galactopyranosyl-(1,6)*-β-D-*glucopyranosyl-(1,4)*-β-D-*glucopyranoside*

^{1}H NMR δ: 1.54, 1.95, 2.01, 2.02, 2.03, 2.04, 2.08, 2.10, 2.11, (s, 30H, 10 × CH_3), 3.54–3.59 (m, 1H, H6′), 3.60–3.65 (m, 1H, H5′), 3.83–3.96 (m, 4H, H6′, H5″, H5, H6), 4.05–4.20 (m, 3H, H6, H6″, H6″), 4.5 (d, *J* = 12.4 Hz, 1H, H1′), 4.52 (d, *J* = 12.0 Hz, 1H, H1″), 4.89 (dd, *J* = 8.2, 8.0 Hz, 1H, H2′), 4.93 (dd, *J* = 9.7, 9.7 Hz, 1H, H4′), 5.05 (dd, *J* = 10.4, 3.4 Hz, 1H, H3″), 5.10–5.30 (m, 6H, H3′, H2″, H1–H4), 5.38 (dd, *J* = 3.2, 1 Hz, 1H, H4″), 7.05 (d, *J* = 2.1 Hz, 2H, Ph), 8.20 (d, *J* = 2.1 Hz, 2H, Ph). ^{13}C NMR δ: 20.5, 20.6, 20.6, 20.7, 20.7, 20.8, 61.2, 61.8, 67.1, 67.3, 68.4, 68.6, 70.7, 70.8, 70.8, 71.0, 71.6, 71.8, 73.0, 73.3, 73.3, 73.4, 75.6, 97.9, 100.2, 101.0, 116.6, 125.8, 143.2, 161.2, 169.0, 169.3, 169.4, 169.5, 169.8, 170.0, 170.0, 170.1, 170.2, 170.3. HRMS (DCI+) *m*/*z*: calcd. for $C_{44}H_{59}N_2O_{28}$ $[M + NH_4]^+$: 1063.3269; found: 1063.3254.

Phenyl* β-D-*galactopyranosyl-(1–6)*-β-D-*glucopyranoside* *(31)

Phenyl β-D-glucopyranoside (7.2 mg, 28 μM) and α-D-galactosyl fluoride (18.2 mg, 100 μM) were dissolved in phosphate buffer (1 mL) containing LacZ E537S (17 μM). After incubation at room temperature for 48 h, with TLC monitoring, the reaction mixture was purified to yield the disaccharide (7.1 mg, 61%).

An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 μM) was used as catalyst to yield the disaccharide (10.0 mg, 85%). ESI-MS *m*/*z*: 441 $[M + Na]^+$.

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