Glycosynthase-based synthesis of xylo-oligosaccharides using an engineered retaining xylanase from *Cellulomonas fimi*†

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Glycosynthases are synthetic enzymes derived from retaining glycosidases in which the catalytic nucleophile has been replaced. The mutation allows irreversible glycosylation of sugar acceptors using glycosyl fluoride donors to afford oligosaccharides without any enzymatic hydrolysis. Glycosynthase technology has proven fruitful for the facile synthesis of useful oligosaccharides, therefore the expansion of the glycosynthase repertoire is of the utmost importance. Herein, we describe for the first time a glycosynthase, derived from a retaining xylanase, that synthesizes a range of xylo-oligosaccharides. The catalytic domain of the retaining endo-1,4-β-xylanase from *Cellulomonas fimi* (CFXcd) was successfully converted to the corresponding glycosynthase by mutation of the catalytic nucleophile to a glycine residue. The mutant enzyme (CFXcd-E235G) was found to catalyze the transfer of a xylobiosyl moiety from α-xylobiosyl fluoride to either *p*-nitrophenyl β-xylobioside or benzylthio β-xylobioside to afford oligosaccharides ranging in length from tetra- to dodecasaccharides. These products were purified by high performance liquid chromatography in greater than 60% combined yield. ^{1}H and ^{13}C NMR spectroscopic analyses of the isolated *p*-nitrophenyl xylotetraoside and *p*-nitrophenyl xylohexaoside revealed that CFXcd-E235G catalyzes both the regio- and stereo-selective synthesis of xylo-oligosaccharides containing, exclusively, β-(1 → 4) linkages.

Introduction

Xylan, the most abundant of the hemicelluloses in plant cell walls, has a linear backbone structure consisting of β-(1 → 4)-linked xylosyl residues.[1,2] *Endo*-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) randomly cleave the glycosidic bond in the xylan backbone to produce xylo-oligosaccharides of varying length.[2] Xylo-oligosaccharides have potential applications because of their many beneficial biomedical and health effects. These include their non-digestibility, high water activity, anti-freezing activity, non-cariogenic nature, their salutary effects on intestinal flora, and their potential as pharmaceuticals.[3] Xylo-oligosaccharides are typically prepared from xylan by chemo-enzymatic hydrolysis,[2,4] and the chemical synthesis of β-(1 → 4)-linked xylo-oligosaccharides using a disaccharide building block has been reported.[5] Recently Eneyskaya *et al.* have reported the enzymatic synthesis of 4-nitrophenyl (PNP) β-(1 → 4)-D-xylo-oligosaccharides (PNPXO) from PNP β-D-xyloside (PNPX), and of 4-methylumbelliferyl β-(1 → 4)-D-xylo-oligosaccharides from 4-methylumbelliferyl β-D-xylobioside using the traditional trans-glycosylation reaction of a retaining β-xylosidase from *Aspergillus* sp.[6,7] However, separation of products was chromatographically challenging. The synthesis of xylo-oligosaccharides using a naturally-occurring glycosyltransferase has also been reported,[8] but its industrial application is restricted due to the cost of substrates and limited availability of the glycosyltransferase.

Glycosynthases are retaining glycosidase mutants in which the catalytic nucleophile has been replaced by an inert amino acid residue.[9] These mutants catalyze the formation of glycosidic bonds when glycosyl fluorides with the opposite anomeric configuration to that of the original substrate, thereby mimicking the glycosyl enzyme intermediate, are employed as substrate donors (Fig. 1). To date, 14 glycosynthases from eight different families have been reported, but glycosynthases originating from retaining xylanases have yet to be reported.[10–12] Nonetheless, two glycosynthase-based enzymatic syntheses of short xylo-oligosaccharides were reported recently. In one case, directed evolution of a glycosynthase from *Agrobacterium* sp. β-glucosidase gave a hyperactive glycosynthase with an expanded substrate repertoire[13] that was able to utilize α-xylosyl fluoride (αXF) as a substrate donor and to successfully transfer xylose to various aryl glycoside acceptors.[14] Most recently, the first glycosynthase from an inverting glycosidase, which hydrolyzes the glycosidic linkage *via* a single displacement mechanism involving acid/base catalysis,[15] has been reported by Honda and Kitaoka.[12] In this instance, the mutation of the general base residue of an inverting xylanase from *Bacillus halodurans* C-125 not only suppressed hydrolytic activity by four orders of magnitude but also allowed transglycosylation *via* the Hehre re-synthesis pathway.[16] However, product polymerization using these enzymes was restricted because of poor substrate recognition of the longer donor or acceptor sugars due to the intrinsic properties of the parent enzymes.[12,14]

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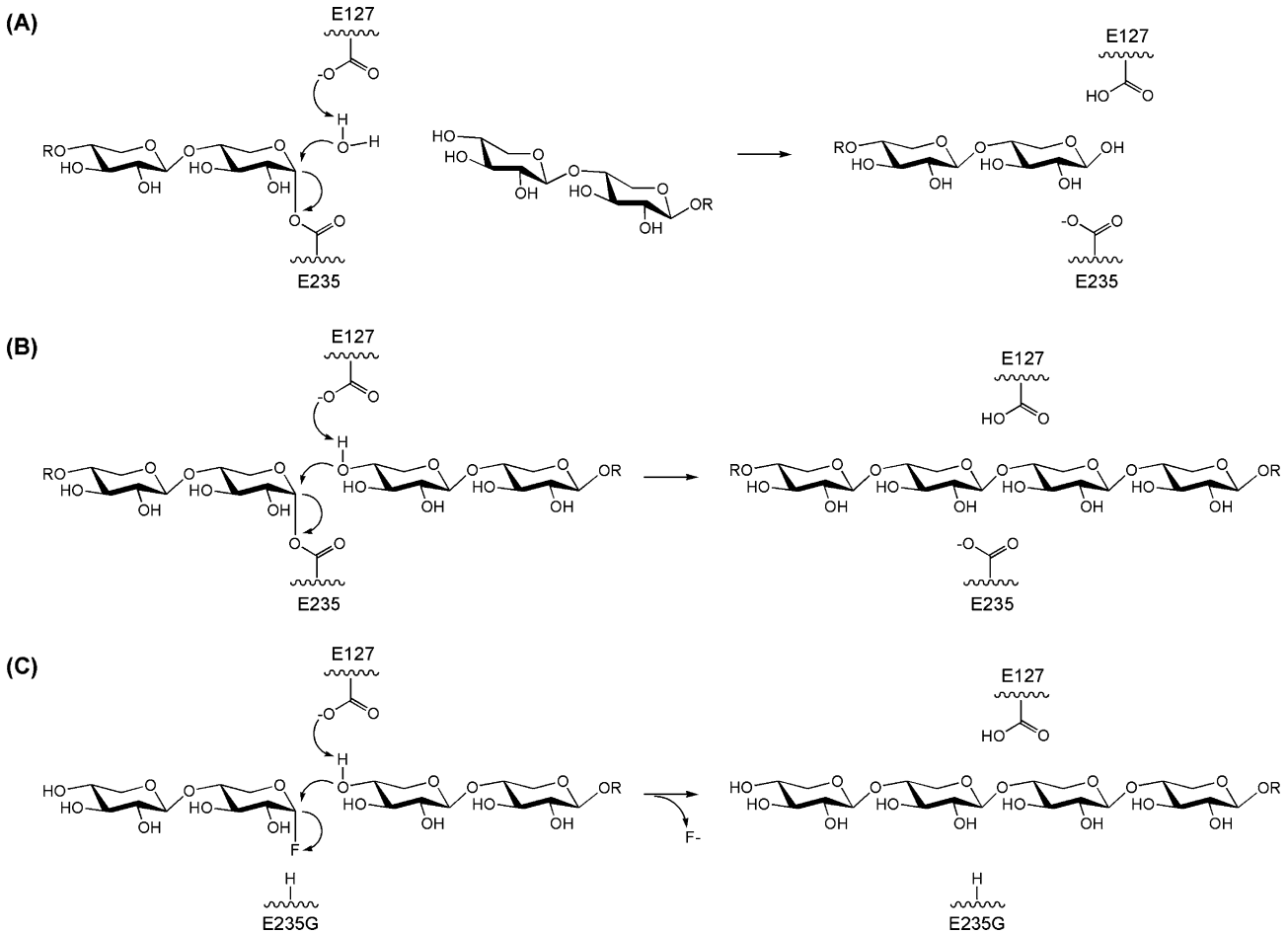

Fig. 1 Mechanisms for the hydrolase and glycosynthase actions of CFX. (A) Glycosidic bond hydrolysis catalyzed by wild-type CFX. (B) Transglycosylation reaction catalyzed by wild-type CFX. (C) Glycosynthase reaction catalyzed by glycosynthase derived from CFX (CFXcd-E235G).

Recently, a new retaining β-1,4-xylanase from *Cellulomonas fimi* (CFX) was identified *via* an activity-based protein profiling methodology for glycosidases.[17] CFX is a member of glycoside hydrolase (GH) family 10 and functions in a similar fashion to another well-characterized GH family 10 glycosidase from *C. fimi* (β-1,4-glycanase, Cex).[17,18] Herein, we describe the chemo-enzymatic syntheses of xylo-oligosaccharides of varying length using glycosynthase technology. Mutation of the catalytic nucleophile in CFX allowed the efficient production of xylo-oligosaccharides, ranging in length from four to twelve xylose units, using α-xylobiosyl fluoride (αX2F) as a donor and aryl xylobiosides as acceptors. To the best of our knowledge, this is the first report of a xylanosynthase derived from a retaining xylanase.

Results and discussion

Construction and catalytic properties of CFXcd

CFX has a xylan-specific carbohydrate binding module (CBM) belonging to CBM-Family 2b[19] at its C-terminus.[17] When the full-length CFX with a His_6-tag at its C-terminus was purified using Ni-NTA affinity chromatography, an additional purification step was needed to remove a 20 kDa peptide that eluted simultaneously, namely the proteolytically cleaved C-terminal CBM. This proteolytic cleavage lowered the purification yield of CFX because the His_6-tag was removed from the recombinant CFX. To overcome this problem we elected to produce the catalytic domain of CFX (CFXcd) with a His_6-tag at its C-terminus. The gene fragment encoding the leader peptide and CFXcd was amplified using pR2CFX$(His)_6$ containing the full-length CFX gene and fused with the His_6-tag by sub-cloning into a pR2TK vector (see also ESI†). A 1 L culture of *Escherichia coli* harboring pR2CFXcd$(His)_6$ yielded about 100 mg of pure CFXcd and no inclusion bodies were detected by SDS-PAGE analysis of the pellet of the cell lysate (data not shown). The molecular mass of CFXcd measured by ESI-MS was 34 411 Da, which matched the predicted molecular weight of the mature CFXcd with the His_6-tag (34 412 Da). The leader peptide of CFX, therefore, has been correctly processed by *E. coli*, with cleavage between the 40th and the 41st amino acid of the pre-mature CFXcd, consistent with previous results.[17]

The catalytic properties of CFXcd for PNP glucoside (PNPG), xyloside (PNPX), cellobioside (PNPC), and xylobioside (PNPX2) as substrates were nearly identical to those of CFX (Table 1). Interestingly, CFXcd displayed a typical transglycosylation pattern upon hydrolysis of higher substrate concentrations of PNPX2, showing higher overall initial rates than those expected for hydrolysis alone (closed circles in Fig. 2). At these concentrations

Table 1 Comparison of kinetic parameters of CFX and CFXcd for hydrolysis of xylo- and cello-configured substrates

Substrate	Enzyme	K_m/mM	k_{cat}/s^{-1}	(k_{cat}/K_m)/mM^{-1} s^{-1}
PNPX2	CFX[a]	1.1 ± 0.1	18 ± 1	16 ± 1
	CFXcd	1.2 ± 0.1	18 ± 2	15 ± 2
PNPX[b]	CFX[a]	—	—	$(1.1 \pm 0.1) \times 10^{-3}$
	CFXcd	—	—	$(6.5 \pm 0.8) \times 10^{-4}$
PNPC	CFX[a]	18 ± 2	2.3 ± 0.2	0.13 ± 0.02
	CFXcd	34 ± 3	3.6 ± 0.4	0.11 ± 0.02
PNPG[b]	CFX[a]	—	—	$(1.2 \pm 0.2) \times 10^{-4}$
	CFXcd	—	—	$(7 \pm 1) \times 10^{-5}$

[a] Data from ref. 17. [b] K_m and k_{cat} not determined because of lack of saturation.

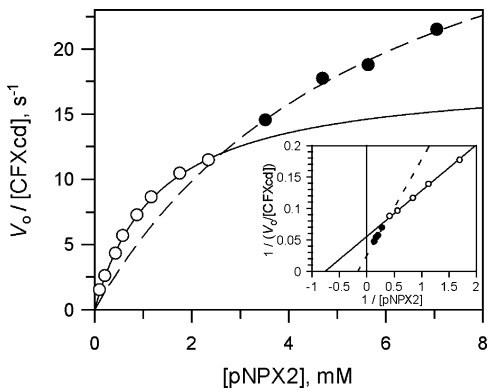

Fig. 2 Hydrolysis and transglycosylation of PNPX2 catalyzed by CFXcd. The solid and dashed lines represent the fits to the Michaelis–Menten equation using initial rates at low concentrations of PNPX2 (*ca.* 0.2–2.5 mM, ○) and high concentrations (*ca.* 3.5–7 mM, ●), respectively. The inset shows the Lineweaver–Burk representation of the same set of data in a range of PNPX2 from 0.5 to 7 mM.

(3.5–7 mM) values of k_{cat} and K_m for the transglycosylation reaction of 39.6 s^{-1} and 6.1 mM, respectively, were estimated by fitting these data to the Michaelis–Menten equation or by analysis of the double reciprocal plot (Fig. 2). The approximate 2-fold increase in k_{cat} relative to that for simple hydrolysis indicates that, for this substrate, deglycosylation was the rate-limiting step and that binding of a second substrate as acceptor leads to enhanced turnover *via* transglycosylation. From these results we concluded that the recombinant CFXcd folds correctly in *E. coli*, displaying not only the same catalytic properties as those of CFX but also typical transglycosylation kinetic behaviour. Although the observation of efficient transglycosylation activity in wild-type glycosidases does not guarantee successful conversion into a glycosynthase,[10,20] these results encouraged us to examine catalytic nucleophile mutants of CFXcd and their potential as glycosynthases.

Construction and selection of active glycosynthases derived from CFXcd

To date, cysteine, alanine, serine, and glycine are the amino acid residues of first choice for the mutations to be made at the nucleophile positions of glycosynthases.[21] Generally, but not always, Ser and Gly are better choices than Ala and Cys.[20–22] Hence, CFXcd mutants bearing Gly or Ser at the catalytic nucleophile position (Glu235) were constructed using site-directed mutagenesis. The glycosynthase ability of the purified CFXcd mutants (CFXcd-E235G and CFXcd-E235S) was directly tested with αX2F as a donor and PNPX2 as an acceptor. TLC analysis of reaction mixtures revealed that only CFXcd-E235G had glycosynthase activity (ESI†).

Catalytic properties of CFXcd-E235G

In order to investigate the catalytic properties of CFXcd-E235G, reactions were carried out using αXF and αX2F as donors and D-xylobiose (X2), PNPX and PNPX2 as acceptors. TLC analysis revealed that, for CFXcd-E235G, donor sugars must contain at least two xylose units [Fig. 3(A), (B)]. This was not surprising because the most favourable binding Gibbs free energy changes for GH10 xylanases are found at the −2 subsite.[23,24] This is completely consistent with the kinetic data in Table 1, where PNPX2 was seen to be a 10^4-fold better substrate than PNPX for CFXcd. Therefore, the −2 subsite of CFXcd-E235G plays a critical role in binding of αX2F and catalysis by the glycosynthase.

Interestingly, the sugars that are able to bind in both the +1 and +2 subsites of the mutant enzyme, such as PNPX,‡ αX2F, and X2, were very poor acceptors for CFXcd-E235G [Fig. 3(B), (C)] unlike the case with other glycosynthases derived from endoglycanases.[22,25] Most of the αX2F in reactions with the poor acceptors was hydrolyzed by CFXcd-E235G, yielding transfer products with low efficiency [lane 4, 6, 9 in Fig. 3(C)]. In contrast, PNPX2, which extends into the +3 subsite,‡ was an efficient substrate for transglycosylation, thereby reducing hydrolysis of the donor, αX2F [lane 5 in Fig. 3(A), (B), (C)]. Therefore, binding of the PNP group of PNPX2 to the +3 subsite of CFXcd-E235G apparently provides a significant contribution to catalysis. These results were unexpected because wild-type GH10 xylanases do not display significant binding interactions at the +3 subsite.[23,24] Indeed, GH10 xylanase from *Streptomyces olivaceoviridis* E-86 (SoXyn10A), which shares 73% amino acid sequence homology with CFXcd, exhibits little contribution from the +3 subsite to the binding of substrates.[23] However, these values of subsite contributions to the substrate binding for the wild-type enzymes were obtained using long substrates (>3 xylose units), which can bind across the glycon and the aglycon subsites, thereby taking advantage of any cooperative effects. Therefore, the +2 subsite of CFXcd-E235G may not display as significant a binding contribution as do other wild-type enzymes because the acceptor sugars only bind to the aglycon subsites. In the case of the good acceptor PNPX2, given the stronger binding provided by the aromatic aglycon moiety,[26] the PNP ring of PNPX3 in the +3 subsite would facilitate the tighter binding of xylobiosyl moieties in the +1 and +2 subsites in a cooperative fashion.

CFXcd-E235G catalyzed very slow hydrolysis of PNPX2, as evidenced by the appearance of PNP in overnight reaction mixtures, according to the kinetic parameters $k_{cat} = 0.02$ s^{-1}, $K_m =$ 1.4 mM. Such hydrolytic activity is uncommon in glycosynthases and, when seen, is often found to arise from very small amounts

‡ The aryl group in aryl glycosides commonly binds well to the subsites of glycosidases.

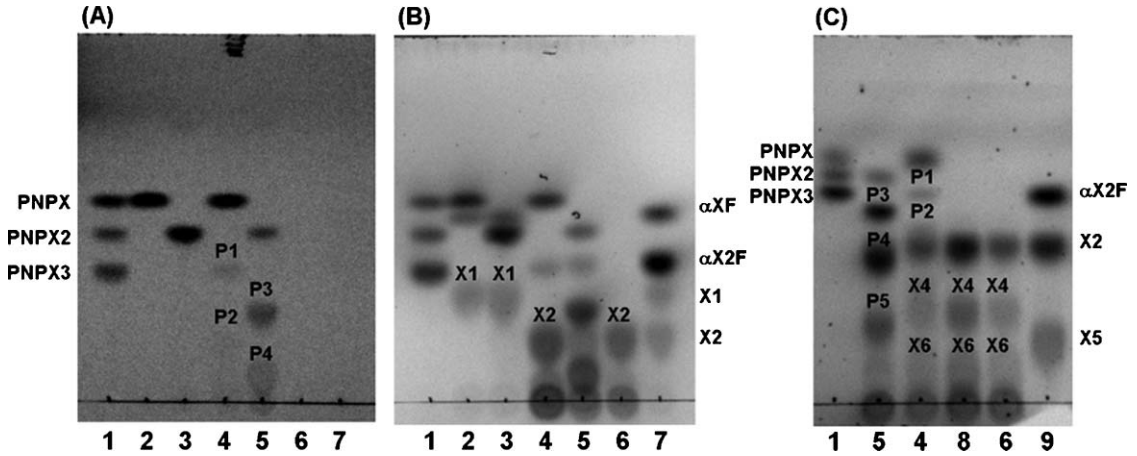

Fig. 3 TLC analysis of the reactions catalyzed by CFXcd-E235G. The compounds were visualized under UV light (A) and by exposure to 10% sulfuric acid in methanol followed by charring (B, C). EtOAc–MeOH–water (7 : 2 : 1) for A and B, and iso-PrOH–EtOAc–water (3 : 1 : 1) for C were used as solvents for TLC. Lane 1: PNPX, PNPX2, and PNPX3 standards; lane 2: reaction mixture of αXF and PNPX; lane 3: reaction mixture of α-XF and PNPX2; lane 4: reaction mixture of αX2F and PNPX; lane 5: reaction mixture of αX2F and PNPX2; lane 6: reaction mixture of αX2F; lane 7: blank reaction mixture of αXF and αX2F; lane 8: reaction mixture of αX2F and X2; lane 9: αX2F, X2 and X5 standards. The concentrations of substrates were 10 mM. The position of standard sugars is indicated on the margins. P1 = PNPX3; P2 = PNPX5; P3 = PNPX4; P4 = PNPX6; P5 = PNPX8; X1 = D-xylose; X2 = D-xylobiose; X4 = D-xylotetraose; X5 = D-xylopentaose; X6 = D-xylohexaose.

of contaminating wild-type enzyme. However, in this instance, incubation of the mutant enzyme with the mechanism-based inactivator 2,4-dinitrophenyl β-D-xylopyranosyl-β-D-(1 → 4)-2-deoxy-2-fluoroxylopyranoside, which inactivates the wild-type enzyme by accumulating a stable glycosyl enzyme intermediate,[17] did not eliminate this hydrolytic activity as would be the case if the activity were a consequence of wild-type contamination. Fortunately, the transglycosylation (glycosynthase) reaction was much faster, thus PNPXO readily accumulated (lane 5 of Fig. 3).

Importantly, as a consequence of the use of a disaccharide donor, and of the irreversibility of the glycosynthase, only products differing in length by two xylose residues were obtained, as is clearly detected by ESI-MS. This property greatly facilitated the ease of chromatographic separation of the xylo-oligosaccharide products, as was revealed by the baseline separations throughout the chromatogram in Fig. 4. This was not the case when wild-type *exo*-glycosidases are used in transglycosylation mode. The reversibility of that reaction results in scrambling of the oligosaccharides so formed. In our case, use of PNPX2 as acceptor resulted in an even series of oligosaccharides ranging in length from tetra- to dodeca-saccharides. Clearly, use of PNPX or PNP β-xylotrioside (PNPX3) as acceptor will provide access to the odd series. Unfortunately, attempts to synthesize cello-oligosaccharides using the D-gluco-configured substrates, α-D-cellobiosyl fluoride[27] and PNPC with CFXcd-E235G were unsuccessful (data not shown). Presumably, the 100-fold lower efficiency (based on the values of k_{cat}/K_m) of this enzyme as a cellulase precludes useful transfer rates (Table 1).

Preparative scale synthesis of PNP β-D-xylo-oligosaccharides

The reaction catalyzed by CFXcd-E235G using αX2F as donor and PNPX2 as acceptor was carried out on a 50 μmol scale. An equimolar ratio of αX2F to PNPX2 was insufficient to drive the reaction to completion since the initial transglycosylation product served as a more effective substrate for successive transfers.

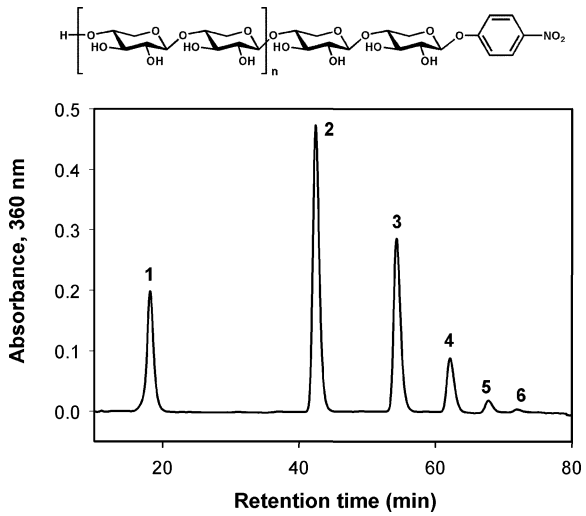

Fig. 4 HPLC analysis of products formed by CFXcd-E235G from αX2F and PNPX2. The eluate was analyzed using a UV detector at 360 nm. Conditions: Tosoh Amide-80 column, 6 mL min^{-1} (CH_3CN–H_2O 80 : 20 to 30 : 70 for 50 min). Peak 1, PNPX2 ($n = 0$); peak 2, PNPX4 ($n = 1$); peak 3, PNPX6 ($n = 2$); peak 4, PNPX8 ($n = 3$); peak 5, PNPX10 ($n = 4$); peak 6, PNPX12 ($n = 5$).

However, addition of further equivalents of the donor over time led to the essentially complete utilization of the acceptor sugar. Therefore, yields of transfer products were reported on the basis of the amount of acceptor employed.

TLC analysis of the reaction between αX2F (100 μmol) and PNPX2 (50 μmol) catalyzed by CFXcd-E235G revealed that reaction had terminated after 24 h (ESI†), yielding a range of oligosaccharides from PNP β-D-xylotetraoside (PNPX4) up to PNP β-D-xylododecaoside (PNPX12), corresponding to five additions of X2. Complete consumption of PNPX2 was never observed, even when reactions were carried out at higher ratios of donor : acceptor and longer reaction times (data not shown).

Table 2 Yields of tranglycosylation products from reactions catalyzed by CFXcd-E235G determined using preparative HPLC

[β-D-Xyl-(1 → 4)-β-D-Xyl-(1 → 4)-]$_n$-β-D-Xyl-(1 → 4)-Xyl-β-R	R = PNP[a]		R = BT[b]	
	Quantity/mg	Yield (%)	Quantity/mg	Yield (%)
$n = 0$	2.8	14	2.5	13
$n = 1$	10.2	30	8.3	26
$n = 2$	9.0	19	10.5	23
$n = 3$	4.5	7	7.1	12
$n = 4$	2.7	4	2.8	4
$n = 5$	1.1	1	0.9	1
Total purification yields of aryl saccharides[c]		75		79
Total percentages of transfer products[d]		81		84

[a] PNP = 4-nitrophenyl; reaction performed with 2 equiv. of xylobiosyl fluoride donor. [b] BT = benzylthio; reaction performed with 3 equiv. of xylobiosyl fluoride donor. [c] Sum of purification yields for aryl saccharides. [d] (Total purification yield of transfer products)/(total purification yields for aryl saccharides) × 100.

This incomplete conversion of substrate probably resulted from product inhibition by the long xylo-oligosaccharides formed, which can occupy all subsites of the enzyme from glycone to aglycone sites. The transfer products were purified by HPLC in a 61% total yield (81% of the total isolated PNPXO, Table 2). The dominant isolated species was PNPX4, corresponding to a single addition, with PNP β-D-xylohexaoside (PNPX6) being the next most abundant. To investigate the structures of transfer products, purified PNPX4 and PNPX6 were dissolved in D_2O and subjected to NMR analyses. ^{1}H NMR data indicated that all of the monosaccharide residues have β-anomeric configurations based on the spin–spin coupling values for the anomeric hydrogen atoms ($J_{1,2} \approx 7.2$–8.8 Hz, Table 3). The assignment of the ^{13}C-signals, given in Table 3, was carried out on the basis of known ^{13}C NMR shifts of a series of PNPXO.[6] The ^{13}C-signals at *ca.* 75–76 ppm in the spectra (Fig. 5) unambiguously confirm the presence of β-(1 → 4)-linkages.[6] Further, the absence of chemical shifts at *ca.* 84–86 ppm, which typically correspond to C-3 of the xylose rings in β-1,3-linked xylo-oligosaccharides,[28] confirm that no β-(1 → 3) linkages form, thereby also indicating that CFXcd-E235G exclusively forms β-(1 → 4)-linked xylopyranose units.

Table 3 ^{1}H and ^{13}C NMR chemical shifts (δ) of PNPX4 and PNPX6 isolated from the reaction mixture of αX2F and PNPX2 in the presence of CFXcd-E235G

Ring[a]	H-1	$J_{1,2}$/Hz	C-1	C-2	C-3	C-4	C-5
PNPX4							
X1	5.24	7.2	99.7	72.3	73.2	75.9	63.0
X2	4.50	8.4	101.5	72.5	73.5	76.2	62.8
X3	4.41	8.0	101.5	72.5	73.5	76.2	62.8
X4	4.45	8.0	101.7	72.6	75.4	69.0	65.0
PNPX6							
X1	5.25	7.3	99.7	72.3	73.2	75.9	63.0
X2	4.50	8.5	101.5	72.5	73.5	76.2	62.8
X3	4.48	8.3	101.5	72.5	73.5	76.2	62.8
X4	4.48	8.3	101.5	72.5	73.5	76.2	62.8
X5	4.48	8.3	101.5	72.5	73.5	76.2	62.8
X6	4.45	8.8	101.7	72.6	75.4	69.0	65.0

[a] X1 = reducing end xylose unit; X2, X3, X4, X5, and X6 = non-reducing xylose units toward the non-reducing end.

Preparative scale synthesis of benzylthio β-D-xylo-oligosaccharides

1-Benzylthio β-D-xylobioside (BTX2) is a non-hydrolysable substrate analogue and acts as a good reactivator for the inactivated GH11 xylanase from *Bacillus subtilis* that has been trapped as its 2-fluoroxylobiosyl-enzyme intermediate.[29] As noted earlier, CFXcd-E235G catalyzes slow hydrolysis of PNPX2, which resulted in a lower yield of PNPXO. It was therefore hoped that a higher total yield of transfer products would be obtained with BTX2 than in the reaction with PNPX2 since the acceptor should not be hydrolyzed in this case. Indeed, BTX2 acts as a better acceptor than PNPX2 and additionally the rate of consumption of αX2F, judged by TLC, was greater than that in the reaction using PNPX2 as acceptor. Presumably, the benzythiol group interacts more strongly at the +3 subsite of the enzyme than does PNP as discussed above. Use of a 3 : 1 molar ratio of αX2F : BTX2 resulted in greater production of hexasaccharide and octasaccharide compared to that seen in the reaction using a 2 : 1 molar ratio of αX2F : PNPX2 (Table 2). However, BTX2 also was not converted completely, and unreacted BTX2 was isolated from reaction mixtures in a 13% yield (16% of the total isolated benzylthio β-D-xylo-oligosaccharides), much as seen in the PNPX2 reaction. Overall, the total yield of transfer products (66%) was similar to that obtained in the PNPX2 reaction (Table 2).

Conclusions

Here, we describe for the first time the enzymatic synthesis of xylo-oligosaccharides using a xylanosynthase (CFXcd-E235G) derived from a retaining xylanase. This expansion of the glycosynthase repertoire allows the regio- and stereo-selective synthesis of β-1 → 4-linked xylo-oligosaccharides in high yields. Owing to the irreversibility of the glycosynthase reaction and the use of a disaccharide donor sugar, products are obtained as a series of xylo-oligosaccharides differing in length by multiples of two xylose units. This greatly simplifies the purification of the products compared to the situation with other enzymatic methodologies for the synthesis of xylo-oligosaccharides.[6] Our methodology, therefore, provides a simple and convenient process for the production of linear xylo-oligosaccharides of defined length.

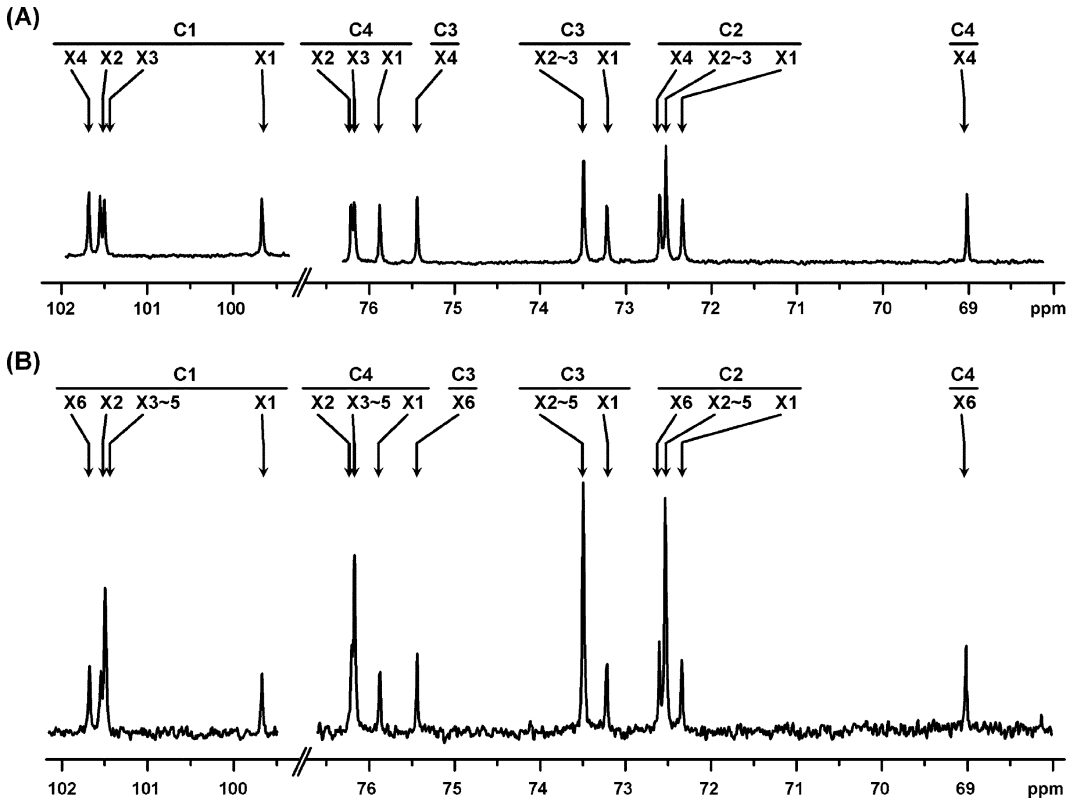

Fig. 5 Partial ^{13}C NMR spectra of PNPX4 (A) and PNPX6 (B) synthesized by CFXcd-E235G. X1 is the reducing end xylose unit, and X2, X3, X4, X5, and X6 refer to the xylose units toward the non-reducing end.

Experimental

Materials and general methods

PNP β-glycosides used as substrates were purchased from Sigma/Aldrich Chemical Co. (Oakville, ON, Canada). D-Xylobiose and D-xylopentaose were obtained from Megazyme (Wicklow, Ireland). αXF, α-cellobiosyl fluoride, PNPX2 and PNPX3 were synthesized according to known methods.[14,27,30,31] The syntheses of PCR primers and the analyses of DNA sequences were carried out by the Nucleic Acid and Proteins Service Unit in the Michael Smith Laboratories at the University of British Columbia. All ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz using a Bruker AV-400 spectrometer. Mass spectra for aryl xylo-oligosaccharides were recorded using a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) equipped with an electrospray ionization ion source (ESI†). High resolution mass spectrometric analysis was carried out by UBC mass spectrometry centre. TLC was performed on aluminium-backed sheets of silica gel $60F^{254}$ (Merck) of thickness 0.2 mm using 7 : 2 : 1 (v/v/v) ethyl acetate–methanol–water or 3 : 1 : 1 (v/v/v) iso-propanol–ethyl acetate–water. The plates were visualized using UV light (254 nm) and/or by exposure to 10% sulfuric acid in methanol followed by charring.

Synthesis of α-D-xylobiosyl fluoride (αX2F)

αX2F was synthesized from *per-O*-acetyl β-xylobiose by fluorination with HF–pyridine using a previously reported method for other glycosyl fluorides.[30] This was followed by deacetylation with catalytic sodium methoxide in dry methanol. The overall yield for the synthesis of αX2F from fully acetylated xylobiose was 62%; mp 174–177 °C (from ethanol). ^{1}H NMR (CD_3OD, 400 MHz, ppm): δ 5.47 (dd, 1 H, $J_{1,2}$ 2.8 Hz, $J_{1,F}$ 53.0 Hz, H-1), 4.31 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1′), 3.87 (m, 2 H), 3.68 (m, 3 H), 3.51 (m, 2 H), 3.31–3.23 (m, 3 H). ^{13}C NMR (CD_3OD, 100 MHz, ppm): δ 108.96 (d, $J_{1,F}$ 225.4 Hz, C-1), 104.24 (C-1′), 77.78, 77.66, 74.44, 73.47, 73.23, 73.06, 71.19, 67.27, 62.73, 62.68. ^{19}F NMR (CD_3OD, 300 MHz, TFA at 0 ppm): δ −77.69 (dd, $J_{F,H-2}$ 26.0 Hz, $J_{F,H-1}$ 53.0 Hz). HRMS ($M + Na^+$): *m/z* calc. for $C_{10}H_{17}FO_8 + Na^+ = 307.0805$; found 307.0804.

Synthesis of benzylthio β-D-xylobioside (BTX2)

Benzylthio β-D-xylopyranoside (BTX) was prepared with minor modifications to our previously published method.[32] The 2,3-*O*-isopropylidene protecting group was incorporated into BTX in the presence of 2-methoxypropene, methanolic hydrogen chloride, and *N*,*N*-dimethylformamide in moderate yield.[33] Coupling of the 2,3-*O*-isopropylidene-protected BTX with 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl trichloroacetimidate[34] followed by acidic work-up gave the partially protected BTX2 in 48% yield. This was deacetylated with a catalytic amount of sodium methoxide in dry methanol. The solvent was evaporated under reduced pressure to yield BTX2 as a colorless oil. ^{1}H NMR (CD_3OD, 400 MHz, ppm): δ 3.08–3.22 (m, 6 H, H-2, H-2′, H-3, H-3′, H-5a, and H-5a′), 3.41 (ddd, 1 H, $J_{4,5a}$ 10.2, $J_{4,5b}$ 5.6 Hz, H-4), 3.58 (ddd, 1 H, $J_{4',5a'}$ 9.9, $J_{4',5b'}$ 5.2 Hz, H-4′), 3.77 (AB q, 2 H, J_{ab} 12.9 Hz, $BnCH_2$), 3.80 (dd, 1 H, $J_{5e,5a}$ 11.3, $J_{5b,4}$ 5.3 Hz, H-5b), 4.02 (dd,

1 H, $J_{5b',5a'}$ 11.6, $J_{5b',4'}$ 5.2 Hz, H-5b′), 4.11 (d, 1 H, $J_{1,2}$ 9.1 Hz, H-1), 4.24 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1′), 7.13–7.26 (m, 5H, Bn); ^{13}C NMR (CD_3OD, 100 MHz, ppm): δ 34.78, 67.24, 68.10, 71.21, 74.23, 74.44, 77.34, 77.75, 78.09, 86.27, 104.06, 128.19, 129.61 (2C), 130.32 (2C), 139.57; HRMS (ESI, M + Na^+): *m/z* calc. for $C_{17}H_{24}O_8S + Na^+ = 411.1089$; found 411.1090.

Construction of pR2CFXcd(His)$_6$ and purification of CFXcd

The gene fragment encoding the catalytic domain of CFX was amplified by PCR using 1 μM of CFX-TOP-Nco-fw primer (5′-GCGAGTGACCATGGCCACGAAACTCCACGCGAC-3′) and CFXcd-END-Not-rev primer (5′-GTCACGTGCGCGG-CCGCATGACCCGCTGAC-3′), 0.2 mM each of the four dNTPs, 5% DMSO, 25 ng of pR2CFX(His)$_6$ [17] as template DNA, and 2.5 U of Pwo polymerase (Roche) in 50 μL 1 × Pwo polymerase buffer. Twenty-five PCR cycles (30 s at 96 °C, 30 s at 65 °C and 45 s at 72 °C) were performed in a thermal cycler (Perkin Elmer, GeneAmp PCR System 2400). The resulting PCR product was digested with NcoI and XhoI and then sub-cloned into pR2TK.[17] The resulting plasmid was designated as pR2CFXcd(His)$_6$. CFXcd was purified from *E. coli* TOP10 cells harboring pR2CFXcd(His)$_6$ by affinity chromatography using Ni-NTA agarose (Qiagen), as previously described.[17] Desalting and the concentration of purified enzyme solutions were carried out using an Amicon Ultra-4 filter unit (10 000 Da cut-off, Millipore). The buffer used in enzyme solutions was exchanged for 100 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0. Protein concentration was determined using the Micro BCA™ protein assay reagent kit (Pierce) using BSA as a standard.

Kinetic analysis of aryl glycoside hydrolysis

Initial rates of hydrolysis of PNPX, PNPG, PNPX2, and PNPC catalyzed by CFXcd in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.5, 0.1% BSA were determined at 37 °C by monitoring the reactions spectrophotometrically at 400 nm as described before.[17] The values of K_m and k_{cat} were determined by fitting the initial velocity curves to the Michaelis–Menten equation using non-linear regression with the program GraFit.

Site-directed mutagenesis to construct CFX-E235G and CFX-E235S

Mutations of the catalytic nucleophile of CFXcd were introduced using a mega primer PCR method. Plasmid pR2CFXcd(His)$_6$ was used as template for the mutagenesis. The 3′-end of the gene fragment for CFX-E235G was amplified with the T7 terminator primer and CFX-E235G-fw (5′-GACGTGCAGATCACCG*GCC*TCGACATCGAG-3′, the mutated codon is underlined), while the primer CFX-E235S-fw (5′-GACGTGCAGATCACCA*GCC*TCGACATC GAG-3′) was used for CFX-E235S. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and then used as mega primers to obtain the full-length genes with the CFX-TOP-Nco-fw primer. The full-length PCR products were digested with NcoI and XhoI, the products separated by agarose gel electrophoresis, and the desired fragments were extracted from agarose gel using a QIAquick Gel Extraction Kit and ligated into the pR2TK vector previously digested with the corresponding restriction enzymes. The resulting plasmids were designated pR2CFXcd-E235G(His)$_6$ and pR2CFXcd-E235S(His)$_6$. The nucleophile mutants of CFXcd were purified by affinity chromatography using Ni-NTA agarose (Qiagen).

Enzymatic synthesis and isolation of xylo-oligosaccharides

To a mixture of αX2F (15 mg, 53 μmol) and PNPX2 (20 mg, 50 μmol) in NaH_2PO_4/Na_2HPO_4 buffer (5 mL of 0.1 M, pH 7.0) was added CFXcd-E235G (1.4 mg) and the mixture then incubated at room temperature. Upon consumption of the donor sugar (*ca.* 10 h), as determined by TLC, an additional 15 mg of αX2F was added to the reaction to give a total added 2 : 1 ratio of αX2F : PNPX2). Benzylthio xylo-oligosaccharides were synthesized in a similar manner by incubating αX2F (15 mg, 53 μmol) and BTX2 (19 mg, 49 μmol) in NaH_2PO_4/Na_2HPO_4 buffer (3 mL of 0.1 M, pH 7.0) in the presence of CFXcd-E235G (1.4 mg). Upon consumption of donor sugar an additional 15 mg of αX2F was added at 3 and 10 h, respectively, to give a total added 3 : 1 ratio of αX2F : BTX2. After 24 h incubation, the reaction mixture was loaded onto a C_{18} SEP PAK cartridge (Waters) to remove non-aryl sugars, enzyme, and salts. The cartridge was washed with 6 mL of water and then the aryl xylo-oligosaccharides were eluted with 6 mL of 50% (v/v) methanol–water. The solvents were evaporated under reduced pressure and the residues loaded onto a preparative Tosoh Amide-80 (2.15 × 30 cm) column. The products were eluted at 6 mL min^{-1} with an acetonitrile–water (70 : 30 to 20 : 80 for 50 min) gradient.

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