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XYLOSYL TRANSFER TO CELLOBIOSE CATALYSED BY AN ENDO-(1→4)-β-D-XYLANASE OF *Cryptococcus albidus*

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ABSTRACT

In transglycosylation reactions catalysed by an endo-(1→4)-β-D-xylanase of the yeast *Cryptococcus albidus*, cellobiose is a relatively good acceptor of xylosyl residues. Three transfer products isolated from a reaction mixture containing phenyl β-D-xylopyranoside, cellobiose, and the enzyme were established by ^{13}C-n.m.r. spectroscopy to be 6′-*O*-β-D-xylopyranosylcellobiose, 6′-*O*-β-D-xylobiosylcellobiose, and 6′-*O*-β-D-xylotriosylcellobiose. When these compounds were treated with the enzyme, the 6-*O*-β-D-xylosidic linkage was hydrolysed slowly in comparison to the rate of hydrolysis of (1→4)-β-D-xylosidic linkages.

INTRODUCTION

Some polysaccharide hydrolases (glycanases) catalyse not only the hydrolysis of glycosidic linkages, but also such reactions as transglycosylation, condensation, and some non-hydrolytic reactions[1–5]. Investigations of such atypical activities usually provide new information on the mechanism of action, the catalytic capabilities, and the active site of the enzymes.

The extracellular endo-(1→4)-β-D-xylanase (EC 3.2.1.8.) of the yeast *Cryptococcus albidus* catalyses the degradation of (1→4)-linked β-D-xylosaccharides and some aryl β-D-xylopyranosides by both transglycosylation and hydrolysis, depending on substrate concentration[6,7]. In accord with the specificity of the enzyme, the transglycosylation reactions generated mainly (1→4)-β-D-glycosidic linkages; however, (1→3)-β-D-xylosyl transfer occurred to a minor extent[8,9]. The observations that phenyl β-D-xylopyranoside was almost as good a xylosyl acceptor as xylobiose[10] and that the β-D-xylanase binding-site accommodated a (1→3)-linked β-D-xylobiosyl residue[9] indicated a certain degree of non-specificity of the enzyme.

We have now examined, as xylosyl acceptors in the transfer reactions of *C. albidus* β-D-xylanase, several, mostly radiolabelled, saccharides and glycosides, of which cellobiose was found to be one of the best acceptors but was xylosylated at HO-6′ and not at HO-4′ as might be expected.

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EXPERIMENTAL

Enzymes. — Endo-(1→4)-β-D-xylanase of *Cryptococcus albidus* was purified as described[11] and 1 unit of activity is defined as the amount of enzyme capable of liberating, from wood xylans, reducing sugars equivalent to 1 μmol of xylose in 1 min. β-D-Xylosidase (exo-β-D-xylanase), a product of *Aspergillus niger*, Strain 14, was partially purified[9] from a crude enzyme preparation kindly donated by Dr. I. Gorbacheva (Bakh Institute of Biochemistry, Academy of Sciences of the U.S.S.R., Moscow). The β-D-xylosidase preparation was free of β-D-xylanase activity, but had considerable β-D-glucosidase activity which, if necessary, could be selectively inhibited by nojirimycin[12,13] (a gift from Professor S. Inouye, Meiki Seika Kaisha, Ltd., Yokohama, Japan).

Substrates. — Xylotriose was prepared enzymically[8] from phenyl β-D-xylopyranoside[14]. [1-^{3}H]Xylobiose (40 Ci/mol), [1-^{3}H]cellobiose (30 Ci/mol), and [1-^{3}H]sophorose (50 Ci/mol) were obtained by catalytic tritiation of unlabelled compounds[15]. D-[U-^{14}C]Xylose and D-[U-^{14}C]lyxose were prepared by molybdate-catalysed epimerisation[16] of D-[U-^{14}C]arabinose (7 Ci/mol), obtained by oxidative degradation of D-[U-^{14}C]glucose 4-nitrophenylhydrazone[17]. D-[U-^{14}C]Glucose (180 Ci/mol) and [U-^{14}C]starch used for the preparation of [U-^{14}C]maltose (specific radioactivity not determined) by beta-amylolysis were obtained from the Institute for Research, Production and Use of Radioisotopes (Prague, Czechoslovakia). D-[U-^{14}C]Galactose (20 Ci/mol) and D-[U-^{14}C]xylose (90 Ci/mol) were obtained from Amersham International (Great Britain). Methyl β-D-xylobioside[19] and methyl β-D-xylotrioside[20] were kind gifts from Dr. P. Kováč.

Chromatography. — T.l.c. was performed on cellulose (Lucefol, Kavalier, Czechoslovakia) with *A*, ethyl acetate–acetic acid–water (17:7:10); p.c. was performed on Whatman No. 1 paper with *B*, ethyl acetate–acetic acid–water (17:7:8); and detection was effected with aniline hydrogenphthalate or silver nitrate and, for 4-nitrophenyl glycosides, u.v. light. Preparative p.c. was performed on Whatman No. 3MM paper with *B* or *C*, 1-butanol–ethanol–water (11:9:11).

Evaluation of the efficiency of acceptors in transglycosylation reactions. — Xylotriose (20mM) was incubated at 30° with *C. albidus* β-D-xylanase (3 U/mL) in the presence of various radioactive saccharides or glycosides (10mM). At intervals, aliquots were subjected to t.l.c. The radioactivity of the separated compounds was used to calculate the amount of acceptor xylosylated.

Preparation of products of xylosyl transfer to cellobiose. — A mixture (40 mL) containing phenyl β-D-xylopyranoside (150mM), cellobiose (100mM), and purified β-D-xylanase (0.06 U/mL) was incubated at 30° for 4 days and then concentrated *in vacuo*. The residue was chromatographed (solvent *B*) on 10 sheets of Whatman No. 3MM paper. The desired products were located by means of guide strips and reference compounds derived from [1-^{3}H]cellobiose (see Results).

Enzymic degradation of cellobiose-containing xylosaccharides. — The sub-

strates (1 and 20mM) were incubated with the appropriate amount of β-D-xylanase at 30°. Aliquots of 20mM mixtures were subjected to t.l.c. Larger aliquots of the mM incubation mixtures were heated at 100° for 2 min, to inactivate the enzyme, and then concentrated *in vacuo* and chromatographed. β-D-Xylosidase degradation was performed with 20mM substrate.

General. — Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter. ^{13}C-N.m.r. spectra of solutions of oligosaccharides in D_2O were recorded with a Jeol FX-100 spectrometer operating at 35.6 MHz in the proton-decoupled mode by using a repetition time of 1.0 s, a pulse width of 20 μs, a sweep width of 4000 Hz, and 8k real data-points. Chemical shifts were measured relative to that of internal methanol (50.15 p.p.m.). Smith degradation and chromatography of polyhydric alcohols were performed on a microscale[21,22]. Radioactivity of compounds on segments of chromatograms was measured in toluene scintillation fluid with a Packard scintillation spectrometer, type 3330.

RESULTS

Specificity of the β-D-xylanase. — Decrease in the radioactivity of compounds tested as xylosyl acceptors in the transglycosylation reactions of xylotriose catalysed by *C. albidus* β-D-xylanase is shown in Table I. The data show that aldopentoses and aldohexoses, including D-xylose, are poor xylosyl acceptors in comparison with xylobiose and phenyl β-D-xylopyranoside. Of the three glucose disaccharides examined, cellobiose, which is structurally closely related to xylobiose, was the best acceptor.

TABLE I

TRANSFER OF XYLOSYL RESIDUES TO RADIOACTIVELY LABELLED SACCHARIDES AND GLYCOSIDES DURING DEGRADATION[a] OF XYLOTRIOSE WITH β-D-XYLANASE OF *C. albidus*

Compound[b]	*Xylosylated product (%)*		
	30 min	*1 h*	*2 h*
D-Xylose	1.8	2.9	2.9
D-Arabinose	<1.0	1.5	1.8
D-Lyxose	<1.0	<1.0	n.d.
D-Glucose	<1.0	<1.0	<1.0
D-Galactose	<1.0	<1.0	2.2
Methyl β-D-xylopyranoside	7.5	11.2	10.5
Phenyl β-D-xylopyranoside	25.7	33.2	42.8
	25.7	38.4	44.7
Xylobiose	24.4	29.7	38.3
Cellobiose	6.8	9.1	7.6
Sophorose	<1.0	<1.0	<1.0
Maltose	<1.0	<1.0	<1.0

[a]Xylotriose, 20mM; β-D-xylanase, 3 U/mL. [b]10mM.

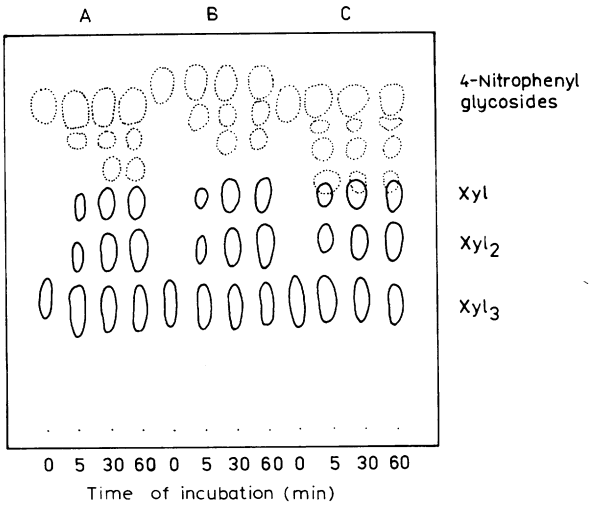

Fig. 1. Transfer of xylosyl residues to *A*, 4-nitrophenyl β-D-glucopyranoside; *B*, 4-nitrophenyl α-D-xylopyranoside; and *C*, 4-nitrophenyl β-D-galactopyranoside (each at 50mM) during degradation of xylotriose (20mM) with *C. albidus* β-D-xylanase (3 U/mL), demonstrated by t.l.c. The spots marked with full lines correspond to reducing saccharides, and those marked with dotted lines to 4-nitrophenyl glycosides.

The determination of radioactivity distribution on chromatograms of the incubation mixtures confirmed that the decrease in the proportion of the radioactivity of the acceptors was accompanied by concomitant increase of radioactivity in the regions corresponding to slower-moving compounds, to xylosylated acceptors, or to the products of their subsequent hydrolysis. However, a substantial part of the radioactivity of phenyl β-D-[U-^{14}C]xylopyranoside appeared in free oligosaccharides, and a considerable part of the radioactivity of [1-^{3}H]xylobiose in D-xylose. The transfer products to methyl β-D-[U-^{14}C]xylopyranoside exhibited the chromatographic mobilities of methyl β-D-xylobioside and methyl β-D-xylotrioside, indicating that (1→4)-β-D-xylosyl transfer was the main type of transglycosylation.

T.l.c. of incubation mixtures containing xylotriose (20mM), 4-nitrophenyl glycoside (50mM), and β-D-xylanase (3 U/mL) (Fig. 1) showed that the enzyme can also transfer xylosyl residues to 4-nitrophenyl β-D-glucopyranoside, β-D-galactopyranoside, and α-D-xylopyranoside. These reactions were not evaluated quantitatively, but the low intensity of the spots corresponding to xylosylated products indicated only a very low extent of xylosylation of the glycosides. 4-Nitrophenol was not liberated.

Isolation of the products of xylosyl transfer to cellobiose. — The relatively high efficiency of cellobiose as a xylosyl acceptor in the transglycosylation reactions of xylotriose allowed the transfer products to be prepared on a larger scale. This was accomplished by using phenyl β-D-xylopyranoside, which is more readily available than xylotriose and gives transglycosylation products[7,8].

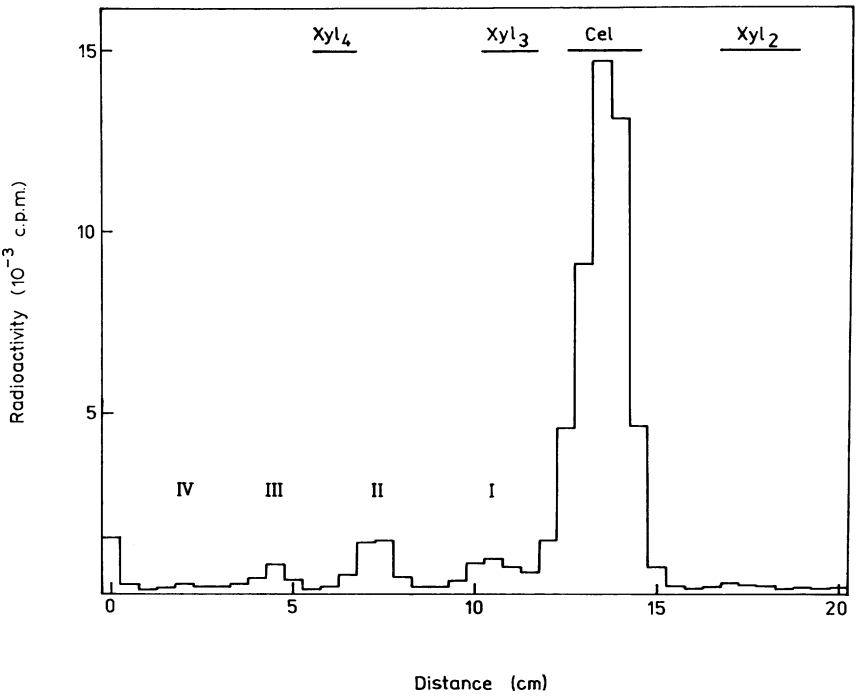

Fig. 2. Distribution of radioactivity on a paper chromatogram of a 24-h incubation mixture containing phenyl β-D-xylopyranoside (150mM), [1-^{3}H]cellobiose (100mM), and β-D-xylanase (0.25 U/mL); I–IV, products of xylosyl transfer to cellobiose.

TABLE II

PAPER-CHROMATOGRAPHIC[a] MOBILITY OF XYLOSYL-TRANSFER PRODUCTS TO CELLOBIOSE AND (1→4)-β-D-XYLOSACCHARIDES

Compound	R_{Xyl}	
	Solvent B	*Solvent* C
Xylobiose	0.73	0.83
Xylotriose	0.47	0.65
Xylotetraose	0.28	0.45
Cellobiose	0.58	
Xylosylcellobiose	0.45	0.63
Xylobiosylcellobiose	0.31	0.40
Xylotriosylcellobiose	0.19	

[a]Whatman No. 1 paper.

When phenyl β-D-xylopyranoside (150mM) and [1-^{3}H]cellobiose (100mM) were incubated with β-D-xylanase (0.25 U/mL) for 24 h, the subsequent distribution of radioactivity on paper chromatograms (Fig. 2) demonstrated that ~20% of the original radioactivity of the cellobiose appeared in four fractions: I (8.0%), II (6.5%), III (1.0%), and IV (0.3%). Fractions II and III had mobilities distinct from those of (1→4)-β-D-xylosaccharides, whereas fraction I co-chromatographed with xylotriose (Table II).

The products of a large-scale incubation mixture (see Experimental) were chromatographed (solvent *B*), and fractions II and III were isolated and re-chromatographed (solvent *C*), to give chromatographically homogeneous, syrupy II (66 mg), $[\alpha]_D^{22}$ −17.5° (*c* 1.2, water), and III (55 mg), $[\alpha]_D^{22}$ −24° (*c* 1.4, water). Fraction I, eluted from the paper together with xylotriose, was treated with β-D-xylosidase to hydrolyse, selectively, xylotriose, which is a much better substrate. The reaction was monitored by t.l.c. and terminated when xylose liberation ceased and when the spot corresponding to xylotriose gave a green-brown colour with aniline hydrogenphthalate (xylotriose, red; xylosylated cellobiose, green-brown). The mixture was then chromatographed (solvent *B*), which separated the xylose from fraction I and allowed the latter to be isolated (16 mg) with m.p. 225–226° (from methanol), $[\alpha]_D^{20}$ −3° (*c* 1, water).

Identification of the products of transfer to cellobiose. — Since cellobiose and cellotriose are not attacked by *C. albidus* β-D-xylanase and D-glucose was not gen-

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFTS OF CELLOBIOSE-CONTAINING XYLOSACCHARIDES

Compound[a]	*Sugar residue or anomer*	*Chemical shifts (p.p.m.)*					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
I	C-α	93.0	72.5[b]	72.7[b]	80.6	71.3	61.3
	C-β	97.0	75.1	75.7	80.4	76.0	61.3
	C′	104.0	74.3	76.7	70.7	76.0	70.1
	C″	104.8	74.3	76.9	70.5	66.4	
II	C-α	93.2	72.4[b]	72.7[b]	80.5	71.2	61.3
	C-β	96.9	75.1	75.6	80.4	76.0	61.2
	C′	103.9	74.2	76.6	70.7	76.0	70.0
	C″	104.6	74.0	74.9	77.6	64.1	
	C‴	103.1	74.2	76.8	70.4	66.4	
III	C-α	93.0	72.4[b]	72.7[b]	80.5	71.2	61.3
	C-β	97.0	75.1	75.6	80.3	76.0	61.2
	C′	103.9	74.2	76.6	70.7	76.0	70.0
	C″	104.6	74.0	74.9	77.5	64.2	
	C‴	103.0	74.0	74.9	77.5	64.2	
	C⁗	103.1	74.2	76.8	70.4	66.4	

[a]I, 6′-*O*-β-D-Xylosylcellobiose. II, 6′-*O*-β-D-Xylobiosylcellobiose. III, 6′-*O*-β-D-Xylotriosylcellobiose.
[b]These resonances may be reversed.

erated during treatment of the phenyl β-D-xylopyranoside–cellobiose mixture with β-D-xylanase, fractions I–III must represent a series of products of xylosyl transfer to cellobiose, differing in the number of xylosyl residues. According to the chromatographic mobility, fraction I appeared to be a trisaccharide, xylosylcellobiose. This was confirmed by hydrolysis of fractions II and III with β-D-xylosidase. A compound corresponding to fraction I was formed after liberation of one xylose residue from fraction II and two xylose residues from fraction III. Fraction I was hydrolysed very slowly by the β-D-xylosidase preparation, affording xylose and glucose, and xylose and cellobiose when nojirimycin was included in the incubation mixture to inhibit β-D-glucosidase.

The structure of cellobiose-containing oligosaccharides was established by ^{13}C-n.m.r. spectroscopy. The spectra were interpreted on the basis of published data for series of D-glucobioses[23], D-glucotrioses[23], D-xylobioses[24], methyl β-D-xylobiosides[24], cellosaccharides[25,26], xylosaccharides[25], and methyl β-glycosides of xylosaccharides[27].

The ^{13}C-n.m.r. spectrum of fraction I (Table III) contained 20 signals, including all of the resonances of cellobiose with the exception of that of C-6′ reported[25,26] to be at 62.4 p.p.m. This was replaced by a resonance at 70.1 p.p.m., which is compatible only with the substitution of cellobiose at HO-6′, as indicated by the ^{13}C-n.m.r. data for isomeric glucobioses and glucotrioses[23]. Other resonances were assigned to the β-D-xylosyl residue. A comparison of the chemical shifts of the signals of the xylose carbons in fraction I with those of the non-reducing, terminal xylosyl groups in various xylosaccharides showed that the upfield position of the C-1 signal of the xylosyl residue in fraction I is to be expected.

Thus, fraction I is 6′-*O*-β-D-xylopyranosylcellobiose. This conclusion was supported by the results of Smith degradation, which gave ethylene glycol, glycerol, and erythritol.

The ^{13}C-n.m.r. spectrum of fraction II contained 26 signals (Table III), and the resonances of C-1α and C-1β of the cellobiose moiety were resolved. The number and the position of xylose resonances indicate that the two xylosyl residues are not magnetically equivalent. The additional resonances of fraction II, in relation to those of fraction I, correspond to an internal D-xylosyl residue β-linked through HO-4. The resonance at highest field (104.6 p.p.m.) was assigned to C-1 of the xylosyl residue linked to O-6′ of cellobiose. The spectrum of fraction II did not contain resonances corresponding to carbons involved in (1→2), (1→3), and α-(1→4) linkages, and therefore fraction II appears to be 6′-*O*-β-D-xylobiosylcellobiose.

The ^{13}C-n.m.r. spectrum of fraction III contained 27 signals. With the exception of the peak at 103.0 p.p.m. assigned to C-1‴ (xylosyl), the chemical shifts of the others were identical with those of fraction II. The presence of two internal xylosyl residues in fraction III was reflected by the intensities of resonances at 74.0, 74.9, 77.5, and 64.2 p.p.m., corresponding to C-2,3,4,5 of the internal xylosyl re-

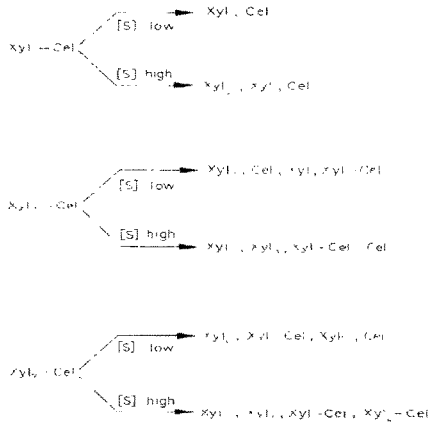

Fig. 3. Initial products of β-D-xylanase digestion of cellobiose-containing oligosaccharides at low (mM) and high (20mM) substrate-concentration [S].

sidues. As shown in Table III, the C-1 resonances of all sugar residues of fraction III were resolved. Thus, fraction III is 6′-*O*-β-D-xylotriosylcellobiose.

Degradation of cellobiose-containing oligosaccharides by β-D-xylanase of C. albidus. — The products of degradation of the oligosaccharides by the enzyme responsible for their synthesis depended on their concentration (Fig. 3). In comparison with xylotriose, mM 6′-*O*-β-D-xylosylcellobiose was hydrolysed extremely slowly, giving D-xylose and cellobiose. A somewhat increased rate of hydrolysis was observed at 20mM, but more transglycosylation than hydrolysis occurred because more xylobiose than xylose was formed.

6′-*O*-β-D-Xylobiosylcellobiose (mM) was cleaved by the enzyme at a rate much higher than that of 6′-*O*-β-D-xylosylcellobiose, to give mainly xylobiose and cellobiose with minor amounts of xylose and 6′-*O*-β-D-xylosylcellobiose. At 20mM, the tetrasaccharide afforded, in the early stage, mainly xylobiose, xylotriose, 6′-*O*-β-D-xylosylcellobiose, and cellobiose. Small proportions of xylose were formed later. Since xylotriose and 6′-*O*-β-D-xylosylcellobiose were not resolved in t.l.c., the colour of the spot (aniline hydrogenphthalate detection) was used to assess the presence of two compounds in the reaction mixture.

6′-*O*-β-D-Xylotriosylcellobiose was a good substrate for β-D-xylanase. Xylobiose and 6′-*O*-β-D-xylosylcellobiose were the main products at low concentrations of substrate and cellobiose and xylotriose were minor products. At 20mM, the reaction mixture contained initially xylobiose, xylotriose, 6′-*O*-β-D-xylosylcellobiose, and 6′-*O*-β-D-xylobiosylcellobiose. Xylose and cellobiose appeared at a later stage.

DISCUSSION

In accordance with the distribution of subsite affinities in the four subsites of the binding site of *C. albidus* β-D-xylanase[10], all of the monosaccharides tested were poor xylosyl acceptors. The enzyme showed only a slight preference in transferring xylosyl residues to xylose. Higher acceptor-efficiency was observed only with such xylose-containing compounds as xylobiose and phenyl and methyl β-D-xylopyranoside, which could interact with both subsites of the aglycon site. For xylobiose and phenyl β-D-xylopyranoside, it should be noted that their glycosidic linkages become subject to hydrolysis as soon as they are glycosylated. Thus, the continuous decomposition of the xylosyl-transfer products may contribute additionally to the high extent of their utilisation as glycosyl acceptors. These considerations may apply, in part, to the transfer products of methyl β-D-xylopyranoside, but not to the products of xylosyl transfer to cellobiose, because the cellobiose moiety is not attacked by the enzyme.

Xylosyl transfer to xylobiose and phenyl and methyl β-D-xylopyranoside mainly involves the formation of β-(1→4) linkages. The surprising finding that 6′-*O*-xylosylation of cellobiose occurred may indicate that the synthesis of the glycosidic linkage can be determined by the properties of the active site of the enzyme and by the structure of the acceptor molecule. Thus, a glycanase may exhibit different specificity with different substrates. The reasons for 6′-*O*-xylosylation of cellobiose may be steric in relation to the enzyme, and conformational in relation to cellobiose, and further work is required to clarify this point.

Examination of the mode and the rate of hydrolysis of the cellobiose-containing oligosaccharides by the enzyme responsible for their synthesis offers some information on the mechanism of their formation and on the specificity of certain subsites in the enzyme binding-site. The low rate of hydrolysis of 6′-*O*-β-D-xylosylcellobiose suggests that this compound is a secondary product of hydrolysis of

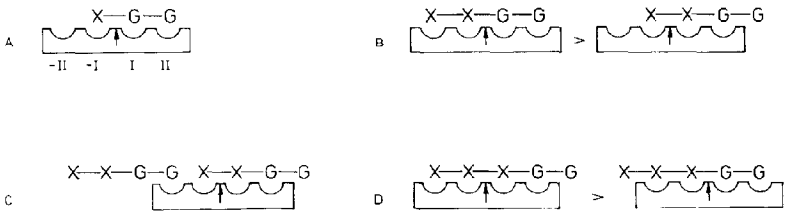

Fig. 4. Productive complexes of β-D-xylanase of *C. albidus* with cellobiose-containing xylosaccharides: A, enzyme–6′-*O*-β-D-xylosylcellobiose complex; B, enzyme–6′-*O*-β-D-xylobiosylcellobiose complexes formed at low concentration of substrate; C, termolecular shifted enzyme–6′-*O*-β-D-xylobiosylcellobiose complex formed at high concentration of substrate; D, enzyme–6′-*O*-β-D-xylotriosylcellobiose complexes formed at low concentration of substrate. Roman numerals mark the enzyme subsites around the catalytic groups (arrow).

higher oligosaccharides rather than a primary product of xylosyl transfer to cellobiose.

6′-*O*-β-D-Xylosylcellobiose forms only a shifted productive complex with the enzyme (Fig. 4A), in contrast to the unimolecular mode of binding of xylotriose[6]. The cellobiosyl moiety directs the substrate to an unconventional position, because the subsite I cannot accommodate a glucosyl residue. A similar, shifted enzyme–substrate complex is formed with 3-*O*-β-D-xylopyranosylxylobiose[9].

6′-*O*-β-D-Xylobiosylcellobiose, at low concentrations, forms two productive complexes with the enzyme (Fig. 4B), of which that with all the subsites occupied preponderates. At high concentrations, the tetrasaccharide seems to be degraded by a mechanism involving transglycosylation which accompanies substrate degradation *via* a termolecular shifted enzyme–substrate complex (Fig. 4C).

The main productive binding of β-D-xylanase with 6′-*O*-β-D-xylotriosylcellobiose, at low concentrations of substrate, involves interaction of the enzyme subsites with all of the xylosyl residues present in the substrate molecule (Fig. 4D). In general, the mechanism of degradation of cellobiose-containing xylosaccharides by *C. albidus* β-D-xylanase is strongly dependent on substrate concentration, as has been demonstrated with (1→4)-β-D-xylosaccharides[6].

6′-*O*-Xylosylated cellobiose has been isolated from enzymic digests of various plant xyloglucans, but, in all cases, the D-xylose was reported[28–30] to be α-linked.

ACKNOWLEDGMENTS

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