Transxylosylation Products of Xylobiose by β -Xylosidase-1 from *Penicillium wortmanni* IFO 7237

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The transxylosylation reaction products of β -xylosidase-1, excreted by *Penicillium wortmanni* IFO 7237 using β -(1 $\rightarrow$ 4)-xylobiose as substrate, have been separated by chromatography on activated charcoal into four fractions, designated as P-1, P-2, P-3, and P-4, respectively. They were further purified by preparative paper chromatography. The characterization and structural analysis were done by measurement of the degree of polymerization (DP) and specific rotation followed by methylation analysis. Moreover, the enzymatic structural analysis of transxylosylation products, with high performance liquid chromatography (HPLC), allowed the confirmation of each structure. The first product, P-1, was β -(1 $\rightarrow$ 3)-xylobiose and the second, P-2, was β -(1 $\rightarrow$ 4)-xylotriose, but, P-3 was *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose or isomeric xylotriose and P-4 was assumed to be *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)]-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose.

β -Xylosidase (EC 3.2.1.37) is widely distributed throughout plants,¹⁾ animals,²⁾ and various microorganisms.^{3~5)} This enzyme catalyzes two reactions, the hydrolysis of xylo-oligosaccharides derived from the action of endo-xylanase on xylan in which the substrates are broken down in the presence of water to their individual constituents, xylose residues, and transxylosylation or a transfer reaction in which β -xylosidase transfers the β -xylosyl moiety from β -xylosyl donors to acceptors. Various microorganisms produce β -xylosidases either extracellularly or intracellularly, and their enzymatic specificities differ from each other according to their sources.⁶⁾ The formation of β -(1 $\rightarrow$ 3)- or β -(1 $\rightarrow$ 4)-xylobiose in the transfer reaction of fungal β -xylosidase purified from commercial hemicellulase with phenyl- β -D-xyloside as substrate was first reported by Claeysens *et al.*⁷⁾ In 1973, Takenishi *et al.* observed a strong activity of the transfer reaction in a β -xylosidase purified from

Aspergillus niger.⁸⁾ Claeysens and coworkers investigated the transfer reaction products of aryl- β -D-xylopyranoside with a purified β -xylosidase from *Penicillium wortmanni* QM 7322. The analysis of transfer products by chemical and enzymatic methods revealed the unequivocal assignment of xylobioses with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages. Although several other homo- and hetero-oligosaccharides could be formed in such a transfer reaction, they did not isolate and identify the oligosaccharides and only reported the above two xylobioses with different linkages.⁹⁾

In our previous papers, we reported on four different types of β -xylosidase from *P. wortmanni* IFO 7237.¹⁰⁾ The purification to homogeneity and physico-chemical and enzymatic properties as well as immunological relationships were studied for each type of β -xylosidase.¹¹⁾ During the experiments, the presence of a strong xylosyl transfer activity was noticed on the paper chromatograms in the re-

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action with various xylooligosaccharides by each type of *P. wortmanni* β -xylosidase. In the course of a comparison of the transfer reaction activities of these four different β -xylosidases, we first did the transfer reaction using β -(1 $\rightarrow$ 4)-xylobiose as substrate and purified β -xylosidase-1 (X-1) of *P. wortmanni* IFO 7237. This paper describes the isolation and identification of transfer reaction products consisting not only of β -(1 $\rightarrow$ 3)-xylobiose but also of higher oligomers than the substrate with dissimilar linkages.

MATERIALS AND METHODS

Materials. The substrate, β -(1 $\rightarrow$ 4)-xylobiose, standard xylooligosaccharides,¹²⁾ and β -(1 $\rightarrow$ 3)-xylobiose¹³⁾ had been prepared in our laboratory.

Enzyme. *P. wortmanni* β -xylosidase-1 (specific activity 22 units/mg protein) was prepared and purified as described in our previous paper.¹⁰⁾ The other type of β -xylosidase (specific activity 13.7 units/mg protein) used in the enzymic structural analysis of transfer products was purified from *Malbranchea pulchella* var. *sulfurea* in our laboratory.¹⁴⁾ The enzyme activity was measured at pH 4.0 or pH 6.7 using phenyl- β -D-xyloside as substrate. The reaction mixture contained 0.5 ml of enzyme solution and 0.5 ml of 0.02 M substrate in McIlvaine buffer (pH 4.0) for *P. wortmanni* or 0.01 M phosphate buffer (pH 6.7) for *M. pulchella*. The reaction was stopped by the addition of 5 ml of 0.05 M Na₂CO₃ after incubation for 15 min at 50°C. Then 1.0 ml of phenol reagent (1 N HCl) was added to the reaction mixture and liberated phenol was measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μ mol of phenol per min under these experimental conditions.

Transxylosylation reaction with xylobiose. The reaction mixture containing 2.0 g xylobiose and 116 units of *P. wortmanni* β -xylosidase-1 in 40 ml of 0.001 M acetate buffer (pH 4.5) was incubated at 45°C for 3 hr. Then the mixture was heated in a boiling water bath for 10 min to inactivate the enzyme and concentrated to 10 ml under reduced pressure at about 45°C. The syrup thus obtained was chromatographed on a charcoal column.

Charcoal column chromatography. The above syrup (10 ml) resulting from the transfer reaction was adsorbed on activated charcoal (Wako Jun Yaku Co.) column (2.6 $\times$ 38 cm) and successively eluted with distilled water and 5%, 10%, 15%, and 30% (v/v) ethanol. The total amounts of eluents were 0.8 l for distilled water and 1.2 l for each ethanol concentration. The flow rate was 50 ml/hr

and 15 ml fractions were collected.

General methods. Total carbohydrate content was measured by the orcinol-hydrochloric acid method.¹⁵⁾ Reducing sugar concentration was estimated by the Somogyi-Nelson method.¹⁶⁾ Specific rotations of purified transfer products were measured with a digital polarimeter model DIP-140 (Japan Spectroscopic Co., Ltd.) at 20°C. DP of transfer products were estimated by a slight modification of the method of Timell based on the orcinol-hydrochloric acid method.¹⁷⁾

Chromatography. Paper chromatography (PPC) was done on Toyo filter paper No. 51A and preparative PPC on Toyo filter paper No. 527 (Toyo Roshi Co., Ltd.). The solvent system used was 1-butanol-pyridine-water (6:4:3, v/v). After removal of the solvent, *p*-anisidine hydrochloride spray was used to detect the positions of sugars on the chromatogram, followed by heating at 130°C for 5 min in a hot-air drier. Samples of transxylosylation products were also analyzed by thin layer chromatography (TLC) on silica gel 60 precoated plates (20 $\times$ 20 cm, 0.2 mm thickness, E Merck), using the solvent system of acetonitrile-water (17:3, v/v). After development by three ascents in the solvent, sugars were detected by spraying with 20% sulfuric acid in methanol and charring for 5 min at 110°C.

High performance liquid chromatography (HPLC). HPLC was done on a column (3.9 $\times$ 150 mm, μ Bond-sphere, Nihon Waters) at 40°C using a column oven (Gaskuro Kogyo, Nihon Dennetsu Keiki) with a Waters Associates M-45 instrument. The column was eluted by degassed acetonitrile-water (70:30, v/v) at 1.0 ml/min and detection was done by a differential refractometer R-401. A recording integrator, Sic Chromatocorder 11 (Sic System Instruments Co., Japan) was used to measure the peak areas.

Methylation analysis. Authentic samples and transfer reaction products were methylated by the Hakomori method¹⁸⁾ and also methylated on a micro-scale by the method of Ciucanu and Kerek.¹⁹⁾ The methylated product were hydrolyzed for 2 hr with 4% sulfuric acid at 100°C and the partially methylated sugars were converted into their alditol acetates after reduction with NaBH₄. The resultant alditol acetates were analyzed by gas liquid chromatography (GLC) on a Shimadzu GC-7A gas chromatograph, fitted with a flame ionization detector, using a glass column (3 $\times$ 1000 mm) packed with 3% ECNSS-M on Gas Chrom Q (100~200 mesh, Nippon Kuromato Kogyo), at a column temperature of 160°C. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min.

Enzymatic analysis. In the enzymatic structural analysis of transfer products, P-1 (1.55 mg), P-2 (4.47 mg), P-3 (2.11 mg), P-4 (0.41 mg), and standard β -(1 $\rightarrow$ 4)xylobiose

(100 mg) were separately dissolved in 0.5 ml of 0.01 M phosphate buffer (pH 6.7) and incubated at 45°C. Each of these substrates was hydrolyzed with 0.16 unit of *Malbranchea* β -xylosidase dissolved in 0.5 ml of the same buffer at 45°C. At various times, 200 μ l of samplings were taken out from the reaction mixture and heated in a boiling water bath for 10 min to stop the reaction. The hydrolysis products were analyzed by TLC as well as HPLC.

RESULTS AND DISCUSSION

Charcoal column chromatography of transxylosylation products of xylobiose

The syrup (10 ml) obtained from transxylosylation reaction of 2.0 g xylobiose with 116 units of *P. wortmanni* β -xylosidase-1 was adsorbed and chromatographed on a charcoal column. Elution was first done with water to remove the hydrolysis product, xylose. Then the column was eluted with 5%, 10%, 15%, and 30% (v/v) ethanol. As shown in Fig. 1, the effluents were separated into six peaks sequentially designated as X₁, X₂, P-1, P-2, P-3, and P-4. The fraction from each peak was collected and concentrated under reduced pressure and used for PPC. The results of PPC showed that the first peak, X₁, contained only xylose and

the second peak, X₂, only xylobiose (data not shown). This X₂ peak was supposed to be a mixture of the remaining substrate and the secondary transfer product xylobiose. The remaining P-1 to P-4 were thought to be transfer reaction products. The yields of six peaks and their chromatographic mobilities on PPC are shown in Table I. Total recovery from charcoal column chromatography of the transfer reaction products was calculated as 89%.

Characterization of transfer reaction products

After fractionation, each of the transfer

TABLE I. FRACTIONATION OF TRANSFER PRODUCTS FROM CHARCOAL COLUMN CHROMATOGRAPHY

Designated peak	Wt. of sugar (mg)	Yield ^a (%)	R _{xyi} in PPC ^b
X ₁	661	30.8	1.00
X ₂	1053	49.0	0.65
P-1	35	1.6	0.99
P-2	130	6.0	0.42
P-3	34	1.6	0.58
P-4	14	0.7	0.38

^a Based on total initial carbohydrate (2 g xylobiose).

^b Toyo filter paper No. 51A; solvent system, 1-butanol-pyridine-water (6:4:3, v/v).

FIG. 1. Charcoal Column Chromatography of Transxylosylation Products.

The reaction mixture contained 2.0 g β -(1 $\rightarrow$ 4)-xylobiose and 116 units of *P. wortmanni* β -xylosidase-1 in 40 ml of 0.001 M acetate buffer (pH 5.4). After incubating at 45°C for 3 hr, the reaction mixture was heated in a boiling water bath for 10 min, concentrated to 10 ml under reduced pressure, and then adsorbed on activated charcoal column (2.6 $\times$ 38 cm). The successive elutions were done with distilled water and 5, 10, 15, and 30% (v/v) ethanol. The size of each fraction was 15 ml and the flow rate of eluent was 50 ml/hr.

FIG. 2. TLC of Transxylosylation Products.

Thin layer chromatogram of transxylosylation products after purified with preparative PPC. S, standard xylo-oligosaccharides; X₁, xylose; X₂, xylobiose; X₃, xylotriose; X₄, xylotetraose; X₅, xylopentaose.

TABLE II. DEGREE OF POLYMERIZATION AND SPECIFIC ROTATION OF PURIFIED TRANSXYLOSYLATION PRODUCTS

Transxylosylation product	DP	[α] _D ²⁰ (degrees)		References
		Observed	Reported	
P-1	2	−18.2 (c 0.39, H ₂ O)	−18.4	20
P-2	3	−48.3 (c 3.33, H ₂ O)	−47.0	21
P-3	3	−44.6 (c 1.07, H ₂ O)	−52 ~ −47	13, 20
P-4	4	ND ^a		

^a ND, not determined.

TABLE III. METHYLATION ANALYSIS OF TRANSXYLOSYLATION PRODUCTS

Alditol acetate of		1,2,3,5-Tetra-O-Me-xylitol	2,3,4-Tri-O-Me-D-Xylp ^a	2,4-Di-O-Me-D-Xylp	2,3-Di-O-Me-D-Xylp	2-Mono-O-Me-D-Xylp	Mode of linkage ^b
Retention time (min)		1.05	3.70	8.00	8.99	22.30	
<i>Saccharides</i>							
Authentic samples	Xylobiose		+		+	X1 $\xrightarrow{\beta}$ 4X	
Original	Xylotriose		+		++	X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X	
	Xylotetraose		+		+++	X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X	
After reduction ^c	Xylobiose	+	+				
	Xylotetraose	+	+		++		
<i>Transxylosylation products</i>							
Original	P-1		+	+		X1 $\xrightarrow{\beta}$ 3X	
	P-2		+		++	X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X	
	P-3		+	+	+	X1 $\xrightarrow{\beta}$ 3X1 $\xrightarrow{\beta}$ 4X	
	P-4		++		+	+	X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X
After reduction	P-3	+	+	+			$\xrightarrow{\beta}$ $\begin{matrix} 1 \\ \uparrow \\ X \end{matrix}$ (OR) X1 $\xrightarrow{\beta}$ 4X1 $\xrightarrow{\beta}$ 4X $\begin{matrix} 3 \\ \uparrow \\ X \end{matrix}$

^a 2,3,4-Tri-O-Me-D-Xylp = 2,3,4-tri-O-methyl-D-xylopyranoside.

^b X denotes D-xylopyranose and the numerals the linkage positions.

^c After reduction with NaBH₄.

+, ++, +++ = approximate molar ratio, average value from three determinations.

product peaks was collected and concentrated under reduced pressure and then purified by preparative PPC. The chromatographically purified transfer reaction products were checked by TLC as shown in Fig. 2. In the characterization of each transfer product, the results from the estimation of DP and $[\alpha]_D^{20}$ are tabulated in Table II. The structural analysis of transfer products was first done by methylation analysis and the results were summarized in Table III.

Hydrolysis of methylated P-1 gave 2,3,4-tri-*O*-methyl-D-xylopyranose and 2,4-di-*O*-methyl-D-xylopyranose in a 1:1 molar ratio indicating the presence of (1 $\rightarrow$ 3)-linkage between the two xylosyl residues of P-1. So, P-1 was supposed to be *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-D-xylopyranose (rhodymenabiose) according to the results of D.P. 2 and $[\alpha]_D^{20}$ -18.2 (Table II). The presence of a 1:2 molar ratio of 2,3,4-tri-*O*-methyl- and 2,3-di-*O*-methyl-D-xylopyranose in the methylation analysis of P-2 was exactly the same as for authentic xylotriose. P-2 was, therefore, identified as *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose which had a DP 3 and $[\alpha]_D^{20}$ -48.3.

Although the same DP of 3 was found with P-3, a different $[\alpha]_D^{20}$ -44.6 was recorded, as shown in Table II. This fact was also supported by the result of methylation analysis of P-3 in which differently methylated sugars were observed than those of P-2 or authentic xylotriose. Hydrolysis of methylated P-3 gave an equal molar ratio of 2,3,4-tri-*O*-methyl-, 2,4-di-*O*-methyl-, and 2,3-di-*O*-methyl-D-xylopyranose. So, it was first supposed to be an isomeric xylotrisaccharide in which (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)-linkages were contained. When P-3 was reduced with sodium borohydride and analyzed in the same way, a methylated sugar alcohol and methylated sugars were found to be 1,2,3,5-tetra-*O*-methyl-xylitol, 2,3,4-tri-*O*-methyl-, and 2,4-di-*O*-methyl-D-xylopyranose in equal molar ratio. These data firmly indicated that the reducing end xylose residue was attached in (1 $\rightarrow$ 4)-linkage. Consequently, P-3 was supposed to be *O*- β -D-xylopyranosyl-(1 $\rightarrow$

3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose.

In the case of structural analysis of P-4, 2,3,4-tri-*O*-methyl-, 2,3-di-*O*-methyl-, and 2-mono-*O*-methyl-D-xylopyranose were observed with a 2:1:1 molar ratio. As the DP of P-4 was 4, it was considered to be a tetraose consisting of a main chain of xylotriose and side chain of a terminal non-reducing D-xylopyranosyl residue which was attached through the C-3 position, either to the central or reducing end D-xylose unit of the main chain. On the basis of these data, P-4 was thought to be *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)]-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose or *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)]-D-xylopyranose.

Enzymatic analysis of transxylosylation products

The enzymatic structural analysis of transxylosylation products was done with purified *Malbranchea* β -xylosidase to confirm the data obtained from methylation analysis. It had already been stated that the purified *Malbranchea* β -xylosidase showed no transfer reaction activity and very high substrate speci-

FIG. 3. TLC of the Products of Hydrolysis of Standard Xylobiose (a) and Transxylosylation Product P-2 (b) by *Malbranchea* β -Xylosidase.

The symbols are the same as Fig. 2.

FIG. 4. HPLC Analysis of the Hydrolyzates of Transxylosylation Products by *Malbranchea* β -Xylosidase. Both of the samples before and after 5 hr hydrolysis were chromatographed by HPLC as mentioned in MATERIALS AND METHODS.

ficity on β -(1 $\rightarrow$ 4)-linkages. Although it hydrolyzed standard β -(1 $\rightarrow$ 4)-xylobiose (Fig. 3a), *Malbranchea* β -xylosidase could not hydrolyze P-1 and P-3 as shown in Fig. 4. It was considered that the existence of a β -(1 $\rightarrow$ 3)-linkage at the non-reducing end rendered P-1 and P-3 stable. On the other hand, the results of enzymatic analysis confirmed that the structures of P-1 and P-3 were *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-D-xylopyranose and *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose.

Malbranchea β -xylosidase easily hydrolyzed P-2 in the normal pattern in which P-2 was degraded into xylobiose and xylose residues within 15 min and completely into xylose at 1 hr (Fig. 3b). So, P-2 was confirmed to be β -(1 $\rightarrow$ 4)-xylotriose. A somewhat different pattern of hydrolysis was observed on P-4 by *Malbranchea* β -xylosidase. As soon as the hydrolysis started, P-4 was broken down into P-3 and xylose residues. No other hydrolysis product was found in 18 hr of reaction in TLC (data not shown), except P-3 and xylose. As the β -xylosidase hydrolyzed the P-4 molecule starting from the non-reducing end xylosyl

residue, it could not hydrolyze P-4 further because of the xylosyl residue with a β -(1 $\rightarrow$ 3)-linkage to the central residue of the main chain. The degradation of P-4 into P-3 and xylose residues in the sample, withdrawn at 5 hr of reaction and analyzed with HPLC, was observed in Fig. 4.

As mentioned in the results of methylation analysis, it was somehow possible that the β -(1 $\rightarrow$ 3)-xylosyl branch might be attached at the C-3 position of the reducing end xylose unit of the main chain in the structure of P-4. If it was so, different hydrolysis products from the enzymatic analysis, for instance xylose and β -(1 $\rightarrow$ 3)-xylobiose (P-1) will be expected in the TLC and HPLC analysis because of the high β -(1 $\rightarrow$ 4)-linkage specificity of *Malbranchea* β -xylosidase. Nevertheless, only xylose and the product with the same *R_f* value as well as retention time to those of P-3 was observed in TLC as well as HPLC. Therefore, we assumed that the structure of the minor transfer product, P-4, was highly probably *O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-[*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 3)]-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose. We also do not exclude the neces-

FIG. 5. The Relationship between Chromatographic Mobility t_R , $\log [(1/R_f) - 1]$ and DP Values of Standard Xylooligosaccharides and Transxylosylation Products.

●, β -(1 $\rightarrow$ 4) series of standard xylooligosaccharides; Δ , transxylosylation products.

sity of further examination of this assumption.

The t_R values, $\log [(1/R_f) - 1]$, of authentic xylooligosaccharides and transxylosylation products obtained from the reaction of xylobiose and *P. wortmanni* β -xylosidase-1, calculated from R_f values of TLC, were plotted against the corresponding DP values. The points fall on two nearly parallel straight lines as shown in Fig. 5. The upper line indicated the authentic xylooligosaccharides with β -(1 $\rightarrow$ 4)-linkages and the lower revealed the transxylosylation products with mixed β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-linkages. The results were in accord with those found in the literature concerning the occurrence of β -(1 $\rightarrow$ 3)-xylobiose and isomeric xylotriose in the hydrolyzates of marine algal xylan (Rhodymenan) by the action of β -(1 $\rightarrow$ 3)-xylanase purified from *Aspergillus terreus*¹³⁾ as well as by a crude extract from sheep rumen bacteria.²⁰⁾ But there had been no report except this one that described the enzymatic synthesis of isomeric xylotriose (P-3) and isomeric xylotetraose (P-4) with a branched structure differing from that of known tetrasaccharides.

Therefore, we concluded that out of four types of β -xylosidase from *P. wortmanni* IFO 7237, β -xylosidase-1 could use the β -(1 $\rightarrow$ 4)-xylobiose as a xylosyl donor as well as a xylosyl acceptor and formed β -(1 $\rightarrow$ 3)-xylo-

biose (P-1), β -(1 $\rightarrow$ 4)-xylotriose (P-2), isomeric xylotriose (P-3), and isomeric xylotetraose (P-4). We supposed that the transfer reaction of *P. wortmanni* β -xylosidase-1 mainly occurred at the C-4 position but also considerably at the C-3 position. The possibility of the formation of β -(1 $\rightarrow$ 3)-xylotriose was not excluded in this study, but it must be in a very minute amount. Also, it was possible that higher oligo-transfer products were synthesized and were discarded in the purification steps by PPC because these were insufficient amounts for further analysis. The comparative elucidation of transxylosylation products by the remaining xylosidase-2, -3, and -4 are also in progress.

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