The Modes of Action of Three Xylanases from Mesophilic Fungus Strain Y-94 on Xylooligosaccharides

Yasushi MITSUISHI, Takashi YAMANOBE and Mitsuo YAGISAWA

*Fermentation Research Institute, Agency of Industrial Science and Technology,
Ministry of International Trade and Industry, 1-1-3 Higashi,
Tsukuba-shi, Ibaraki 305, Japan*

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The modes of action of three thermostable endo-xylanases (Xn-A, Xn-B, and Xn-C) from the mesophilic fungus strain Y-94 on xylooligosaccharides and their alditols (DP 2~8) were studied. None of the enzymes could hydrolyse xylobiose. Oligosaccharides upwards from xylotetraose were immediately hydrolysed by the endo-xylanases, but xylotriose was slowly hydrolysed. Xn-A hydrolysed xylotriose more slowly than the other two. From the dependency of the molecular activity (*ko*) on the chain length of the substrates, it was suggested that the three xylanases had the same subsite size (5 xylose units). Analysis of the frequency distribution of bond cleavage of oligosaccharide-alditols showed that no enzymes could attack the first bond from the non-reducing end of oligosaccharides. The other bonds were hydrolysed by these enzymes by endo-type action. The action pattern for xylopentaitol suggested that the catalytic site was located between the second and third subsite from the non-reducing end, since the substrate was mainly hydrolysed to xylobiose and xylotriitol.

In our previous paper, we described the purification of three thermostable endo-xylanases (Xn-A, Xn-B, and Xn-C) from the culture supernatant of the mesophilic fungus strain Y-94.¹⁾ There were some differences in physicochemical properties among the three xylanases but no significant differences in the hydrolytic activities to the insoluble xylans. Thus, we attempted to elucidate the mode of action of endo-xylanase on xylooligosaccharides, and to clarify differences in these three xylanases.

The mode of action of α -amylase on maltooligosaccharides was investigated in detail.^{2~4)} Some investigators have also reported on the mode of action of endo-xylanases on xylooligosaccharides,^{5~11)} but no quantitative investigation like those on α -amylase has so far been made.

This paper deals with a quantitative study of the modes of action of three xylanases on xylooligosaccharides.

MATERIALS AND METHODS

Enzyme preparations. Three purified endo-xylanases from the mesophilic fungus strain Y-94 were prepared as previously reported.¹⁾

Preparation of xylooligosaccharides and corresponding alditols. Xylooligosaccharides (xylobiose through xylooctaose) were prepared as follows. Eight grams of hardwood xylan (kindly supplied from Sanyo Kokusaku Pulp Co., Ltd.) were suspended in 800 ml of 0.1 N HCl and incubated for 20 min in a boiling water bath. The partially hydrolysed mixture was then poured into 200 g of ice and neutralized by the addition of 5.4 g of Na₂CO₃. Insoluble materials were removed by centrifugation (3,000 $\times$ g, 10 min). Ten grams of activated carbon powder were added to the clarified supernatant. The carbon powder was filtered and washed thoroughly with water. The absorbed xylooligomers were then eluted three times by 300 ml of 60% ethanol from the carbon powder. The filtrates were mixed and concentrated under reduced pressure at 45°C. To remove acid oligosaccharides, the water solution of the hydrolysate was passed through a column (4 $\times$ 11 cm) of Dowex 1 $\times$ 8 (acetate form). The non-absorbed fraction was concentrated and put onto a column (4 $\times$ 150 cm) of Bio-Gel P-2 (Bio-Rad Laboratories) and eluted by water at 50°C. Carbohydrates were detected by a differential

FIG. 1. Chromatogram of Xylooligosaccharides Obtained from Controlled Hydrolysis of Hardwood Xylan on Bio-Gel P-2.

X2 through X8 show the elution position of xylobiose through xylooctaose. The properties of the fractions obtained were listed in Table I.

TABLE I. SOME PROPERTIES OF XYLOOLIGOSACCHARIDES

Fraction ^a	Yield (%)	DP ^b	[α] _D ^c	
			Authors	Kusakabe <i>et al.</i> ^{12,13)}
X2	0.3	1.80	-24.3	-25
X3	1.2	2.66	-45.5	-51
X4	2.0	4.19	-60.7	-63
X5	1.9	5.17	-73.7	-73
X6	1.8	6.23	-77.2	-78
X7	1.2	7.50	-81.5	-84
X8	0.9	8.01	-87.3	—

^a Fractions (X2~X8) correspond to the peaks in Fig. 1.

^b Degree of polymerization (DP) was calculated as follows. Oligosaccharide-alcohol (see MATERIALS AND METHODS) was completely hydrolysed by 1N HCl (100°C, 4 hr). The resulting xylose and xylitol were isolated by HPLC as described in the text. DP = (xylose + xylitol)/xylitol.

^c Optical rotation power ([α]_D) was measured by a DIP-360 apparatus (Nihon Bunko).

refractometer (Shodex SE-11, Showa Denko). Figure 1 shows the separation of xylooligomers by the column of Bio-Gel P-2. Isolated xylooligomer fractions were collected and concentrated. Each fraction was rechromatographed twice on the same column, concentrated, and lyophilized. Table I shows the yield, degree of polymerization (DP), and optical rotation power ([α]_D) of each fraction.

The corresponding alditols of xylooligosaccharides were prepared as follows. Fifty mg of purified xylooligomers (xylobiose through xylooctaose) in 1.5 ml of water were

FIG. 2. Chromatogram of Xylooligosaccharides and Corresponding Alditols (Xylitol through Xylohexaose) on Shodex Ionpak KS-801.

Ten μ l of sample (containing 50 μ g of each oligosaccharide) was analyzed by HPLC. The peak of chromatogram are as follows. 1, xylitol; 2, xylose; 3, xylobiitol; 4, xylobiose; 5, xylotriitol; 6, xylotriose; 7, mixture of xylo-tetraitol and xylotetraose; 8, mixture of xylopentaitol and xylopentaose; 9, mixture of xylohexaitol and xylohexaose.

reduced by NaBH₄ for 3 h at room temperature. After reduction, NaBH₄ was decomposed by the addition of 3 ml of a cation exchange resin (Dowex 50 W $\times$ 2) and sodium ion was removed. The filtrate was then evaporated five times with methanol. These preparations had no reducing power toward the Somogyi reagent.¹⁴⁾

High-performance liquid chromatography (HPLC) of xylooligomers and alditols. Carbohydrate oligomers were analyzed with a Nihon Bunko high-performance liquid chromatograph (880 PU) with an automatic sampler (KSST 601, Kyowa Seimitsu), a differential refractometer (Shodex SE-11), and a System Instruments 7000A integrator. A Shodex Ionpak KS-801 column (300 $\times$ 8 mm, 2 column, Showa Denko) was used, and the column was eluted with degassed water at 80°C. Samples of 10 μ l were injected into the HPLC and eluted at a velocity of 0.6 ml/min. Figure 2 shows the elution profile of the mixture of xylooligosaccharides and corresponding alditols.

Enzyme reaction. All the reactions were done at 60°C and pH 4.9. The enzyme preparations were buffered at this pH with 10 mM acetate buffer. Enzyme activity was measured as described previously.¹⁾

The action of endo-xylanases on xylooligosaccharides and alditols was studied using a reaction mixture of 0.4 ml consisting of 1 mg of substrate and 0.4 unit of enzyme. The mixture was incubated for 5 h and the hydrolysates were

analyzed by HPLC.

To measure the kinetic parameters of each xylanase preparation, 1 ml of reaction mixture (containing 0.05 unit of enzyme and various concentrations of xylooligosaccharide) was incubated for 5 min and the net reducing power resulting from enzyme reaction was measured by the Nelson-Somogyi method.^{14,15} The amount of the three xylanases (e_0) used was 4.7×10^{-3} (Xn-A), 3.1×10^{-3} (Xn-B), and 4.6×10^{-3} μ M (Xn-C).

To calculate the frequency distribution of bond cleavage for individual alditols of xylooligosaccharides of DP 4~8, HPLC was used. The reaction system was the same as for the hydrolysis of xylooligosaccharides except that the incubation period was 2 min so as to examine the initial reaction. The products were separated by HPLC and the quantity of each isolated product was calculated from its peak area on the HPLC chromatograms using an integrator. To obtain the quantitative distribution of products, the molar ratio of a given product to all the products was calculated.

RESULTS

Actions of the three xylanases on xylooligosaccharides

Table II shows the degree of hydrolysis and product distribution (weight base) of xylooligosaccharides (DP 2~8). All three xylanases completely hydrolysed the xylooligosaccharides upwards from xylopentaose and produced mainly xylobiose and xylotriose. On the other hand, the oligosaccharides of DP 3~4 were hydrolysed to various degrees by the three xylanases but xylobiose was not hydrolysed at all. The degree of hydrolysis of xylotriose by Xn-A, Xn-B, and Xn-C was 6.8, 26.8, and 29.2%, respectively. Xylotetraose was also hydrolysed by Xn-A, Xn-B, and Xn-C to the degree of 92.8, 98.5, and 98.5%, respectively. Xn-B and C produced xylose

TABLE II. DEGREE OF HYDROLYSIS AND PRODUCT DISTRIBUTION

Enz	Sub	DH ^a (%)	Products distribution (% w/w) ^b							
			X	X2	X3	X4	X5	X6	X7	X8
A	X2	0.0	0.0	100.0	—	—	—	—	—	—
	X3	6.8	2.5	4.3	93.2	—	—	—	—	—
	X4	92.8	0.0	83.1	9.3	7.6	—	—	—	—
	X5	98.8	0.0	53.5	44.2	1.2	1.2	—	—	—
	X6	99.0	0.0	57.2	37.8	2.9	1.1	1.0	—	—
	X7	100.0	0.0	73.5	25.8	0.7	0.0	0.0	0.0	—
	X8	100.0	0.0	60.9	36.8	2.3	0.0	0.0	0.0	0.0
B	X2	0.0	0.0	100.0	—	—	—	—	—	—
	X3	26.8	12.7	24.1	63.2	—	—	—	—	—
	X4	98.5	0.0	92.9	5.6	1.5	—	—	—	—
	X5	100.0	6.8	65.4	27.8	0.0	0.0	—	—	—
	X6	100.0	6.6	69.4	24.1	0.0	0.0	0.0	—	—
	X7	100.0	9.7	67.9	22.3	0.0	0.0	0.0	0.0	—
	X8	100.0	9.2	67.5	23.3	0.0	0.0	0.0	0.0	0.0
C	X2	0.0	0.0	100.0	—	—	—	—	—	—
	X3	29.2	11.6	17.5	70.8	—	—	—	—	—
	X4	98.5	0.0	86.8	11.7	1.5	—	—	—	—
	X5	100.0	6.9	61.0	32.0	0.0	0.0	—	—	—
	X6	100.0	5.0	69.7	25.3	0.0	0.0	0.0	—	—
	X7	100.0	9.3	68.0	22.7	0.0	0.0	0.0	0.0	—
	X8	100.0	8.5	66.0	25.5	0.0	0.0	0.0	0.0	0.0

The reaction was done at 60°C for 5 hr using 1 mg substrate and 0.4 unit enzyme in 0.4 ml of the reaction mixture.

^a Degree of hydrolysis (DH) was calculated from the amount of residual substrate.

^b Product distribution was calculated from the HPLC chromatogram and expressed as wt% of isolated products in total hydrolysates.

Abbreviations: Enz, enzyme; Sub, substrate; X-X8, xylose-xylooctaose; A, Xn-A; B, Xn-B; C, Xn-C.

FIG. 3. s/v versus s Plots of Xn-A Catalyzed Hydrolyses of Xylooligosaccharides.

X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; X7, xyloheptaose; X8, xylooctaose.

TABLE III. KINETIC PARAMETERS OF THE THREE ENDO-XYLANASES

Substrate	Xn-A		Xn-B		Xn-C	
	K_m (mM)	ko^a (s^{-1})	K_m (mM)	ko (s^{-1})	K_m (mM)	ko (s^{-1})
X4	1.24	350	0.63	330	0.62	170
X5	0.90	610	0.39	450	0.48	320
X6	0.54	510	0.27	410	0.40	320
X7	0.47	520	0.25	440	0.18	290
X8	0.29	480	0.24	470	0.17	290

K_m and V_{max} were obtained from Fig. 3.

^a Molecular activity, ko , is expressed as V_{max}/e_o where e_o is the molar concentration of the enzyme. For abbreviations, see Table II.

from higher oligosaccharides upwards from xylopentaose but Xn-A produced no xylose from them.

Kinetic parameters of the three endo-xylanases

Differences in the dependencies of enzyme action on the chain length of xylooligosaccharides were studied for the kinetic parameters, Michaelis constant (K_m), and molecular activity (ko). The K_m value was calculated from s/v versus s plots. Figure 3 shows the case of Xn-A. The s/v versus s plots were linear for all the substrates. The values of K_m and ko of three xylanases are listed in Table III. The K_m values of all enzymes decreased

TABLE IV. DEGREE OF HYDROLYSIS OF OLIGOSACCHARIDE-ALDITOLS

Substrate	Degree of hydrolysis (% w/w)		
	Xn-A	Xn-B	Xn-C
X2OH	0.0	0.0	0.0
X3OH	0.0	19.4	14.7
X4OH	19.7	98.2	98.1
X5OH	96.8	100.0	100.0
X6OH	100.0	100.0	100.0
X7OH	100.0	100.0	100.0
X8OH	100.0	100.0	100.0

Calculation of the degree of hydrolysis was done as described in the footnote of Table II.

Abbreviations: X2OH ~ X8OH, xylobiitol ~ xylooctaitol.

FIG. 4. HPLC Chromatograms of Initial Products from Xylooligosaccharide-alditols by Xn-A.

The peaks of the chromatograms are as follows. 1, xylobiitol; 2, xylobiose; 3, xylotriitol; 4, xylotriose; 5, xylotetraitol; 6, xylopentaitol; 7, xylohexaitol; 8, xyloheptaitol; 9, xylooctaitol. Substrates are follows: X4OH, xylotetraitol; X5OH, xylopentaitol; X6OH, xylohexaitol; X7OH, xyloheptaitol; X8OH, xylooctaitol.

with increasing chain length but ko became almost constant for DP more than 5. Among the three enzymes, Xn-A had higher molecular

Xn-A	Xn-B	Xn-C
(0.0) (21.1) (49.9) (54.4) (60.8)	(2.4) (23.9) (46.4) (58.3) (61.7)	(3.9) (29.5) (43.6) (54.4) (61.3)

FIG. 5. The Frequency Distribution of Bond Cleavage during the Initial Reaction of Three Endo-xylanases on Xylooligosaccharide-alditols.

(): The degree of hydrolysis of substrate. ○, xylose unit; ●, xylitol.

FIG. 6. HPLC Chromatograms of Hydrolysates Obtained from Prolonged Incubation of Xylotriitol with Three Endo-xylanases.

The peaks of the chromatograms are as follows. 1, xylitol; 2, xylobiose; 3, xylotriitol.

activity but had lower affinity toward the oligosaccharides.

Actions of three xylanases on xylooligosaccharide-alditols

Table IV shows the degrees of hydrolysis of xylooligosaccharide-alditols. Compared to the hydrolysis of the corresponding xylooligosaccharides (see Table II), xylobiitol was hardly hydrolysed by any enzymes. Xylotriitol was not hydrolysed by Xn-A.

FIG. 7. HPLC Chromatograms of Hydrolysates Obtained from Prolonged Incubation of Xylotriitol with Three Endo-xylanases.

The peaks of the chromatograms are as follows. 1, xylitol; 2, xylobiitol; 3, xylobiose; 4, xylotriitol; 5, xylotetraitol.

Frequency distribution of bond cleavage of xylooligosaccharide-alditols

Figure 4 shows the HPLC chromatograms of the products from the hydrolysis of oligosaccharide-alditols (DP 4~8) by Xn-A in the 2 min reaction. The cleavage patterns were obtained from chromatogram peak data as described in MATERIALS AND METHODS. The values are indicated in Fig. 5 along with the results for the other two enzymes. The degree

of hydrolysis indicated in parentheses increased with the DP of the substrates. The hydrolysis rate of xylo-tetraitol was very low in short reactions (2 min).

The data showed all three enzymes cleaved the inside bond of the substrates by endo-type action, but no enzyme could attack the first bond from the non-reducing end of the substrates.

Product distribution of smaller oligosaccharide-alditols

As shown in Table IV and Fig. 5, xylo-triitol and xylo-tetraitol were slowly hydrolysed by xylanases. Thus, the product distribution of a prolonged reaction (5 hr) of given substrates was analyzed. Figures 6 and 7 show the HPLC chromatograms of the hydrolysates of xylo-triitol and xylo-tetraitol by the three enzymes.

As shown in Fig. 6, Xn-A did not hydrolyse xylo-triitol at all and the other two partially hydrolysed the substrate and produced xylobiose and xylitol. In the case of xylo-tetraitol (Fig. 7), Xn-A could partially hydrolyse the substrate but the other two could almost completely hydrolyse it. The hydrolysates were xylobiose, xylobiitol, xylo-triose, and xylitol. These data indicated that the three enzymes could cleave the first bond from the alditol side of xylo-tetraitol.

DISCUSSION

We studied the action of three thermostable endo-xylanases from the mesophilic fungus strain Y-94 on xylooligosaccharides and the corresponding alditols. HPLC analysis using Shodex Ionpak KS-801 column was useful for isolating the enzyme products, because this column clearly separated xylooligosaccharides and the corresponding alditols in the range of DP 1~3 (Fig. 2).

The product distribution analysis provided detailed information of the enzyme reactions with xylooligomers. Under the tested conditions, none of the xylanases could hydrolyse xylobiose (Table III) or hydrolyse the first bond from the non-reducing end of the oligo-

saccharides upwards from xylo-triose (Fig. 5). Therefore, xylobiose might be one of the final products. From the data of the frequency distribution of bond cleavage, it was clearly shown that the three enzymes acted as typical endo-type enzymes toward oligosaccharides (Fig. 5), and higher xylooligomers were hydrolysed to xylobiose and xylo-triose by subsequent hydrolyses. In higher oligosaccharides upwards from xylopentaose, it could not be seen whether the enzymes hydrolysed the first or second bond from the reducing end of the substrates (Fig. 5). Enzymes may possibly hydrolyse these bonds to a small extent, since part of the xylo-tetraitol was hydrolysed to xylo-triose and xylitol by the three enzymes (Fig. 7).

According to Nitta *et al.*,³⁾ the subsite size of endoglycanase can be directly estimated from the dependency of *ko* on the chain length of the substrate. In these three endo-xylanases, the values of *ko* became constant at DP 5 (Table III). The fact suggested the size of the subsite was 5 xylose units for all three xylanases. Moreover, frequency distribution analysis of bond cleavage showed the xylopentaitol was mainly hydrolysed to xylobiose and xylo-triitol by the three enzymes (Fig. 5). The frequency of bond cleavage of intact xylopentaose was not directly estimated by the analysis of xylopentaitol. However, xylopentaose was mainly cleaved to xylobiose and xylo-triose in the first stage of hydrolysis. Thus these data suggested that the catalytic site was located between the second and third subsites from the non-reducing end.

Among three enzymes, there were no significant differences in the mode of hydrolysis of higher oligosaccharides upwards from xylopentaose. Differences were noted only when the enzymes reacted with xylo-triose and xylo-tetraitol (Tables II and IV). In the hydrolysis of xylo-triose, Xn-A hydrolysed this oligosaccharide more slowly than the other two. Therefore, the subsite affinity of Xn-A should be significantly different from the other two.

The role of each enzyme in the hydrolysis of xylan has yet to be found, but the study of the

action of the three xylanases on xylooligosaccharides explains why the final products of xylan are mainly xylobiose and xylose with a small amount of xylotriose.¹⁾ The mechanism is as follows. At first, high molecular weight xylan is hydrolysed rapidly by endo-type reactions of the enzymes and the resulting higher xylooligomers are also hydrolysed to xylotriose and xylobiose. Xylotriose is then slowly hydrolysed and finally xylobiose and xylose accumulate in the solution.

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