Note

Chemical Modification of Xylanases from *Streptomyces* sp.

Masaki MARUI, Kotoyoshi NAKANISHI,* and Tsuneo YASUI

Seitoku Junior College of Nutrition, 1–4–6 Nishi-shinkoiwa, Katsushika-ku, Tokyo 124, Japan
* *Department of Brewing and Fermentation, Tokyo University of Agriculture, 1–1–1 Sakuragaoka, Setagaya-ku, Tokyo 156, Japan*
Received July 10, 1992

The amino acid sequence and the primary structure of xylanase have been reported for numerous microorganisms,[1] and for some bacteria, the nucleotide sequence of the gene for the active site of the enzyme has been examined.[2,3] Although the properties of the xylanases from *Streptomyces* have been well investigated,[4] the active site was not described. *Streptomyces* sp. No. 3137 produces three xylanases designated as X-I, X-II-A, and X-II-B, induced by xylan or methyl β-xyloside. X-I is different from X-II-A and X-II-B in physico-chemical and immunological properties as described previously,[4] and the two X-II enzymes are similar except for the isoelectric point (10.1 and 10.3). This paper deals with the chemical modification of the three xylanases from *Streptomyces* sp. induced by methyl β-xyloside, and concludes that xylanase activity is related to some carboxyl groups and not to tryptophan residues.

The culture conditions for *Streptomyces* sp. No. 3137, the induction of xylanases, and the purification procedures of the xylanases were described previously.[4] The purified xylanase samples used throughout this experiment had specific activities of 160, 100, and 160 units/mg of protein for X-I, X-II-A, and X-II-B, respectively. Xylanase activity is measured by the method described previously,[4] that is, incubating 0.2 ml of diluted enzyme solution with 1.0 ml of 2% xylan suspension and 0.8 ml of McIlvaine's buffer (pH 5.5) for 10 min at 55°C, and measuring the released reducing sugar by a modification of Somogyi's method.[5] One unit of enzyme activity is defined as the amount causing production of 1 μmol of reducing sugar as a xylose equivalent per minute, under optimal conditions. Chemical modifications were done using several reagents under reaction conditions which were generally similar to those used for cellulase[6] and lysozyme.[7-9] The different functional groups of the enzymes were modified with the following reagents: carboxyl groups, with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or

triethyloxonium fluoroborate (TEOFB); indole groups, with *N*-bromosuccinimide (NBS) or 2-hydroxy-5-nitrobenzylbromide (HNBB); imidazole groups, with diethylpyrocarbonate (DEP), iodoacetate or methylene blue; guanidino groups, with 2,3-butanedione; phenol groups, with iodine ion or *N*-acetylimidazole; and thioether groups, with chloramine T. Phenylalanine methylester, aminomethane sulfonic acid, and TEOFB were purchased from the Aldrich Chemical Company (U.S.A.). The other chemicals used were of reagent grade and commercially available. The reaction conditions followed during these modifications are summarized in the Table, which also shows the effects of chemical reagents on xylanase activity.

The chemicals that were found to affect xylanase activity were EDC with 250 mM aminomethane sulfonic acid for amidation of carboxyl groups, TEOFB for esterification of carboxyl groups, and NBS at high concentration (0.5 mM) for oxidation of indoles. The others had no effect on the enzyme activity, leaving more than 90% of the original activity.

The degree of xylanase inactivation was investigated with EDC at various concentrations (10–100 mM). The semi-logarithmic plots of X-I or X-II-B activity in various EDC concentration as a function of inactivation time showed a linear relationship (Fig. 1). These data indicate that the inactivation reaction proceeded in the form of apparent-first-order kinetics with respect to active enzyme concentration at excess amount of EDC. The apparent rate constant, k_{app} (min^{-1}), was calculated from Fig. 1. The formula of this reaction was assumed to be as below.

$$\text{active enzyme} + n\text{EDC} \rightarrow \text{inactivated enzyme}$$

So, k_{app} equals $k[\text{EDC}]^n$, where k (liter/mol min) is the rate constant of above reaction. Figure 2 shows the linear relationship of $\log(k_{app})$ and $\log(\text{EDC concentration})$ with a slope (n) equal to 1. This indicates that one molecule of EDC binds to one molecule of the xylanases when the

Table Effects of Chemical Reagents on Xylanase Activities

Reagents	Residual activity (%)			Reagent concn. (mM)	Protein concn. (μM)	Reaction conditions
	X-I	X-II-A	X-II-B			
EDC[a]	5	ND[b]	5	100	200	pH 4.75, 25°C, 60 min.
TEOFB	10	ND	10	200	900	pH 4.5, room temp., 20 min.
NBS	95	100	100	0.1	20	0.1 M Acetate b.,[c] pH 4.0, 25°C, 30 min.
	<5	<5	<5	0.5	20	
HNBB	100	100	100	40	250	0.2 M CaCl₂, pH 4.9, 25°C, 30 min.
DEP	100	100	100	1	40	50 mM Phosphate b., pH 6.0, 25°C, 75 min.
Iodoacetate	100	100	100	10	200	0.1 M Citrate b., pH 6.0, 25°C, 30 min.
Methylene blue	91	ND	95	25 μM	100	25 mM Phosphate b., pH 7.0, 10°C, 60 min.
2,3-Butane dione	100	ND	100	10	100	50 mM Borate b., pH 8.5, 20°C, 60 min.
Iodine	100	ND	100	2.2	80	0.2 M Tris b., pH 8.5, 4°C, 60 min.
N-Acetylimidazole	100	ND	100	5	200	50 mM Barbital b., pH 7.5, 25°C, 60 min.
Chloramin T	90	ND	100	1.7	100	0.1 M Tris b., pH 8.5, 25°C, 20 min.

[a]　EDC, containing aminomethane sulfonic acid (0.25 M) and NaCl (0.7 M).
[b]　ND, not determined.
[c]　b., buffer.

Abbreviations: DEP, diethylpyrocarbonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HNBB, 2-hydroxy-5-nitrobenzylbromide; NBS, *N*-bromosuccinimide; TEOFB, triethyloxonium fluoroborate.
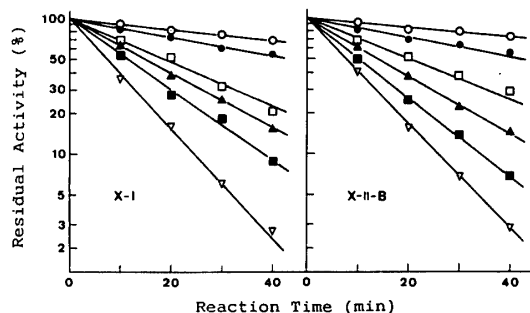
Fig. 1. Effects of EDC on Xylanase Activities.

The reaction mixtures contained 0.2 mM X-I or X-II-B, 0.25 M aminomethane sulfonic acid, 0.7 M NaCl, and EDC, of which final concentrations were 10, 20, 40, 60, 80, and 100 mM, at pH 4.75. Samples of the reaction mixture incubated at 25°C for 10, 20, 30, and 40 min were diluted 30-fold with 0.1 M phosphate buffer (pH 4.75) to stop the reaction, and were used for assaying the enzyme activity. Symbols: ○, 10 mM; ●, 20 mM; □, 40 mM; ▲, 60 mM; ■, 80 mM; ▽, 100 mM.
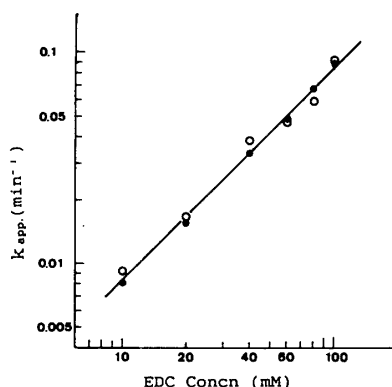

Fig. 2. Relationship between k_{app} and EDC Concentration.

The apparent-first-order rate constant of inactivation (k_{app}) was obtained from the data in Fig. 1. Symbols: ○, X-I; ●, X-II-B.

inactivation occurs.

The carboxyl group protection of the xylanase substrate from the EDC modification was examined. The presence of the substrate xylotriose protected the carboxyl groups from modification by EDC so that 40% activities of the two xylanases were retained after 40 min (Fig. 3). In the presence of the non-substrate xylobiose or no sugar, the remaining activities were found to be less than 5%, which indicates that xylobiose does not protect the carboxyl groups. These data show that the carboxyl groups participate in forming the active site of the xylanases.

X-I was modified with EDC and phenylalanine methylester, and the amino acid composition of native and modified X-I were compared to recognize that carboxyl groups were modified by EDC and to identify how many of the modified carboxyl groups there were. The mole percentage of phenylalanine residue increased from 2.8% to 4.8%, due to phenylalanine methylester modified carboxyl groups of X-I, of which the activity decreased by about 5% remaining. The number of modified carboxyl groups (P) was calculated by the following formula; $(11 + P)/(401 + P) = 4.8\%$. The number 11 is phenylalanine residues of the native X-I and the number of 401 is all the amino acid residues in it. P is approximately 9, so the modified carboxyl groups were 9 in a molecule of X-I.

Carbodiimide reacts with sulfhydryl groups of cysteines, aromatic hydroxyl groups of tyrosines, and carboxyl groups alike. In the case of EDC affecting an enzyme activity, it would be possible that cysteine and tyrosine residues have a relationship to the enzyme activity like a carboxyl group does. Due to the absence of modification of cysteine and tyrosine residues with iodoacetate and N-acetylimidazole treatments, respectively, it is concluded that these two amino acids were not related to the enzyme activity. The results of modification with EDC and TEOFB, which reduced the enzyme activity to 10%, suggest that one or more carboxyl groups are in the active site of xylanases. X-I and X-II-B were similarly affected by chemical modification particularly by EDC
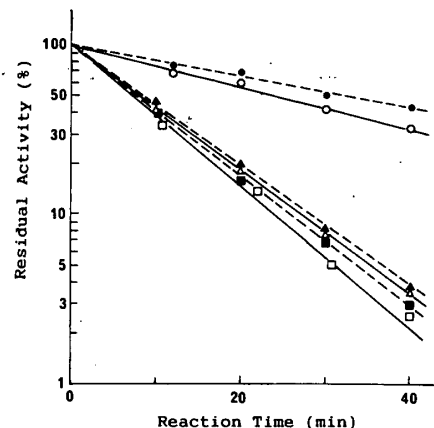

Fig. 3. Effects of Xylobiose or Xylotriose on the Reaction of Xylanases with EDC.

Xylanase (0.1 mM) was treated with 0.1 M EDC, 0.25 M aminomethane sulfonic acid, 0.7 M NaCl, pH 4.75, 25°C, with and without 0.1 M xylotriose or 0.1 M xylobiose. At intervals, samples of the reaction mixtures were diluted 15-fold with McIlvaine buffer (pH 5.5) and then were assayed for enzyme activity.

Symbols:	xylanase	presence of		absence of sugars
		xylotriose	xylobiose	
	X-I	○	△	□
	X-II-B	●	▲	■

and TEOFB.

Although NBS at 0.12 mM did not affect the enzyme activities of X-I and X-II-B, tryptophan residues were obviously modified under these conditions. The number of modified tryptophan residues was estimated to be two for X-I and one for X-II-B at 0.12 mM NBS by the method of Spande and Witkop.[10] At 0.5 mM of NBS, the activities of three xylanases were reduced to less than 5% on account of the destruction of protein which means the cleavage of peptide bonds by the high concentration of NBS, but not the modification of the active sites of the enzymes. HNBB, which is also known to modify tryptophan residues, did not affect the activity of the three enzymes.

It was reported that *Bacillus* xylanase had some tryptophan residues in the active site according to the results of crystallography and xylanase-specifying nucleotide sequences,[11] and to the study of tryptophan modification.[12] However, it is suggested here that tryptophan residues may have no relationship to the activity of *Streptomyces* xylanase and that carboxyl groups are related to the active site. Significant differences between *Streptomyces* and *Bacillus* were observed in the xylanases.

References

1) W. A. Wood and S. T. Kellogg, "Methods in Enzymology," Vol. 160, Academic Press, Inc., San Diego, California, 1988, pp. 632–684.
2) N. R. Gilkes, B. Henrissat, D. G. Kikburn, R. C. Miller, Jr., and R. A. J. Warren, *Microbiol. Rev.*, 55, 303–315 (1991).
3) H. Tsujibo, T. Sakamoto, K. Miyamoto, T. Hasegawa, M. Fujimoto, and Y. Inamori, *Agric. Biol. Chem.*, 55, 2173–2174 (1991).
4) M. Marui, K. Nakanishi, and T. Yasui, *Agric. Biol. Chem.*, 49, 3399–3407 (1985).
5) M. Somogyi, *J. Biol. Chem.*, 160, 61–68 (1945).
6) P. L. Hurst, P. A. Sullivan, and M. G. Shepherd, *Biochem. J.*, 167, 549–556 (1977).
7) T.-Y. Lin and D. E. Koshland, Jr., *J. Biol. Chem.*, 244, 505–508 (1969).
8) S. M. Parsons, L. Jao, F. W. Dahlquist, C. L. Borders, Jr., T. Groff, J. Racs, and M. A. Raftery, *Biochemistry*, 8, 700–712 (1969).
9) K. Hayashi, T. Imoto, G. Funatsu, and M. Funatsu, *J. Biochem.*, 58, 385–387 (1965).
10) T. F. Spande and B. Witkop, "Methods in Enzymology," Vol. XI, Academic Press Inc., New York, 1967, p. 498.
11) H. Okada, *GBF Monogr.*, 12, 81–86 (1989).
12) V. Deshpande, J. Hinge, and M. Rao, *Biochim. Biophys. Acta*, 1041, 172–177 (1990).