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Mode of Action and Active Site of Xylanase II from *Trichoderma koningii* ATCC 26113

Kim, Hyun-Ju, Sa-Ouk Kang*, and Yung Chil Hah

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

The action mode of xylanase II from *Trichoderma koningii* ATCC 26113 on xylan and related oligosaccharides (xylotriose, xylotetraose, and arabinoxylotriose) indicated that xylanase II is an endo-enzyme and also has trans-xylosidase activity. The ^{1}H-NMR studies of the reaction products formed by xylanase II revealed that all the hydrolysis products of xylooligosaccharides by the enzyme have only β-1,4-xylosidic linkage(s). Chemical modification of the enzyme with iodoacetamide showed that two cysteine residues per molecule of the enzyme was essential for the activity. Modification of the enzyme with *N*-bromosuccinimide demonstrated that four of the eight tryptophan residues were involved in its active site.

KEY WORDS □ **xylanase II, endo-enzyme, trans-xylosidation, cysteine, tryptophan, *Trichoderma koningii***

Xylan is a major component of hemicellulose, which is the most abundant naturally occurring polymers second only to cellulose. It is composed of β-1,4-linked D-xylose polymer having side chains of 1,3-linked L-arabinofuranose and 1,2-linked D-glucopyranose, or its 4-*O*-methyl ether. Since the structure of xylan is variable, several enzymes are involved in complete hydrolysis of xylan. Chief among the enzymes are endo-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37).

Many microbial xylanases have been reported from bacteria (2, 3, 22, 25, 38), yeast (4, 29), and fungi (14, 15, 26, 31, 36). Especially, endo-xylanases from *Trichoderma*, which is one of the best producer of the enzyme, have been widely investigated (1, 8, 9, 24, 33). However, in most of the studies on xylanases from *Trichoderma*, substrates for the enzymes have been limited to xylan. Studies of the action pattern of polysaccharide hydrolases using their natural polymeric substrates are complicated by the fact that the substrate molecule suffers repetitive attack by the enzyme (5). In this respect, the investigations using xylooligosaccharides as substrates would clarify the accurate action mechanism of xylanases. This work has been difficult to develop, due to the preparation of pure component oligosaccharides. In addition, the structures of the reaction products released by microbial xylanases have been demonstrated in only a few cases (12, 23).

Xylanases from *Trichoderma koningii* have been reported (41) but their action mode on oligosaccharides has not been described in detail. Therefore, the exact action mechanism of the enzymes is still obscure. Previously, xylanase I (19) and II (21) were purified from *T. koningii* and the action pattern and active site of xylanase I were investigated (20). We now report the action mode of a second enzyme, named as xylanase II, from *T. koningii*, on xylan and related oligosaccharides. This paper also describes chemical modification studies which reveal involvement of cysteine and tryptophan residues at its active site.

MATERIALS AND METHODS

Microorganism and growth conditions

Trichoderma koningii ATCC 26113 was cultivated for the enzyme production, as described previously (21).

Chemicals

D-Xylose, L-arabinose, oat-spelt xylan, and larchwood xylan were purchased from Sigma (U.S.A.). CMC (carboxymethyl cellulose) from Wako (Japan), Avicel from Fluka (Swiss). Xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), arabinoxylobiose (AX_2), and arabinoxylotriose (AX_3) were produced by the enzymatic hydrolysis of oat-spelt xylan. The separation of oligosaccharides was performed according to the method proposed by Miller *et al.* (28) with some modifications. All the other

chemicals used were of the highest quality generally available.

Enzyme assay

Xylanase activity was estimated by incubating a 0.5 ml reaction mixture containing 1% xylan (wt/vol), as described previously (21). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of reducing sugars per min.

Enzyme purification

Xylanase II was purified according to the method described previously (21).

Analysis of reaction products by HPLC

The reaction products of enzymatic hydrolysis of xylan and related oligosaccharides were analyzed by HPLC. Hydrolyzates were centrifuged in a microcentrifuge (Eppendorf, Germany) at 12, 000×rpm for 30 sec in order to remove any solids and the supernatants were filtered through 0.45 μm-filter (Millipore, U.S.A.). These filtrates were analyzed on the μ-Bondapak NH_2 column (Waters, U.S.A.) using 76% acetonitrile as eluant at a flow rate of 1.5 ml/min. The sugar products were detected with a Waters R 401 differential refractometer.

^{1}H-NMR spectroscopy

For ^{1}H-NMR studies, each of the reference sugars (xylose, xylobiose, xylotriose, xylotetraose, arabinose, arabinoxylobiose, and arabinoxylotriose) previously desiccated over P_2O_5 *in vacuo* for several days, was dissolved in 0.5 ml of D_2O (Sigma, U.S.A.). Then the solvent was removed *in vacuo*, and the residue was dissolved again in 0.5 ml of D_2O. This procedure was repeated once more. Finally, the sample solutions were prepared in 5-mm tube for ^{1}H-NMR spectroscopy.

In order to elucidate the structures of reaction products released from xylotriose by the enzyme, the reaction mixture which consisted of 500 μl of 50 mM xylotriose in 50 mM acetate buffer (pH 5.0) and 40 μl of 1 μM xylanase was incubated at 40°C for 1.3 h and then the reaction was stopped by heating for 10 min. The products were separated by a column of charcoal-celite as described by Miller *et al.* (28) with some modifications. The procedure of sample preparation for ^{1}H-NMR studies was just the same as in the case of the reference sugars. The ^{1}H-NMR spectra was taken with a Bruker FT-NMR spectrometer at 200.1 MHz with a 5-mm insert at a probe temperature of 20°C.

Chemical modification

For the investigation of the pH profile of xylanase II, the enzyme was incubated with various concentrations of arabinoxylotriose at a given pH. Buffers used were boric acid, citric acid, and trisodium orthophosphate buffer.

Modification experiments were performed according to the methods described previously (20).

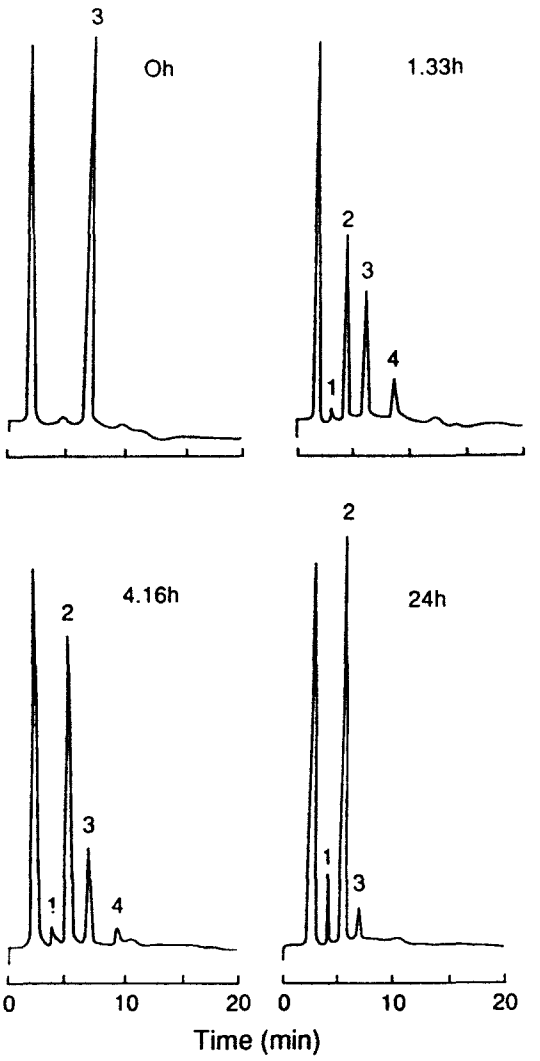

Fig. 1. *HPLC of reaction products of xylotriose hydrolyzed by the purified enzyme.*
Conditions: flow rate, 1.5 ml/min; eluant, 76% acetonitrile. 1, Xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose.

RESULTS

Action mode of xylanase II on xylan and related oligosaccharides

The hydrolysis products of oligosaccharides with the purified enzyme were identified by HPLC. Xylose and oligosaccharides were eluted in the order of $X_1>X_2>AX_1>X_3>AX_2>X_4>AX_3$ on HPLC with time course. Xylobiose and arabinoxylobiose were not atacked by the enzyme. The enzyme degraded X_3 to X_2 and a very small

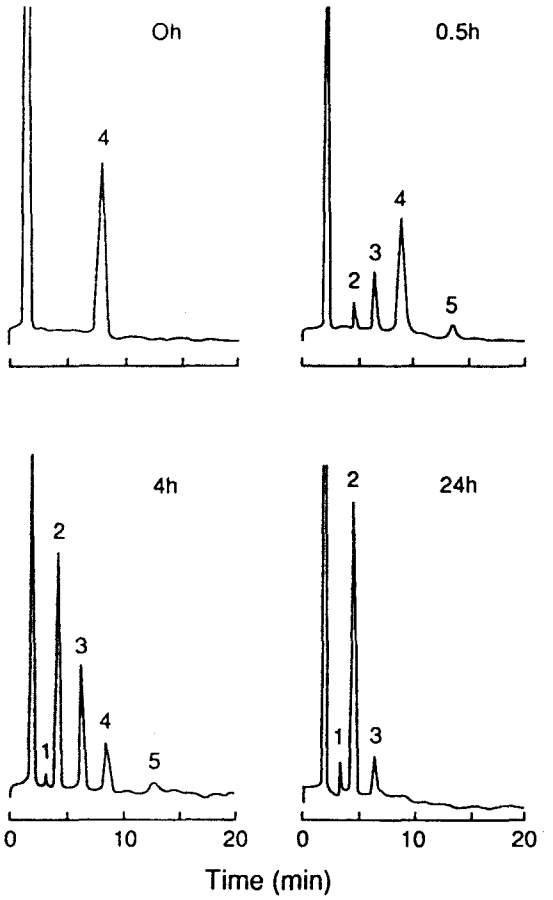

Fig. 2. *HPLC of reaction products of xylotetraose hydrolyzed by the purified enzyme.*
Analysis conditions were the same as in Fig. 4. 1, Xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose; 5, xylopentaose.

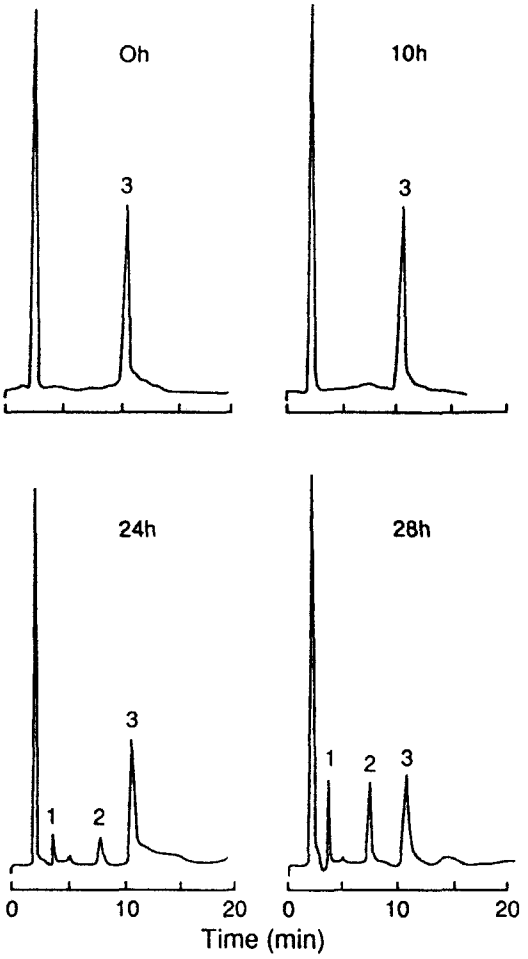

Fig. 3. *HPLC of reaction products of arabinoxylotriose hydrolyzed by the purified enzyme.*
Analysis conditions were the same as in Fig. 4. 1, Xylose; 2, arabinoxylobiose; 3, arbinoxylotriose

amount of X_1 after 24 h incubation (Fig. 1). At earlier stages of the hydrolysis, only a trace amount of X_1 was detected, while X_2 and X_4 increased continuously. With time course, X_4 increased and then decreased. Throughout the reaction, the molar concentration of X_2 was much higher than that of X_1. This result supports the view that the xylosidic bonds in X_3 are hardly attacked by the enzyme. The principle reaction is deduced that a donor X_3 is joined to an acceptor X_3 via a xylotriosyl-enzyme intermediate by trans-xylosidase activity of the enzyme, yielding X_6 which can be quickly hydrolyzed to X_4 and X_2. Fig. 2 shows HPLC chromatogram of the reaction products of X_4 by the enzyme. After 0.5 h incubation, X_2, X_3, and X_5 were accumulated and X_1 was not produced. A trace amount of X_1 was released after 4 h reaction. At early stage of the incubation, the molar concentration of X_3 was much higher than that of X_1. Therefore, it is probable that more X_4 are split into X_2 than into X_3 and X_1. Most of X_3 are probably released from X_6 which was formed from X_2 and X_4 via xylobiosyl- or xylotetraosyl-enzyme intermediate, *i.e.*, trans-xylosidation. In addition, it is deduced that X_5 was also produced from X_3 and X_2 by trans-xylosidation and then degraded to smaller oligosaccharides with time course. The enzyme yielded AX_2 and X_1 from AX_3 on prolonged incubation (Fig. 3). The results of hydrolysis on xylan and related oligosaccharides clearly indicate that xylanase II is an endo-enzyme and also has trans-xylosidase activity.

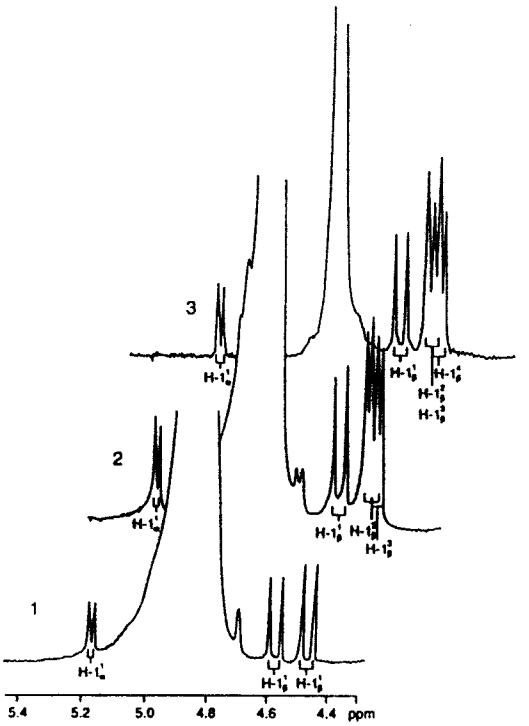

Fig. 4. *'H-NMR spectra of reaction products of xylotriose by the purified enzyme.*
The ^{1}H-NMR spectra was taken with a Bruker FT-NMR spectrometer at 200.1 MHz. 1, X_2^* (xylose dimer); 2, X_3^* (xylose trimer); 3, X_4^* (xylose tetramer).

Analysis of reaction products of xylanase II by ^{1}H-NMR spectroscopy

To elucidate the structures of reaction products of xylotriose by the enzyme, ^{1}H-NMR studies were carried out (Fig. 4). Previously, the hydrolysis products of xylotriose by the purified enzyme were identified by HPLC. As shown in Fig. 1 (1.33 h incubation), the chromatogram exhibited four separated peaks located at the positions of xylose, xylobiose, xylotriose, and xylotetraose, respectively. The first peak can be readily assigned to xylose. There are some possibility that the reaction products contain xylosidic linkages other than β-1,4-xylosidic linkage. Therefore, it is not clear that the second, third, and fourth peaks indicate xylobiose, xylotriose, and xylotetraose. The three peaks of reaction products are designated as X_2^*, X_3^* and X_4^*, respectively. The ^{1}H-NMR spectra of the three reaction products are shown in Fig. 7. The spectra of X_2^*, X_3^* and X_4^* are almost completely consistent with those of X_2, X_3, and X_4. The chemical shifts of H-1 protons and coupling constants, J(1, 2) of each

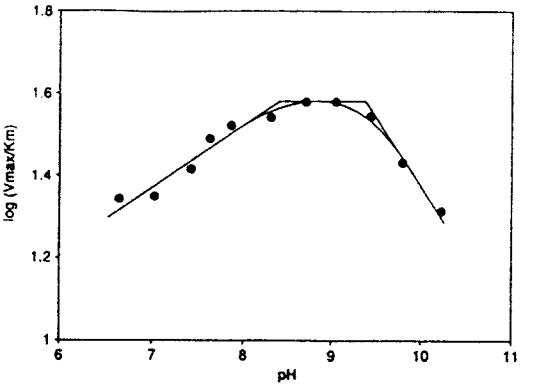

Fig. 5. *Dependence of the kinetic parameters on pH for the hydrolysis of arabinoxylotriose by xylanase II.*
The kinetic parameters were derived from Lineweaver-Burk plots of initial rates of hydrolysis.

reaction products are the same as those of corresponding reference oligosaccharide. Therefore, the reaction products can be clearly identified to be xylobiose, xylotriose, and xylotetraose, respectively.

Active site

The pH profile of the steady-state kinetic parameters for arabinoxylotriose by xylanase II is shown in Fig. 5. The bell-shaped pH profile curve of xylanase II indicates that two groups with apparent pK_a values of 8.4 and 9.4 are implicated. The result suggests that cysteine may be involved in the active site of xylanase II.

As shown in Fig. 6A, xylanase II was modified by iodoacetamide and the time course of inactivation of the enzyme was linear. After 40 min incubation of 10 M xylanase II with 30 mM iodoacetamide, the residual activity against arabinoxylotriose was 25%. Analysis of a log (k) versus log [iodoacetamide] plot on xylanase II provided a reaction order of 1.7 with respect to iodoacetamide (Fig. 6B). The result exhibits that at least two essential cysteine residues are present in xylanase II.

The time course of inactivation of xylanase II with iodoacetamide and reactivation by the addition of cysteine or dithiothreitol is shown in Fig. 7. The enzyme inactivated by iodoacetamide was completely reactivated with 10 mM cysteine or 10 mM dithiothreitol and recovered to about 95% of residual activity by the treatment of 5 mM cysteine. However, the addition of 1 mM cysteine after inactivation did not restore enzyme activity.

Fig. 8A showed that *N*-bromosuccinimide had effect on xylanase II activity. The successive addition of NBS revealed a decrease in

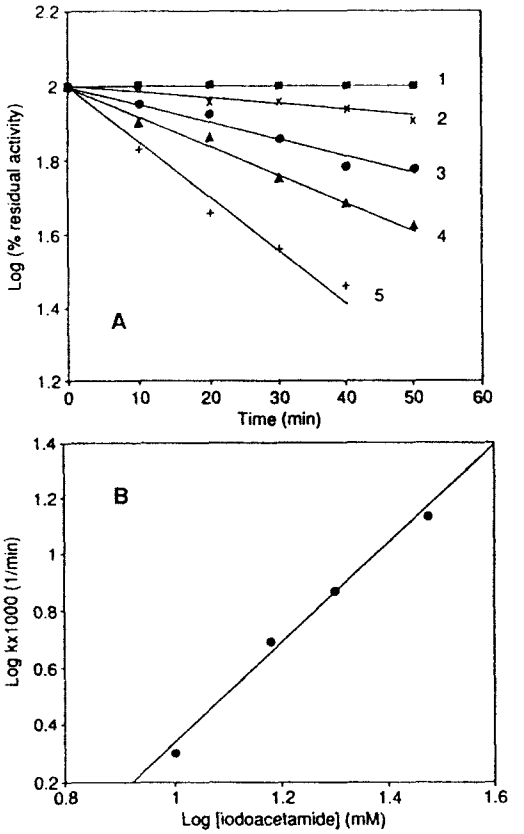

Fig. 6. *(A) Inactivation of xylanase II by various concentrations of iodoacetamide and (B) apparent order of reaction with respect to iodoacetamide concentration.*
(A) 1, No treatment; 2, 10 mM; 3, 15 mM; 4, 20 mM; 5, 30 mM; enzyme (10 μM) in 0.1 M sodium phosphate buffer, pH 7.0, at 25°C.
(B) The pseudo first-order rate constants (k) were calculated from the slopes of the data of (A).

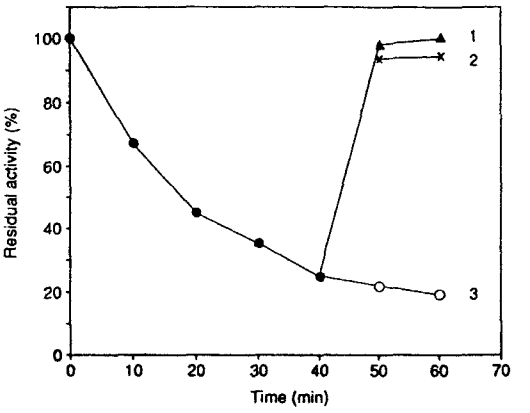

Fig. 7. *Reactivation of xylanase II after inactivation by iodoacetamide.*
Inactivation of 10 μM enzyme by 30 mM iodoacetamide and reactivation after 40 min with cysteine or dithiothreitol (DTT). 1, 10 mM cysteine or 10 mM DTT; 2, 5 mM cysteine; 3, 1 mM cysteine.

absorbance at 280 nm. When 8-fold molar excess of NBS over xylanase II was added, the enzyme was completely inactivated. A plot of the residual enzyme activity as a function of the number of modified tryptophan residues showed that eight tryptophan residues per molecule of xylanase II was oxidized by NBS (Fig. 8B).

An estimation of the number of essential tryptophan residues in xylanase II is provided by an analysis of the kinetics of inactivation. As shown in Fig. 9A, after 30 min incubation of 7.25 μM xylanase II with 0.45 mM NBS, residual activity was 14%. Logarithmic plots of residual activity as a function of time of inactivation for the various concentrations of NBS were linear, showing that the inactivation process obeys pseudo-first-order kinetics. A plot of log (k) versus log [NBS] on xylanase II was linear and the order of reaction was 4.5 (Fig. 9B). From these results, it is suggested that at least four of the eight tryptophan residues are essential in the catalysis of xylanase II.

DISCUSSION

A xylanase, designated as xylanase II, was purified from the culture filtrate of *T. koningii*. It showed approximately 10-fold increase in specific activity. Our enzyme was different from the endoxylanase 2 isolated from *T. koningii* by Wood and McCrae (41) in molecular weight, isoelectric point, and action patterns. The molecular weight and isoelectric point of our enzyme were 21 kDa and 9.4, respectively. These values are appreciably higher than those of endoxylanase 2 previously isolated from the strain, which were estimated to be 18 kDa (Mr) and 7.3 (pI). Our xylanase has relatively high isoelectric point. Isoelectric point above 9 has been found in several xylanases from *Trichoderma pseudokoningii* (1), *Bacillus circulans* (13), *Streptomyces roseiscleroticus* (16), *Trichoderma harzianum* (37), and *Trichoderma viride* (39).

The enzyme degraded oat-spelt and larchwood xylan randomly and produced mainly lower oligomers than xylotetraose as end products. Xylanase II did not release arabinose from arabinoxylan. According to Dekker's classification

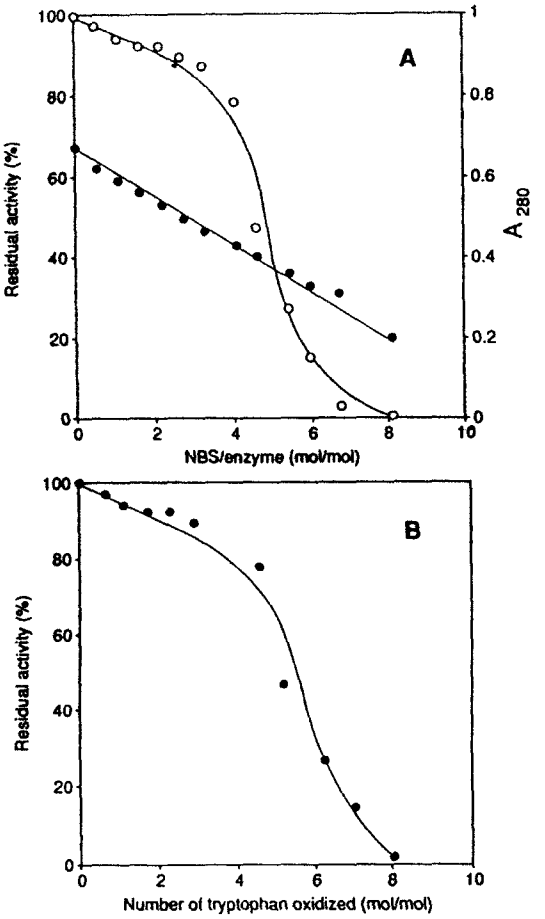

Fig. 8. *N-Bromosuccinimide (NBS) oxidation of tryptophan residues in xylanase II.*
Oxidation of the enzyme (1.85×10^{-5} M) was accomplished with the stepwise addition (10 μl) of NBS (2×10^{-4} M). After each addition, the residual activity (○) and absorbance at 280 nm (●) were measured. (B) Estimation of the number of tryptophan residues oxidized by NBS in xylanase II. Oxidation of tryptophan residues in the enzyme was carried out as described in (A). The number of tryptophan residues oxidized was calculated as described in the Materials and methods.

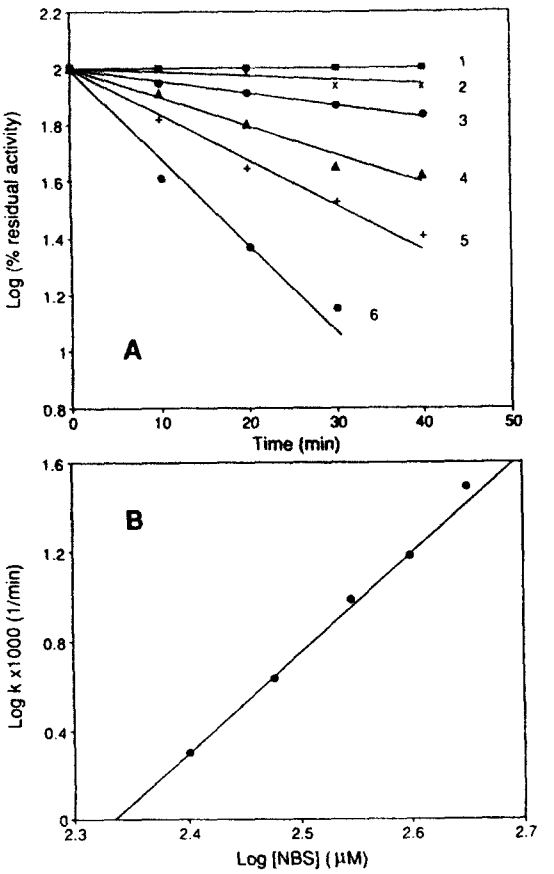

Fig. 9. *(A) Inactivation of xylanase II by various concentrations of NBS and (B) apparent order of reaction with respect to NBS concentration.*
(A) 1, No treatment; 2, 0.25 mM; 3, 0.3 mM; 4, 0.35 mM; 5, 0.4 mM; 6, 0.45 mM; enzyme (7.25 μM) in 0.1 M sodium acetate buffer, pH 4.4, at 25°C.
(B) The pseudo first-order rate constants (k) were calculated from the slopes of the data of (A).

of xylanases (10), the enzyme belongs to the group of "nondebranching xylanases". The nondebranching group of xylanases are by far the most common and degrade heteroxylans randomly (10). Such enzymes have been reported for fungal xylanases from *Sporotrichum dimorphosporum* (7), *Ceratocystis paradoxa* (11), and *Aspergillus niger* (15).

Xylobiose and arabinoxylobiose were not attacked by the enzyme. It is clear that our enzyme requires more than two xylosyl residues to express its activity. On the other hand, endoxylanase 2 reported by Wood and McCrae did not attack xylotriose as well as xylobiose. The production of X_4 from X_3 and of X_5 from X_4 demonstrates that xylanase II has trans-xylosidase activity, which was found in several xylanases of fungi and bacteria (30, 32, 40). All the results on the hydrolysis of oligosaccharides by xylanase II reveal that the enzyme is an endo-xylanase. After prolonged incubation with AX_3, xylanase II produced AX_2 and X_1. Generally, an exo-enzyme degrades oligosaccharides by successive removal of terminal xylose unit from the nonreducing end.

Since xylanase II is an endo-enzyme, the production of large amount of AX_2 and X1 from AX_3 indicates that α-L-arabinosyl branch residue presents on the nonreducing end of D-xylosyl chain of the oligosaccharide. In the hydrolysis of X_3 and X_4, xylanase II showed trans-xylosidase activity. However, in the hydrolysis of AX_3, such activity of the enzyme was not observed. These results demonstrate that arabinosyl containing xylooligosaccharides can not be used as acceptor or donor by the enzyme for trans-xylosidation. In the action of xylanase II, trans-xylosidation is indispensible process. It is deduced that a donor substrate is joined to an acceptor substrate via substrate-enzyme intermediate and then readily degrades to smaller oligosaccharides. The action mode of the enzyme on AX_3 shows that this enzyme slowly attacks the first β-D-xylosidic bond from the reducing end of AX_3, while it can not attack the second β-D-xylosidic bond from the reducing end of the substrate.

The structures of the hydrolysis products of xylotriose by the purified enzyme were identified by ^{1}H-NMR spectroscopy. The chemical shifts of H-1 protons and coupling constants, J(1, 2) of each reaction products are the same as those of corresponding reference oligosaccharide. From the results, it can be concluded that all the reaction products (including trans-xylosylated products) generated by the action of xylanase II on xylooligosaccharides have only β-1,4-xylosidic linkage(s).

In the pH profile curve of xylanase II, two ionizations were observed with apparent pK_a values of 8.4 and 9.4. The result suggests that cysteine may be operative in the catalytic mechanism of enzyme action.

Modification of xylanase II by iodoacetamide with gradient concentration showed inhibition of enzyme activity was linear, providing cysteine residue is involved in the active site of this enzyme. Plots of percentage residual activity as a function of time at various concentrations of iodoacetamide indicate that the inactivation process exhibits pseudo-first-order kinetics with respect to time at any fixed concentration of inhibitor.

Analysis of a log (k) versus log [iodoacetamide] yielded a reaction order of 1.7. Thus, it is revealed that the loss of enzyme activity results from reaction of two cysteine residues per molecule of xylanase II.

The sulfhydryl group of cysteine is in general the most reactive of any amino acid. As with most other functional group in proteins, the reactivity of cysteine is a reflection of the nucleophilic nature of the thiol group. It ionizes at slightly alkaline pH and reacts rapidly with alkyl halides, such as iodoacetamide, to yield the alkyl derivative. Generally, the dissociation value for sulfhydryl group in proteins is about 8.5. The pK_a values of cysteine in xylanase II from *T. koningii* are 8.4 and 9.4. Therefore, it is suggested that the substrate is hydrolyzed by nucleophilic attack of cysteine thiol anion with pK_a value of 8.4. However, the role of each cysteine residue remains unclear. Investigations concerning the function of essential cysteine residues are needed.

The loss of enzyme activity by iodoacetamide and reactivation by cysteine or dithiothreitol were investigated. The enzyme inactivated by iodoacetamide were completely reactivated with 10 mM cysteine or dithiotheitol. The results clearly indicate a competitive displacement of iodoacetamide by high concentrations of thiol and involvement of cysteine residues at the active site of xylanase II from *T. koningii*.

Fig. 8A shows that the effect of NBS on xylanase II. The absorbance decrease in the enzyme is accordent with the conversion of tryptophan residues from the indole to the oxindole (35).

There are several aspects in the use of NBS for the modification of tryptophan residues in proteins which should be considered. The reaction should be performed at mildly acidic pH. Under such condition, reaction is restricted to modification of tryptophan residues. At pH values close to neutrality, there is the increased possibility of modification of amino residues other than tryptophan (35). As the reaction on xylanase II with NBS was carried out at pH 4.4, modification of tryptophan residues in the enzyme by NBS can be assumed.

A plot of log (k) versus log [NBS] was linear and the order of reaction was 4.5. The result suggests that four tryptophan residues are invloved in active site of xylanase II. Keskar *et al.* (18) have reported that one tryptophan residue is essential in the function of xylanase from *Streptomyces*. Tryptophan residues have been shown to play an essential role in the binding sites of cellulase and lysozyme, which are functionally related to xylanase (6, 17).

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초 록: *Trichoderma koningii* ATCC 26113에서 분리된 Xylanase II의 작용양상과 활성부위

김현주 · 강사욱* · 하영칠 (서울대학교 자연과학대학 미생물학과, 서울대학교 분자미생물학연구센터)

Xylan과 관련 다당류(xylotriose, xylotetraose, arabinoxylotriose)에 대한 *Trichoderma koningii* ATCC 26113에서 분리된 xylanase II의 작용양상은 xylanase II가 endo-enzyme이고 trans-xylosidation의 활성을 가지고 있다고 보여진다. Xylanase II에 의해 형성된 반응산물을 ^{1}H-NMR분광법으로 분석한 결과는 본 효소에 의해 얻어진 xylooligosaccharides의 가수분해산물은 모두가 β-1,4-xylosidic linkage만을 가지고 있는 것으로 판명되었다. 본 효소를 iodoacetamide로 화학적으로 변형시켰을 때 효소 mole당 cysteine 잔기가 두개가 활성에 필요한 것으로 보여졌으며, *N*-bromosuccinimide 로 처리하였을 때는 활성부위에 tryptophan 잔기가 여덟개 존재하는 것으로 판명되었다.