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Mode of Action and Chemical Modification of an Alkaline Xylanase (CX-III) from Alkalophilic *Cephalosporium* sp. RYM-202

Myoung Kyu Kang,† Pil Jae Maeng, and Young Ha Rhee*
Department of Microbiology, Chungnam National University, Taejeon 305-764, Korea
†Department of Environmental Technology, Tonghae Junior College, Tonghae, Kangwon-Do 240-150, Korea

호알카리성 *Cephalosporium* sp. RYM-202로부터 분리된 alkaline xylanase (CX-III)의 작용 양상 및 화학적 변환

강명규† · 맹필재 · 이영하*
충남대학교 자연과학대학 미생물학과
†동해전문대학 환경공업과

ABSTRACT: The hydrolysis products formed from birchwood xylan by the action of an alkaline xylanase (CX-III) from alkalophilic *Cephalosporium* sp. RYM-202 were xylobiose and xylooligosaccharides polymerized with more than 4 sugar molecules. This enzyme was not active on xylobiose but readily attacked xylotriose accumulating xylobiose as a major product. The predominant end-products from xylotetraose by CX-III were xylobiose and xylotriose. These results indicate that the enzyme is typically endo-type xylanase possessing transglycosidase activity. Chemical modification of CX-III with *N*-bromosuccinimide revealed that two tryptophan residues per molecule of CX-III were essential for its catalytic activity on xylan. On the other hand, iodoacetamide and diethylpyrocarbonate did not influence the activity of the enzyme, suggesting that cysteine and histidine residues are not involved in the active site of this alkaline xylanase.

KEYWORDS: Action mode, Alkaline Xylanase, Alkalophilic *Cephalosporium* sp., Chemical Modification.

The major component of hemicellulose is xylan, which is a heterogeneous polymer consisting of a backbone of β-1,4-linked D-xylopyranosyl residues that often contain arabinosyl, methylglucuronosyl, and acetyl substituents (Biely, 1985). Xylanolytic enzyme systems include at least xylanases (β-1,4-endoxylanases, EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). Xylanases cleave internal glycosidic bonds within the xylan backbone, while β-xylosidases release xylosyl residues from the nonreducing ends of xylooligosaccharides (Wong *et al.*, 1988).

In addition to possible applications in the biocoversion of hemicellulosic material to sugar, alcohol, and other useful products (Kuhad and Singh, 1993; Wong and Saddler, 1992), there has been an increasing interest in applying xylanases in the pulp and paper industry during recent years. The use of xylanases for the prebleaching of kraft pulp has been considered one of the most important new industrial applications of the microbial enzymes (Paice *et al.*, 1992; Senior *et al.*, 1991). Since xylan is soluble in alkaline solution and many bleaching processes are per-

*Corresponding author

formed at a highly alkaline pH, xylanase active and stable at highly alkaline pHs are preferable for pulp applications (Horikoshi, 1996; Viikari *et al.*, 1994). An additional criterion in the use of xylanases for pulp and paper applications is that they must be cellulase-free, because cellulase activity in xylanase destroys pulp properties by degradation of cellulose fibers (Lundgren *et al.*, 1994).

Although alkaline xylanases have been purified and characterized from several bacteria including *Bacillus* spp. (Honda *et al.*, 1985; Okazaki *et al.*, 1985; Nakamura *et al.*, 1993) and a *Streptomyces* sp. (Rhyum *et al.*, 1993), there are few reports about the enzymatic properties of alkaline xylanases from fungi. To our best knowledge, there have been only two reports so far on alkali-tolerant xylanases from fungal strains, even though they were examined only in an unpurified state (Bansod *et al.*, 1993; Chandra Raj and Chandra, 1995).

Recently we have isolated an alkalophilic fungal strain, *Cephalosporium* sp. RYM-202, that grows optimally at pH 9.5-10.0 (Kang *et al.*, 1993). In addition to its multiple carboxymethyl cellulases (Kang and Rhee, 1995), this organism can produce extracellularly multiple xylanases when wheat bran is a carbon source. In previous papers, we reported on the purification and characterization of three cellulase-free xylanases from *Cephalosporium* sp. RYM-202, CX-I, CX-II, and CX-III, which exhibited high activity and stability at alkaline pH (Kang *et al.*, 1995, 1996). These alkaline enzymes were shown to have very exceptional properties that could be compared with those of xylanases from other microbial origins. The aim of the study presented here was to examine action patterns of CX-III which was one of the alkaline xylanases purified from *Cephalosporium* sp. RYM-202 on xylan and xylooligosaccharides. On the basis of chemical modification experiments, we also describe the determination of essential amino acid residues involved at active site of this xylanase.

Materials and Methods

Xylanase preparation

An alkaline xylanase (CX-III) that was purified to homogeneity by a series of column chromatographies from a culture supernatant of *Cephalosporium* sp. RYM-202 was used in this study (Kang *et al.*, 1995). Xylanase activity was determined by measuring reducing sugars by the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixture consisting of 0.9 ml of 0.5% (W/V) birchwood xylan in 50 mM sodium phosphate buffer (pH 7.5) and 0.1 ml of suitably diluted enzyme was incubated at 50°C for 20 min. One unit of xylanase was defined as the amount of enzyme liberating 1 μmol of xylose equivalent per min under the assay conditions.

Chemicals

Birchwood xylan, xylose, *N*-bromosuccinimide (NBS), diethylpyrocarbonate, and iodoacetamide were supplied from Sigma Chemical Co. (USA). Charcoal (Darco G-60) was purchased from Aldrich (USA). Xylooligosaccharides (xylobiose, xylotriose, and xylotetraose) were prepared from the enzymatic hydrolyzates of birchwood xylan according to the modified method of Miller *et al.* (1960). The birchwood xylan (12g, 1% W/V) was incubated with 120 units of a purified xylanase, CX-II (Kang *et al.*, 1996), from *Cephalosporium* sp. RYM-202 in 50 mM sodium phosphate buffer (pH 7.0) at 50°C for 15 h. The reaction was stopped by heating at 100°C for 10 min. After centrifuge, the supernatant was concentrated at 40°C by rotary evaporation. The enzyme hydrolyzates were passed th-

rough a column of 50% charcoal-50% celite, using ethanol-water linear concentration gradient. Oligosaccharides in the effluent were estimated by DNS method (Miller, 1959). Fractions containing each xylooligosaccharide were separately pooled and concentrated by rotary evaporation at 40°C. If necessary, the concentrates were diluted with water. After the addition of a half volume of chloroform, the supernatant phase was collected and lyophilized.

Enzymatic hydrolysis of xylan and xylooligosaccharides

For analyses of degradation products from xylan, CX-III (10.8 units) was incubated with 4.5% (W/V) birchwood xylan in 0.1 ml of 50 mM sodium phosphate buffer (pH 7.5) at 50°C for 24 h. The aliquots at different time intervals were analyzed by thin-layer chromatography (TLC). A 2 μl portion of each sample was spotted onto TLC silica gel plate 60 F_{254} (Merck, Germany) and chromatographed in a solvent system containing chloroform/acetic acid/water (6:7:1, V/V) at room temperature. Sugars on TLC plates were detected by the ethanol-sulfuric acid spray method (White and Kennedy, 1986). Similar mixtures and hydrolysis conditions were performed to determine the hydrolysis products from xylooligosaccharides (xylobiose, xylotriose, and xylotetraose). The aliquots were withdrawn at time intervals and analyzed using high-performance liquid chromatography (HPLC). Hydrolyzates were centrifuged in a microcentrifuge (MSE, England) at 13,000 rpm for 30 sec in order to remove any solids, and the supernatants were filtered through 0.45 μm Millipore filter (Waters, USA). These filtrates were analyzed on the Shim-pack CLC-NH_2 column (Shimadzu, Japan) by injecting a 10 μl portion with 76% acetonitrile as eluant at a flow rate of 2 ml/min. The sugar products were detected with a refractive index detecter (Shimadzu model: RID-6A, Japan) and the elution pattern and retention time of the sugars were registered on a Shimadzu CR-7A data module.

Modification of xylanase with chemical inhibitors

To evaluate the effect of *N*-bromosuccinimide (NBS) on the xylanase activity, the following procedures were performed. The purified CX-III (1 μM, 160 μl) was incubated with 40 μl NBS (5 to 25 μM) dissolved in 0.1 M sodium acetate buffer (pH 4.4) at 25°C. The final concentrations of NBS in the reaction mixture were corresponded to 1 - 5 μM. Aliquots (25 μl) were taken at time intervals, diluted in 100 μl of 0.2 M sodium phosphate buffer (pH 7.0), and the residual activities were determined. Reaction procedures for iodoacetamide and diethylpyrocarbonate were carried out in a similar manner, except that the final concentration of inhibitors increased up to 10 mM. In all cases, controls were performed without enzyme, but containing inhibitor to check for any subsequent interference in the assay.

Results and Discussion

Action mode of CX-III on xylan

The thin-layer chromatogram of hydrolysate of birchwood xylan by CX-III is shown in Fig. 1. The enzyme degraded xylan at random, and accumulated a large amount of xylobiose (X_2) and xylooligosaccharides with degree of polymerization higher than 4, even though xylotriose (X_3) was also detectable at the early stage of hydrolysis. After 6 h of incubation time, a trace amount of xylose (X_1) was detected. These results suggest that the enzyme is an endo-type xylanase producing X_2 as a predominant end

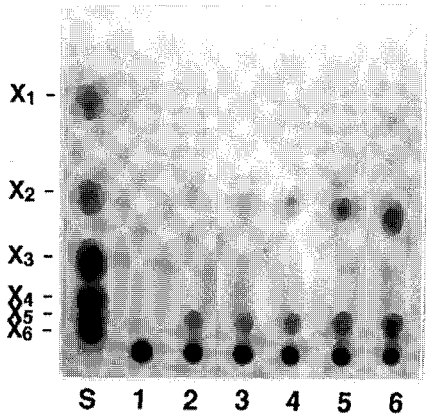

Fig. 1. TLC analysis of hydrolysates formed by the action of CX-III from birchwood xylan. One hundred microliters of 4.5% (W/V) birchwood xylan in 50 mM sodium phosphate buffer (pH 7.5) were incubated with 10.8 U of CX-III at 50°C for 0 (lane 1), 0.2 (lane 2), 0.5 (lane 3), 1.5 (lane 4), 6 (lane 5), and 24 (lane 6) hr. Xylooligosaccharide standards (lane S) included xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5), and xylohexaose (X_6).

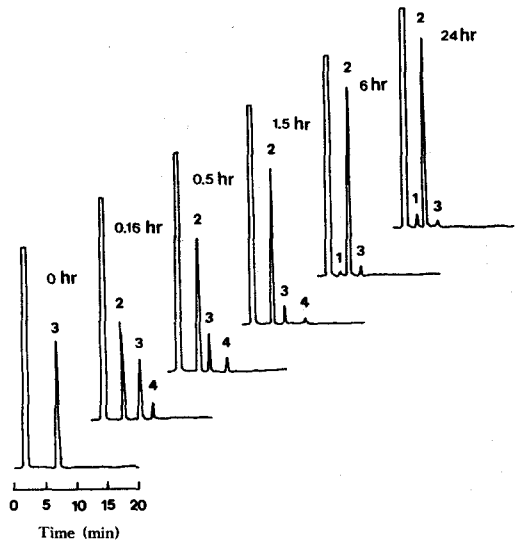

Fig. 2. HPLC analysis of reaction products from xylotriose by the action of CX-III. The enzyme (10.8 U) was incubated with 121 mM xylotriose in 50 mM sodium phosphate buffer (pH 7.5). 1, xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose.

product of xylan hydrolysis, along with X_3 and higher xylooligosaccharides as intermediates. The hydrolysis pattern of this enzyme was quite similar to thoses of CX-I and CX-II from *Cephalosporium* sp. RYM-202 (Kang *et al.*, 1996) and alkaline xylanase from a *Bacillus* sp. (Nakamura *et al.*, 1993), but significantly different from those of fungal xylanases from *Trichoderma viride* (Ujiie *et al.*, 1991), *T. reesei* (Tenkanen *et al.*, 1992), *T. harzianum* (Tan *et al.*, 1985), and *Aureobasidium pullulans* (Li *et al.*, 1993), in which X_1 and X_2 were the main hydrolysis products from xylan.

In the present study, it is noteworthy that the amount of higher xylooligosaccharide corresponding to xylopentaose (X_5) increased throughout the hydrolysis of xylan. The accumulation of X_5 in the hydrolysates could be due to the low activity of the xylanase against this xylooligosaccharide. Unlike other xylanases that hydrolyze X_5 (Honda *et al.*, 1985; Nakamura *et al.*, 1993; Murai *et al.*, 1994), those from *T. reesei* (Tenkanen *et al.*, 1992) and *A. pullulans* (Li *et al.*, 1993) were reported to be incapable of hydrolyzing X_5. The strange action patterns of CX-III cannot be explained on the basis of the experiments performed in this work, and thus further studies on the kinetics of this xylanase against the higher xylooligosaccharides need to be done.

Action mode on xylooligosaccharides

Xylooligosaccharides were hydrolyzed by CX-III at pH 7.5, and the hydrolyzates at time intervals were analyzed by HPLC. Xylobiose was not hydrolyzed by the xylanase (data not shown). On the other hand, the enzyme readily attacked xylotriose (X_3) and finally accumulated X_2 as a major product and X_1 as a minor product (Fig. 2). At the initial

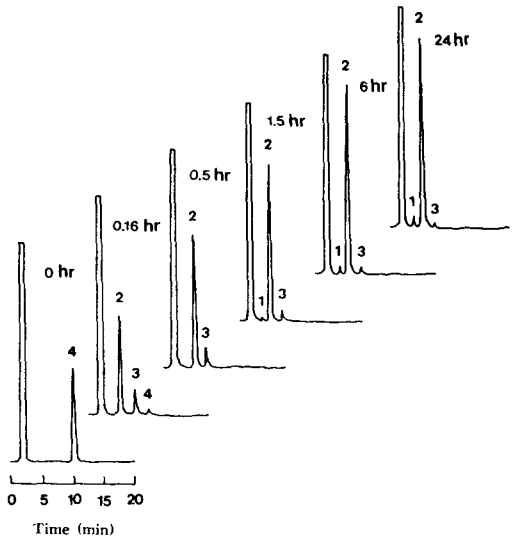

Fig. 3. HPLC analysis of reaction products from xylotetraose by the action of CX-III. The enzyme (10.8 U) was incubated with 92 mM xylotetraose in 50 mM sodium phosphate buffer (pH 7.5). 1, xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose.

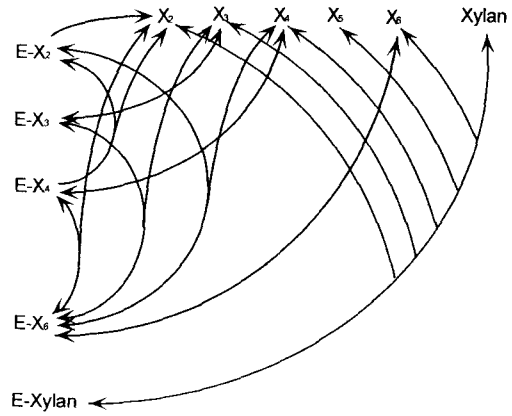

Fig. 4. Hypothetical degradation pathways of xylan and xylooligosaccharides by CX-III. E, xylanase (CX-III); X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_6, xylohexaose; E-Xn, enzyme-xylooligosaccharide intermediate.

stage of the reaction, no detectable amount of X_1 was released from X_3 while some xylotetraose (X_4) was detected. Throughout the reaction, the amount of X_2 was extremely larger than that of X_1. These results strongly support the view that X_2 was produced from X_3 via some other way than simple hydrolysis, *i.e.*, either of two xylosidic bonds in X_3 was not directly split by the xylanase. Therefore the most probable explanation for this pattern of degradation is that a donor X_3 molecule was joined to an acceptor X_3 by transglycosidation activity of the enzyme to yield xylohexaose (X_6) which was quite rapidly hydrolyzed into three X_2 molecules or into X_2 and X_4. Xylotriose has been reported to be the smallest xylooligosaccharide that is hydrolyzed by most of characterized xylanases derived from xylanolytic fungi, such as *Aspergillus niger* (Vrsanska *et al.*, 1982) and *Trichoderma* spp. (Beldman *et al.*, 1987; Wong and Saddler, 1992; Kim *et al.*, 1994).

The main hydrolysis products from xylotetraose were xylobiose and xylotriose, and after a prolonged incubation, the latter was further hydrolyzed into X_2 without any significant accumulation of X_1 (Fig. 3). Since the production of xylotriose from xylotetraose is not possible without the formation of xylose at the early stage of the reaction, the results confirm that X_4 molecules were mainly split into X_2 molecules and that the majority of X_3 molecules was released from X_6 molecules previously formed from X_2 and X_4 by transglycosidation. In this study, however, a noticeable peak corresponding to X_6 was not detected in the chromatogram, which was presumably due to very rapid hydrolysis of X_6 into two X_3 molecules or into X_2 and X_4. In contrast to X_5, X_6 is considered to be highly susceptible to the action of CX-III.

Consequently, on the basis of the present results it is obvious that the enzyme is an endo-type xylanase possessing transglycosidase activity. A plausible degradation pathways of

xylan and xylooligosaccharides by CX-III are depicted in Fig. 4. The transglycosidase activities have already been observed in xylanases of different microbial origins, including *Bacillus* spp. (Okazaki *et al.*, 1985; Honda *et al.*, 1985), *A. niger* (Vrsanska *et al.*, 1982), *Cryptococcus albidus* (Biely *et al.*, 1981) and *Trichoderma* spp. (Beldman *et al.*, 1987; Wong *et al.*, 1988; Wong and Saddler, 1992; Kim *et al.*, 1994).

Chemical modification of xylanase

Chemical modification of an enzyme leads to an alteration of its surface structure and can be used to identify the essential catalytic and substrate-binding residues (Lundblad and Noyes, 1984). In a previous report, all of the xylanases, including CX-III, from *Cephalosporium* sp. RYM-202 were shown to be completely inhibited by 1 mM NBS, which indicates that the presence of tryptophan is essential for the action of these enzymes on xylan (Kang *et al.*, 1995, 1996). Complete inactivation by NBS has already been reported for xylanases of different microbial strains (Nakamura *et al.*, 1993; Tsujibo *et al.*, 1992; Keskar *et al.*, 1989). The involvement of tryptophan residues at the substrate-binding region of the xylanases is a characteristic property which may be conserved among many different microbial xylanases (Deshpande *et al.*, 1990). In this study, the effect of various concentrations (1 to 5 μM) of NBS on the activity of CX-III was examined. As shown in Fig. 5A, the enzyme was severely inactivated by NBS even at lower concentrations. It retained less than 5% of its original activity after preincubation of the xylanase (1 μM) with 5 μM NBS for 30 min. The logarithmic plots of residual activity as a function of reaction time at different concentrations of NBS were linear, which suggests the inactivation process obeys pseudo-first-order kinetics with respect to time at any fixed concentration of NBS. Applying the analysis of inactivation kinetics described by Levy *et al.* (1963), the logarithm of observed pseudo-first-order rate constants (k) calculated from the slope of logarithm plots of the residual activity against the time of reac-

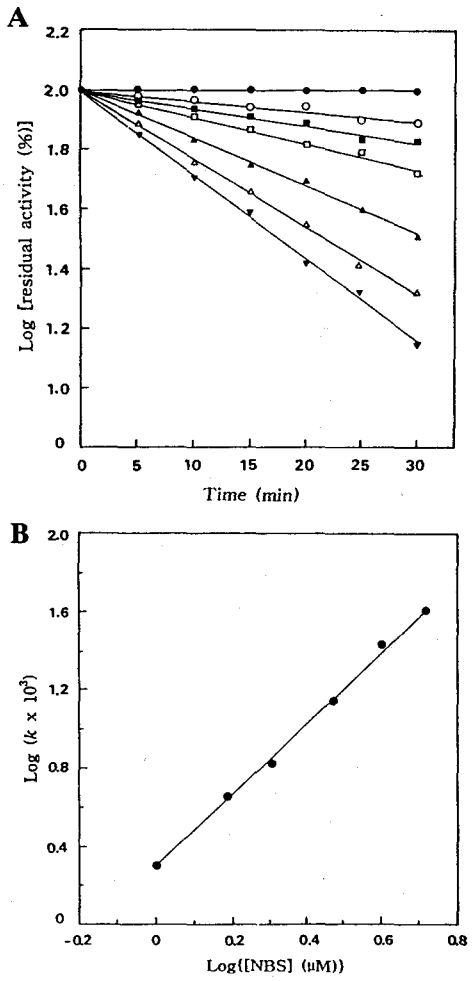

Fig. 5. (A) Inactivation of CX-III by different concentrations of NBS (●, 0 μM; ○, 1 μM; ■, 1.5 μM; □, 2 μM; ▲, 3 μM; △, 4 μM; ▼, 5 μM). (B) Apparent order of reaction with respect to NBS concentrations. The logarithm of pseudo-first-order rate constant (k) calculated from the slopes of the data in (A) was plotted against the logarithm of NBS concentration.

tion was plotted against the logarithm of the NBS concentration (Fig. 5B). The plot of log (k) versus log[NBS] for the xylanase was linear, and the order of reaction that was estimated from the slope of the plot was 1.92. This value revealed that the loss of enzyme activity by NBS resulted from reaction of two tryptophan residues per molecule of CX-III. In other words, an average of two tryptophan residues per molecule of the xylanase play an essential role for the activity on xylan. This is a good evidence for the involvement of the tryptophan residues at the active site of xylanases. The number of tryptophan residues essential at active (substrate-binding) site has been reported to be one for xylanase from thermotolerant *Streptomyces* (Keskar *et al.*, 1989) and four for xylanase from *T. koningii* (Kim *et al.*, 1994).

The effects of iodoacetamide and diethylpyrocarbonate, which are the chemical reagents for protein modification that preferentially modify cysteine and histidine residues, respectively, on the activity of CX-III were also examined. These chemicals did not affect the activity of the xylanase even at increased concentrations up to 10 mM (Table 1). These results suggest that cysteine and histidine residues are not involved in the active site of CX-III. It has previously been observed that CX-III is completely inhibited by 1 mM Hg^{2+} (Kang *et al.*, 1995). Heavy metal ions are generally considered to inactivate enzymes by forming covalent salts with cysteine or histidine residues. Since the present result reveals that CX-III is devoid of cysteine and histidine residues in the active site, the inhibitory effect of Hg^{2+} on the activity of CX-III could be ascribed to interaction with tryptophane residues rather than with thiol groups. On the other hand, in the cases of many xylanases (exhibiting acidic or neutral pH optima) from a *Chainia* sp. (Deshphande *et al.*, 1990), a *Streptomyces* sp. (Keskar *et al.*, 1989) and *T. koningii* (Kim *et al.*, 1994), the presence of one or two cysteine residues at active site has been reported to be essential for the enzyme activity.

Table 1. Effect of iodoacetamide and diethylpyrocarbonate on the enzymatic activity of CX-III

Reagent	Concentration (mM)	% Relative
None		100
Iodoacetamide	2	100
	10	101
Diethylpyrocarbonate	2	103
	10	100

Tryptophan residues have also been shown to play an essential role in the catalytic mechanism of cellulase and lysozyme, which are functionally related to xylanase (Hurst *et al.*, 1977; Clarke, 1987). By contrast, the involvement of cysteine residues at the active site of cellulase and lysozyme has not hitherto been reported. Since there is no other report demonstrating the essential catalytic and substrate-binding residues for alkine xylanase, it is unclear whether non-critical role of cysteine and histidine residues are general to alkaline xylanases or not. However, the results obtained in the present investigation show a similarlity of catalytic mechanism of CX-III to those established for cellulase and lysozyme, and support a close relationship between xylanase, cellulase and lysozyme (Hurst *et al.*, 1977; Gilkes *et al.*, 1991).

적 요

호알카리성 진균 *Cephalosporium* sp. RYM-202가 생산하는 alkaline xylanase (CX-III)의 작용에 의해 xylan 기질로부터 생성되는 주요 가수분해 산물은 xylobiose와 중합도가 4 이상인 xylooligosaccharides이었다. 이 효소는 xylobiose에 대한 분해능을 가지고 있지 않지만 xylotriose로

부터는 xylobiose를, xylotetraose로부터는 xylobiose와 xylotriose를 주산물로 형성하였다. 이러한 결과들은 CX-III가 transglycosidase 활성을 소유하는 전형적인 endo-type xylanase임을 보여준다. *N*-bromosuccinimide에 의한 CX-III의 화학적 변환 실험 결과 효소 1분자 당 2개의 tryptophan 잔기가 활성에 관여하는 것으로 나타났다. 그러나 iodoacetamide 및 diethylpyrocarbonate에 의한 효소활성의 저해효과는 나타나지 않음으로써 이 효소의 활성부위에 cysteine과 histidine 잔기가 필수적이지 않음이 확인되었다.

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