Purification and Characterization of Xylanase from *Bacillus licheniformis*.

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Bacillus licheniformis Xylanase의 정제와 특성

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Three kinds of xylanases, X-C, X-I, and X-II, were separated from culture filtrate of an alkalophilic bacteria, *Bacillus licheniformis* OR-1. Their molecular weights were estimated to be 29,000, 50,000, and 34,000, respectively. They were most active at pH 6.0-6.5, and at temperature of 50°C. Mercurc ion and p-chloromercurybenzoate inhibited the xylanase activity of X-C and X-II remarkably, whereas X-I was not affected. Xylanase X-I hydrolyzed barley straw xylan liberating xylose, xylobiose, and arabinose, while X-C and X-II produced only xylobiose and xylotriose.

D-Xylanase (1.4-β-D-xylan xylanohydrolase EC 3, 2, 1, 8) has been found in many imcroorganisms such as *Aspergillus niger* [1-6], *Ceratocystis paradoxa* [7, 8] *Tricoderma viride* [9, 10], *Penicillin janthinellum* [11]. It has been reported that some of these fungi produced several different types of xylanase, which has similar enzymatic characteristics [1-8].

However, such multiplicity of the enzyme has not been observed in xylanase from bacteria such as *Bacillus* sp. [12, 13], *B. subtilis* [14, 15], *B. pumilus* [16], *B. polymyxa* [17], and *Streptomyces xylophagus* [18]. Bacterial xylanase showed optimum pH higher then that of fungal xylanase.

During the course of studies of microbial xylanase system we have separated three kinds of xylanase from an alkalophilic bacteria identified as *Bacillus licheniformis*. Their enzymatic characteristic particularly in their reactivity on the substrate appeared different from those of fungal xylanase. In this report, the enzymatic characteristics of xylanases from *B. licheniformis* are described.

Materials and Methods

Isolation and culture of microorganism

The strain OR-1 used in this experiment was isolated from soil. Rotten plant tissue or soil sample were extracted with small amounts of sterile water and plated on agar plate containing medium composed of 0.5% xylan 0.2% $NaNO_3$, 1.0% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KC1, 0.001% $FeSO_4$, and 0.1% yeast extract. pH of the medium was adjusted to be 9.0. Well grown colonies after 5 days culture at 30°C were transferred to liquid medium. The flasks which showed high xylanase activity at pH 8.0 after 3 days were selected and used for the further study. The strain was cultured in large scale for the crude enzyme preparation.

Enzyme assay

D-xylanase activity was determined with 0.25% solution of barley straw xylan as a substrate in 50mM phosphate buffer (pH 6.5) at 40°C. Reducing sugar was assayed by the method of Somogyi-Nelson method [19]. Barley straw xylan was prepared according to the procedure described previousely [6]. One unit of xylanase was defined as the amount of the enzyme which releases reducing sugar equivalent to 1 umole of D-xylose per minute under the specified condition.

Protein determination

Protein was estimated according to the method of

Lowry et. al[20] using crystaline bovine serum albumin as a standard.

Enzymatic hydrolysis of D-xylan

Enzymatic hydrolysis of 1% xylan solution was carried out with the concentration of 1 unit enzyme per ml substrate solution at 30°C.

Paper chromatography

Sugar composition of hydrolyzed xylan solution was analyzed by paper chromatography on Whatman No. 1 filter paper. The developing solvent was composed of n-butanol : pyridine : water = 6:4:3 (v/v)[21]. Sugar spots on the paper were detected by aniline hydrogen phthalate method[22].

Materials

Xylan from larchwood X-3875, Lot 48C-00541 was purchased from Sigma Chemical Co., U.S.A. DEAE-Sephadex and CM-Sephadex were obtained from Pharmacia Fine Chemicals. All other Chemicals used were reagent grade.

Results

Characteristics of the strain

A bacterial strain OR-I with high xylanase activity was isolated from soil. This bacterium is endospore-forming rod-shaped (0.5p0.7 x 1.5-2 um) and gram positive (Fig. 1). It is aerobic or facultative anaerobic and motile. It produced acid and gas in glucose media. Other physiological characteristics of the strain are summarized in Table 1: From these experimental results of morphological and physiological characteristics, the strain OR-1 can best be identified as *Bacillus licheniformis*[23].
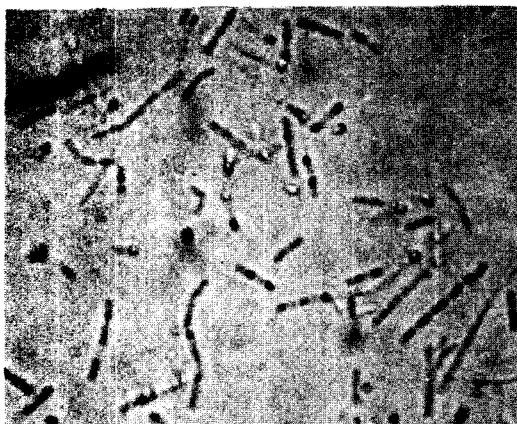

Fig. 1. Microscopic view of *Bacillus Lichenifor-mis* OR-1 (x600)

**Table 1. Taxonomic characteristics of the strain OR-I**

Characteristics	Description
Cell structure	Rods: with 0.5-0.7 μm length 1.5-2.0 μm some in chains
Endospore formation	One at the center of a sporangial cell
Gram staining	Positive
Motility	Motile
Acid formation	Positive with xylose and glucose
Gas formation	Positive with glucose
Starch hydrolysis	Positive
Cell growth	Positive in 7% NaCl in anaerobic agar in citrate agar
Reduction of litomas milk	Peptonization and milky color

Partial purification of xylanase

Ammonium sulfate fractionation: Culture filtrate which was employed as crude enzyme solution was fractionated for xylanase by the addition of ammonium sulfate to 50% saturation. The precipitate was dissolved in 2mM acetate buffer (pH 5.0) containing 1mM EDTA and 10mM mercaptoethanol (Buffer I). The buffer I was used through-out the purification procedures. Insoluble precipitate was discarded by centrifugation.

Ion exchange chromatography on CM-Sephadex C-25: Clear supernatant was desalted on the Sephadex G-25 column (3.3 x 52cm) which was equilibrated with Buffer I. The desalted xylanase fraction was applied on the CM-Sephadex C-25 column (3.2 x 30cm) in Buffer I. Two fraction of xylanases, were obtained as shown in Fig. 2. The bound fraction was eluted out by NaCl salt gradient. The fractions with high xylanase activity (No. 93-99) were pooled for the further purification and assigned as the X-C fraction.

Gel filtration on Sephadex G-100: Franction X-C was further purified by gel filtration on Sephadex G-100 which was equilibrated with 50mM phosphate buffer (pH 6.5), containing 1mM EDTA and 10mM mercaptoethanol (Buffer 2) and was proved to be homogeneous by disc gel electrophoreis (Fig. 3 and 4). The non-binding xylanase fraction on CM-Sephadex column was applied on the DEAE-Sephadex after adjusting pH to 8.0 with 0.1M
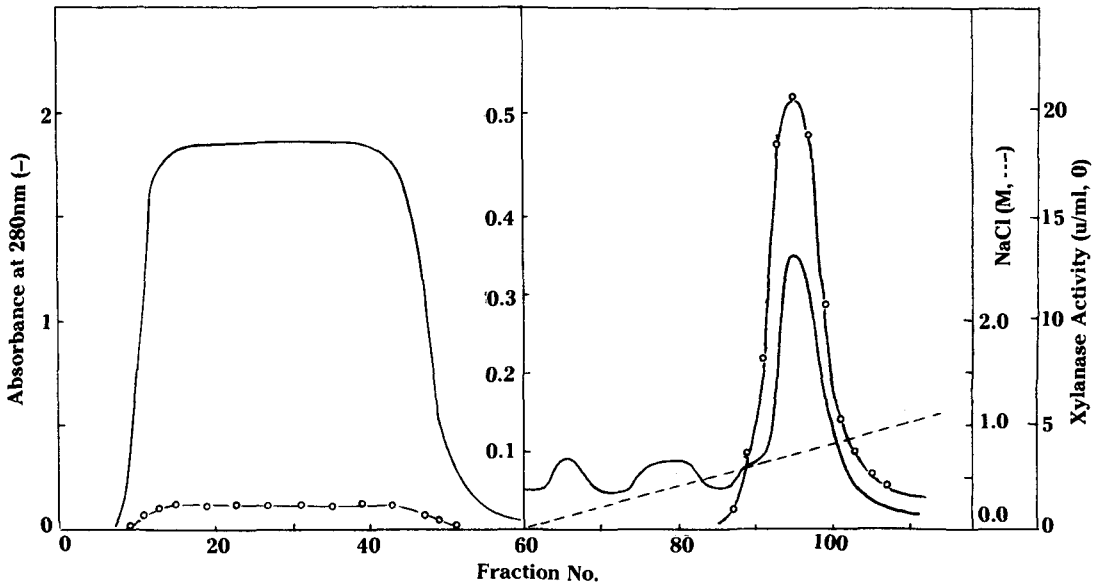
Fig. 2. **Ion exchange chromatogram of ammonium sulfate fractionation of D-xylanase from** *B. lichenifor-mis* OR-1.

CM-Sephadex C-25 column was equilibrated with 2mM acetate buffer (pH 5.0). Elution was carried out by linear salt gradient elution.

NaOH. The fraction did not bind also on DEAE-Sephadex under the experimental condition mentioned above. Other proteins were, however, removed by this anion exchanger.

Xylanase fraction passed by through DEAE-Sephadex A-25 column was concentrated by freeze drying and applied on Sephadex G-100 column equilibrated with Buffer 2. Two fractions of xylanases, X-I and X-II, were obtained as shown in Fig. 5. The fractions 50-55 and 59-65 with high xylanase activity were pooled and assigned as xylanase X-I and X-II, respectively. Purifica-
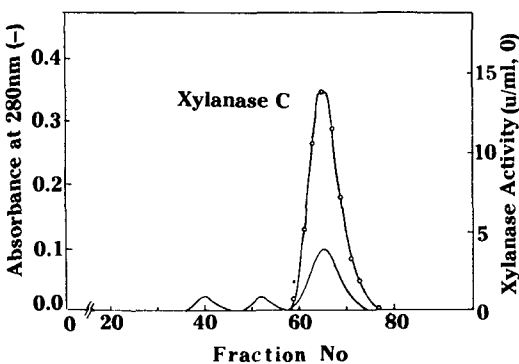

Fig. 3. **Gel filtration chromatogram of xylanase X-C on Sephadex G-100 ion exchange chromatography.**

Fig. 4. **Polyacrylamide disc gel electrophoresis of xylanase X-C.**

tion procedures for xylanase X-C, and X-II were summarized in Table 2. They were purified 39.5, 2.9, and 19.5 fold with respect to the crude extract, respectively. The xylanase X-C fraction was proved to be homogeneous by disc gel electrophoresis whereas X-I and X-II fractions were not completely pure. But, three kinds of xylanase were separated from crude enzyme in this experiment.

Molecular weights

Molecular weights of the xylanase fraction X-C, X-I,

Table 2. Purification of xylanases from *Bacillus licheniformis* OR-1.

Procedure	Total protein (mg)	Total activity (u)	Specific activity (u/mg)	Purification (fold)	Yield (%)
1. Crude extract	2370	2422	1.0	1	100
2. AmSO₄ fractionation (0-50% saturation)	363	1802	5.0	5	74.4
3. CM-Sephadex chromatography (fraction X-C)	49.7	879	17.7	17.7	36.3
4. DEAE-Sephadex chromatography (fraction X-I & II)	39.5	363	9.2	9.2	15.0
5. Gel filtration on Sephadex G-100					
fraction X-C	15.5	612.2	29.5	39.5	25.3
X-I	16.8	48.8	2.9	2.9	2.0
X-II	9.9	193.2	19.5	19.5	8.0

and X-II were estimated to be 29,000, 50,000, and 34,000, respectively, by the method of gel filtration on Sephadex G-100 (Fig. 5).
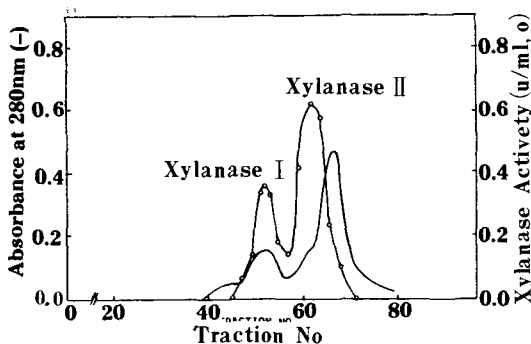
pH effect

Optimum pH's for there xylanase were investigated to be 6.0 for X-I and 6.5 X-C and X-II (Fig. 7). The enzymatic activity of X-C and X-II was decreased rapidly above the optimum pH, whereas X-I showed only a slight decrease above pH 6.0.
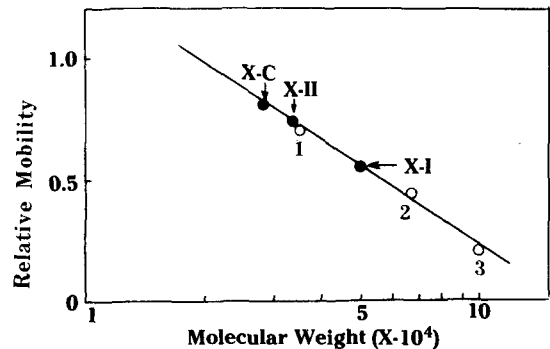
Temperature effect

All the xylanase fraction X-C,I, and II showed similar activity profile at various temperature. The highest activity of these enzyme fraction appeared at 50°C (Fig.8).
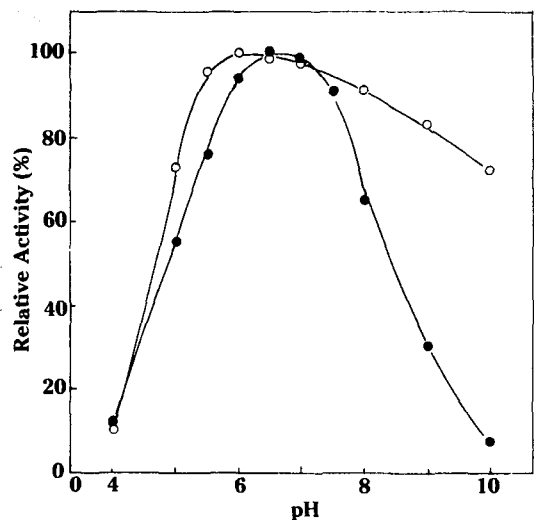
Metal ion effects

Mercuric ion affected most remarkably on the xylanase activity of X-C and X-II fractions while other

Fig. 6. Molecular weights of xylanase fractions by gel filtration on Sephadex G-100.

Fig. 5. Gel filtration chromatogram of xylanase X-I and II on Sephadex G-100.

Fig. 7. Effect of pH on the xylanase activity.
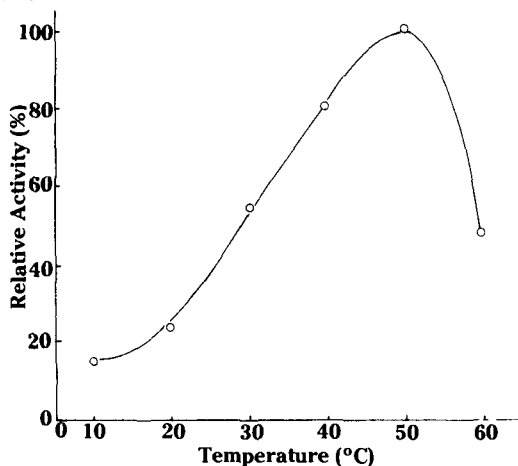**O: xylanase I, O: xylanase X-C and X-II**

Fig 8. Effects of temperature on xylanase activity.

Table 4. Degree of hydrolysis of xylan by xylanase from _B. licheniformis_ OR-1

Enzyme fractions	Degree of hydrolysis (%)	
	barley straw xylan	larchwood xylan
Xylanase X-C	12.0	11.0
X-I	22.9	24.4
X-II	11.3	11.2
Crude xylanase + ß-xylosidase	45.8	41.2

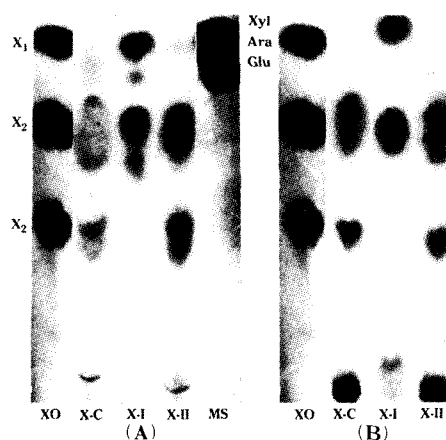One u/ml of each enzyme preparation was used.

Fig. 9. Paper chromatogram of reaction products of xylanases.

Barley straw xylan (A) and larchwood xylan (B). XO: xylose, xylobiose and xylotriose standards, MS: xylose and arabionose standards

metals did not (Table 3). The complete inactivation of these two fractions was occured with 1mM of p-chloromercurybenzoate within 30 min. The results suggest that these xylanases are sulfhydryl enzymes.

Hydrolytic pattern of the enzymes

Degrees of hydrolysis of 1% xylan solution by these enzymes were tabulated in Table 4. The crude xylanase which contained all the three xylanase fractions and ß-xylosidase hydrolyzed the xylan solution to the degree of 45.8 and 41.2% for barley straw and larchwood xylan, respectively. As seen in Fig. 9, hydrolytic pattern of xylanase X-C and X-II were similar to each other. They produced xylobiose and xylotriose as final products. They could not hydrolyze xylotriose to xylose and xylobiose. They liberated only a small amount of D-xylose. They also could not liberate arabinose side chain from barley straw xylan.

The xylanase X-I fraction, however, hydrolyzed xylotriose to xylose and xylobiose as final products. It liberated more xylose than did the other xylanase fractions. It also liberated arabinose from barley straw xylan.

Table 3. Effects of metal ion on xylanases activity

Metal ion	Relative Activity (%)		
(1mM)	X-C	X-I	X-II
K⁺	104.6	100.0	95.2
Na⁺	97.3	98.1	101.6
Mg⁺⁺	94.6	100.8	98.4
Ca⁺⁺	86.5	95.0	103.2
Hg⁺⁺	32.4	94.2	24.8
EDTA	100	100	100

Discussion

The presence of multiple fractions of xylanase with similar enzymatic characteristics has been observed frequently in fungi[1-8]. Such mutiplicity of xylanase, however, has not been reported in bacterial system [14-18]. In the present study, it was observed that _Bacillus licheniformis_ OR-1 preduced at least three kinds of xylanase. Their optimum pH was 6.0-6.5 as was other bacterial xylanase.

The experiment using SH-modifying agents such as mercuric ion or p-chloromercurybenzoate revealed that SH group of xylanase X-C and X-II was required for the enzymatic activity, whereae xylanase X-I was not affected by these thiol agents at all.

Xylanase produced by this alkalophilic bacteria degraded 1% xylan solution to the degree of 45.8% for

bacteria degraded 1% xylan solution to the degree of barley straw xylan and 41.2% for larchwood xylan. It was also observed that barley straw xylan was hydrolyzed less by xylanase from bacteria than that from *Aspergillus niger* KG-79 (53.9%) while the former did better on larchwood xylan than the later (28.0%). Such changes in degree of hydrolysis could be resulted from differences in enzyme characteristics and/or differences in the reaction pH for the bacterial enzyme (pH 6.5) and the fungal one (pH 4.5). Differences in the reaction pH may cause conformation changes of xylan[6] in such way as to alter the subceptibility of the substrate to the enzymes.

Xylanase X-C and X-II from *B. licheniformis* OR-I hydrolyze xylotriose to and xylose xylobiose, and also could liberate arabinose from xylan to some extent. It is shown that enzymatic properties of xylanase X-C and X-II are very similar to each other except their molecular weights. The xylanase fractins appear different from those repoduced from *A. niger*[6], whereas xylanase X-I demonstrated similar characteristics as those from *A. niger*.

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