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Purification and Characteristics of Xylanases from Produced Thermophilic Alkalophilic *Bacillus* K 17

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高温, 알칼리성 *Bacillus* K17이 生成하는 Xylanase의 精製 및 特性

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The culture filtrate of thermophilic alkalophilic Bacillus K 17 strain contained two types of xylanases were purified by ammonium sulfate fractionation, DEAD-Sephadex A-50 column chromatography, CM-Sephadex C-50 column chromatography and Sephadex G-100 gel filtration. The purified enzymes were found to be homogeneous by sodium dodecyl sulfate and disc polyacrylamide gel electrophoresis. Xylanase I and II were characterized with respect to molecular weight, optimal temperature and pH, thermal and pH stability, and Michaelis constant. Xylanase II was more active and stable, and showed greater substrate affinity and molecular weight than xylanase I. The activities of xylanases I and II were inhibited by Cu^{++}, Ag^{+}, Hg^{++} and Fe^{++}. Xylanase I hydrolyzed xylan to yield xylobiose and higher amount of xylooligosaccharides, but xylanase II produced xylose other than xylobiose and xylooligosacchrides.

Xylan, a polymer of D-xylose linked by β-1.4-xylosidic linkage, is one of the commonest materials in agricultural wastes, accounting for 30% of the dry weight in some case. Xylan can be degraded to xylose by the sequential action of xylanase (1.4-β-D-xylan xylanohydroase; EC 3.2.1.8) and β-xylosidase (1.4-β-D-xylan xylohydrolase; EC 3.2.1.37). The xylanases produced by many microorganisms have been purifed to homogeneity and their properties were examined.[1-7] In the provious study,[8] we isolated five alkalophilic and thermophilic *Bacillus* that produced thermostable and alkalostable xylanase under alkaline conditions at high temperature. In subsequent investiagations on the purification of the enzymes, we have found that the xylanases from the strain K17, selected as the potent producer of xylanase among the isolates, was composed of two components. This paper deals with the purification and characterization of two xylanase from the strain K17.

Key word: Xylanase

Materials and Methods

Microorganism and cultivation

Thermophilic alkalophilic *Bacillus* K17 strain, isolated in

the previous study,[8] was used. For xylanase production, the strain was cultivated at 45°C for 48hr using an alkaline medium consisting of : wheat bran 10.0g, yeast extract 5.0g, polypeptone 5.0g, K_2HPO_4 1.0g, $MgSO_4$ $7H_2O$ 0.2g and Na_2CO_3 10.0g in 1liter of water. Sodium carbonate was sterilized separately and added to the medium.

Xylanase assay

Xylanase activity was determined by measuring the reducing sugar liberated from larchwood xylan[8] as the substrate. The reaction mixture, consisting of 0.5ml of 0.5% xylan solution in 50mM phosphate buffer (pH 6.5) and 0.05ml of appropriately diluted enzyme solution, was incubated at 60°C for 10min.

The reducing sugar was measured by the 3.5-dinitrosalicylic acid method.[9] One unit of xylanase was difined as the amount of enzyme that liberated 1 μmole of xylose in one minute.

Protein measurement

Protein concentration was measured by the method of Lowry et al[10] with bovine serum albumin as the standard.

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in a 7.5% acrylamide gel by the method of Davis.[11] Weber and Osborn's method[12] of sodium dodecyl sulfate (SDS) polyacrylamide disc gel electrophoresis was used for the molecular weight determination of the enzyme protein. Bovine albumin (molecular weight 66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), trypsin inhibitor (20,100) and α-lactoalbumin (14,200) were used as the molecular weight marker proteins. Protein staining was performed with Amidoblack 10B.

Thin layer chromatography of xylan hydrolyzates

Xylan was hydrolyzed with the xylanases according to the method described in the previous study.[8] The hydrolyzates were chromatographed on the silica gel plate with a solvent system of n-butanol-acetic acid-water (6:4:3 by volume) and detected by 2N H_2SO_4 containing 0.1% orcinol.

Enzyme purification

All purification steps were carried out at 4°C

Step 1. Ammonium sulfate fractionation.
The culture broth was centrifuged at 8,000 rpm for 15 min to remove cells and wheat bran debris. To the supernatant, ammonium sulfate was added to 80% saturation. After standing for one day, the resulting precipitate was collected by centrifugation at 10,000 rpm, dissolved in 50 mM phosphate buffer (pH. 6.5) and dialyzed for 48hr against several changes of the same buffer. The resultant insoluble materials were removed by centrifugation.

Step. 2. DEAE-Sephadex A-50 chromatography.
The dialyzed enzyme solution was charged onto a DEAD-Sephadex-A50 column (2.2 × 80cm) equilibrated with 50 mM phosphate buffer (pH 6.5). The column was washed with the same buffer until component I had been completely eluted. Component II was eluted with a linear gradient of 0 to 0.5M Nacl in 50 mM phosphate buffer (pH 6.5).

Step 3. CM-Sephadex C-50 chromatography.
The pooled nonadsorbed active fraction (component I) in step 2 was applied to a CM-Sephadex C-50 column (2.2 × 60cm) equilibrated with the 50 mM phosphate buffer. The column was washed with a linear gradient of 0 to 0.3 M Nacl in the 50mM phosphate buffer.

Step 4. Gel filtration.
Components I and II were purified by gel filtration on Sephadex G-100 column (2.2. × 40 cm) with 50 mM phosphate buffer.

Chemicals

Xylooligosaccharides were donated by Dr. T. Akiba of the Institute of Physical and Chemical Research, Japan, and molecular weight marker proteins and larchwood xylan were purchased from Sigma Chemical Co. (St. Louis USA). DEAD-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-100 were purchased from Parmacia Fine Co. (Sweden). Other chemicals used were of analytical grade and obtained commercially.

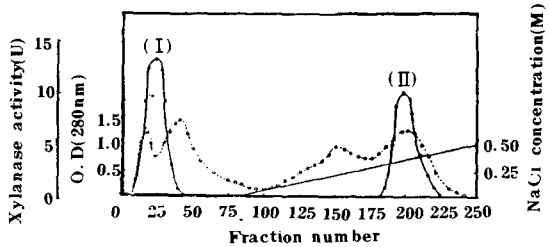

Fig. 1. Column chromatogram of xylanases from strain K17 on DEAE-Sephadex A-50.

Elution was carried out with 50 mM phosphate buffer (pH 6.5) for xylanase peak I and with a linear gradient of NaCl in the same buffer for xylanase peak II.
Column dimension was 2.2×80 cm and flow rate was 20ml/hr with 5ml/tube fraction.
○---○ ; O. D (280 nm), ●—● ; xylanase, — ; NaCl concentration.

Table 1. Summary of the purification procedure of xylanase I and xylanase II from strain K 17.

Purification step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
1. Culture fluid	1,800	12,240	2,114	5.8	100.0
2. $(NH_4)_2SO_4$ precipitation	200	8,400	476	17.6	68.6
3. DEAE-Sephadex					
Xylanase I	150	5,700	198	29.0	46.6
Xylanase II	150	4,050	167	24.3	33.1
4. CM-Sephadex					
Xylanase I	120	4,080	25	163.0	33.3
5. Sephadex G-100					
Xylanase I	80	1,364	6	227.0	11.1
Xylase II	80	1,016	9	113.0	8.3

Fig. 2. Polyacrylamide disc gel electrophoresis of the purified xylanases I and II from strain K17.

A: xylanase I
B: xylanase II

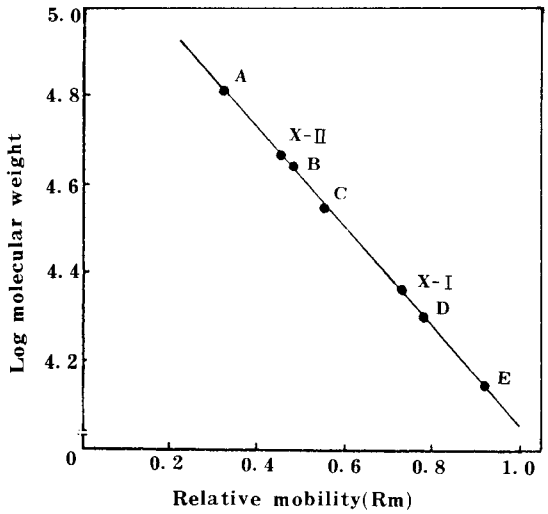

Fig. 3. Determination of the molecular weight of the purified xylanases I and II from strain K 17 by SDS-polyacrylamide gel electrophoresis.

A: bovine albumin (M. W 66,000), B: egg albumin (M. W 45,000), C: glyceraldehyde-3-phosphate dehydrogenase (M. W 36,000), D: trypsin inhibitor (M. W 20,100) E: lactoalbumin (M. W 14,200), X-I: xylanase I, X-II: xylanase II.

Results

Purification of xylanase

The xylanases from K17 strain were separated into two active component (xylanase I and xylanase II) on DEAD-Sephadex A-50 as shown in Fig. 1.

Xylanase I was eluted at the void volumn without adsorption on the DEAE-Sephadex A-50 column, whereas xylanase II was adsorbed and eluted at a concentration of about 0.35 M NaCl. The enzyme purification is summarized in Table 1. Xylanase I and II were purified 30-and 20-fold in specific activity with yield of 11.1% and 8.3%, respectively.

The homogeneity of the purified xylanases was examined by disc and SDS polyacrylamide gel electrophoresis. Fig. 2 shows the polyacrylamide disc gel electrophoretic patterns of the two enzymes.

Molecular weight determination.

Relative electrophoretic mobilities of the purified xylanases and of the reference proteins on SDS polyacrylamide gel electrophoresis were plotted versus their molecular weights. The mobility of the purified xylanase I corresponded to a molecular weight of 23,000, and that of the purified xylanase II to molecular weight of 48,000 (Fig. 3).

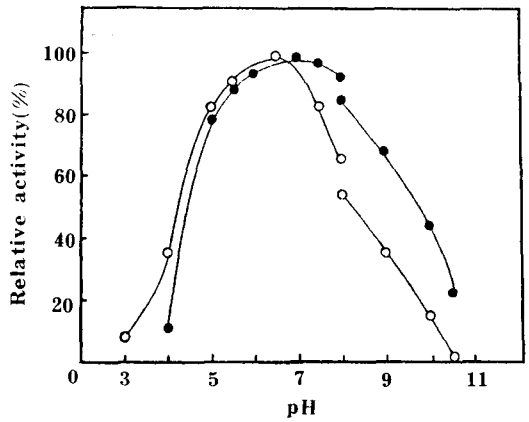

Fig. 4. Optimal pH of purified xylanases I and II from strain K17.

○—○;xylanase I, ●—●;xylanase II

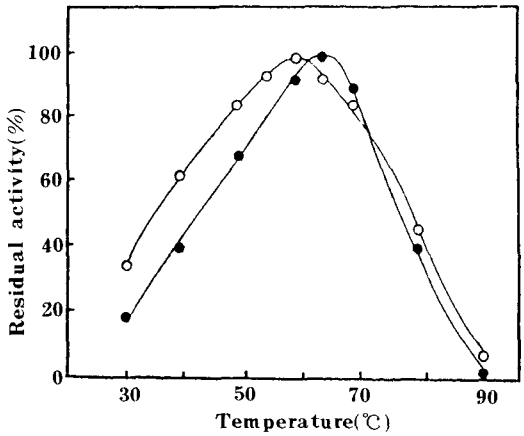

Fig. 6. Optimal temperature of purified xylanases I and II from strain K17.

○—○;xylanase I, ●—●;xylanase II

Effect of pH

Optimum pH The activities of kylanase I and II were measured at various pHs in McIlvaine buffer (pH 3.0-pH 8.0) and McIlvaine-NaOH buffer (pH 8.0-pH 10.5).

Xylanase I had sharp pH activity curve with optimal ativity at pH 6.5. On the other hand xylanase II had broad pH curves with relatively high activities in the alkaline region (Fig. 4).

pH stability Xylanases I and II were incubated at various pHs in McIlvaine and McIlvaine-NaoH buffer for 30 min at 45°C and the remaining activities were assayed. Xylanaes I was stable at pH 5.0 to 9.0, while xylanase II was stable at pH 60. to 10.0. The remaining activity at pH 11.0 of xylanase II was 60% and that of xylanase I was absent (Fig. 5).

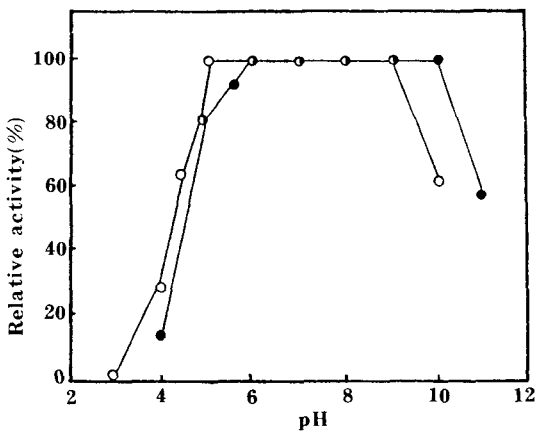

Fig. 5. pH stability of purified xylanases I and II from strain K17.

○—○;xylanase I, ●—●;xylanase II

Effect of temperature

Optimum temperature Fig. 6 shows the effect of temperature on the enzyme activities. The optimum temperature of xylanase I was 60°C and that of xylanase II was 65°C.

Thermal stability The thermal stabilities of xylanses I and xylanase II were examined by incubation in McIlvain buffer (pH 6.5) at various temperatures. The residual activities were measured at the interval of 10min. Xylanase I and II were stable up to 60°C for 60 min. The thermal stability of xylanase II was higher than that of xylanase I (Fig. 7).

Effect of various compounds

After the enzyme solutions were mixed with 50 mM phosphat buffer (pH 6.5) containing the test reagents at the final concentration of 5 mM and preincubated at 30°C for 30 min. The residual activities were measured. As shown in

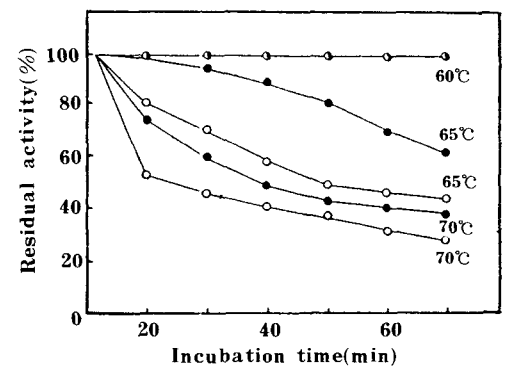

Fig. 7. Thermal stability of purified xylanases I and II from strain K17.

○—○; xylanase I, ●—●; xylanase II

Table 2. Effect of various compounds on the activity of purified xylanases I and II from strain K 17.

Compound (5 mM)	Xylanase I Activity (U)	Xylanase I Relative activity (%)	Xylanase II Activity (U)	Xylanase II Relative activity (%)
Control	3.83	100.00	2.17	100.00
$MnSO_4 \cdot 4H_2O$	3.74	97.65	1.82	83.87
ICH_2COOH	3.83	100.00	2.09	96.31
EDTA*·$2H_2O$	3.45	90.07	1.62	74.65
*p*CMB**	3.37	87.98	2.00	92.17
$CoCl_2$	3.34	87.21	1.68	77.42
$CuCl_2 \cdot 2H_2O$	2.21	57.70	0.98	45.16
$AgNO_3$	1.44	37.59	2.03	93.55
$FeSO_4 \cdot 7H_2O$	1.53	39.94	0.83	38.25
$HgCl_2$	0	0	0	0

*Ethylenediaminetetraacetic acid disodium salt.
*Sodium *p*-chloromercuric benzonate.

Table 2, Hg^{++} Ag^{+}, Cu^{++} and Fe^{++} showed inhibitory effect on the enzyme activites.

Kinetic constant

Activities of xylanases I and II were measured in McIlvaine buffer at pH 6.5 with various xylan concentrations at 60°C for 10 min. The Michaelis constants were estimated from Lineweaver-Burk plots.[13] The results are shown in Fig. 8 and 9. The *Km* values of xylanase I and II were quite different: the *Km* value of xylanase I was 4.67 mg-xylan/ml,

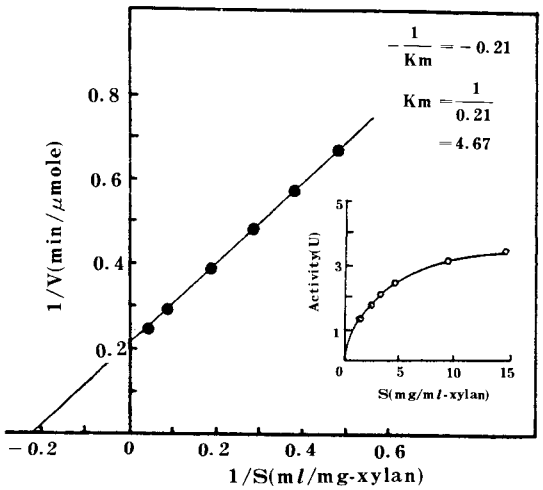

Fig. 8. Lineweaver-Burk plot of xylanase I from strain K 17.

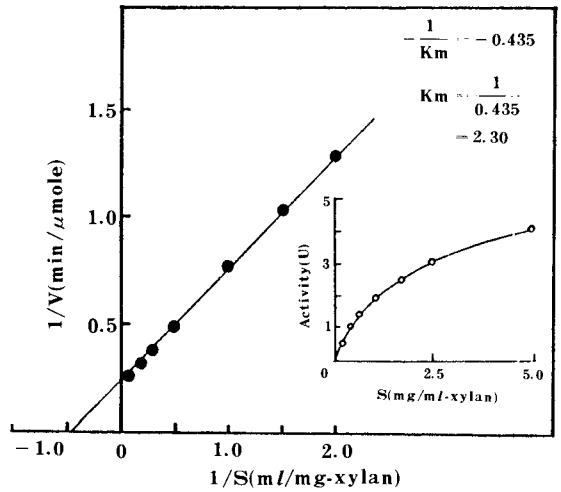

Fig. 9. Lineweaver-Burk plot of xylanase II from strain K17.

and that of xylanase II was 2.30 mg-xylan/ml.

Thin layer chromatography of xylan hydrolyzates

To 1 ml of 1% xylan solution was added 0.1 ml(5 units) each of the two enzymes and the mixture was incubated at 60°C for 24 hr. The reaction mixtures were chromatographed on the silica gel plate. The results are shown in Fig. 10. With xylanase I, the final products of hydrolysis were xylobiose, xylotriose, and xylotetraose, but xylose was not produced. This result suggests that xylanase I is a typical endo-xylanase. On the other hand, with xylanase II, the final products were xylose, xylobiose and xylotriose. The degree of hydrolysis by xylanase I and II were about 22% and 30%, respectively, at 24 hr of reaction time. The hydrolysis products with xylanase I and II were completly degraded to xylose by the addition of β-xylosidase (2.5 units).

Discussion

Xylanases from thermophilic alkalophilic *Bacillus* K17 strain were found to consist of two active components; namly xylanases I and II. Distinct differences in properties between xylanases I and II were observed in the molecular weight, optimal pH and temperature, *Km* values and the hydrolysis products: the molecule weight of xylanase I and II are 23,000 an 47,000; optimal pH values are 6.5 and 7.0; optimal temperature are 60°C and 65°C; *Km* values are 4.67 and 2.30 mg-xylan/ml; and hydrolysis products with xylanase I are xylobiose, xylotriose, xylotetraose and those with xylanase II are xylose, xylobiose, xylotriose, respectively.

Xylanase II is more active and stable in alkaline region

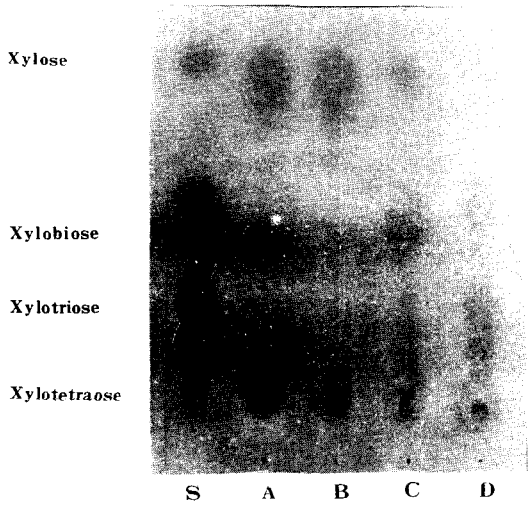

Fig. 10. Thin layer chromatogram of the xylan hydrolyzate by purified xylanase Ⅰ, Ⅱ and β-xylosidase from strain K17.

S; standard
A; hydrolysis products with xylanase Ⅱ + β-xylosidase
B; hydrolysis products with xylanase Ⅰ + β-xylosidase
C; hydrolysis products with xylanase Ⅱ
D; hydrolysis products with xylanase Ⅰ

than xylanase I. The optimal pH values of xylanase I and II (pH 6.5 and 7.0 respectively) were similar to those of other alkalophilic xylanases produced by *Bacillus* strains.[14, 15] Xylanase II was more active and stable at the relatively higher temperature than xylanase I. *Bacillus circulens* WL-12 strain[16] was reported to produce xylanase A and B with the *Km* values of 8 mg xylan/ml and 4 mg xylan/ml, respectively. The *Km* values of the xylanases obtained in this laboratory showed to be the same order with those values.

In the previous report,[8] we reported that hydrolysis products with crude xylanase were xylose and xylobiose, but in this work the hydrolysis with the mixture of xylanases I and II were found to yield xylotriose and xylotetraose other than xylose and xylobiose (data not shown). The difference in the hydrolysis products between crude enzyme and the purified xylanase may be due to the presense of ß-xylosidase in the crude enzyme solution. Lately, we also purified the extracellular ß-xylosidase from *Bacillus* K17 strain, which will be reported elsewhere.

Some xylanases produced by other rmicroorganisms have reported to show multienzyme system.[16] We found that thermophilic alkalophilic *Bacillus* K17 strain produced two types of xylanase in an alkaline medium. There are several possible mechanisms by which the xylanases are produced in the cells of *Bacillus* K17 strain. 1) the two xylanases are directly produced by two independent genes of *Bacillus* K17 strain, 2) Proteolytic enzymes process the enzyme molecules during cultivation. To clarify this cloning of xylanase genes of *Bacillus* K17 will be performed in an *E. coli* system.

요 약

고온, 알칼리성 *Bacillus* K17이 생성하는 extracellular xylanase를 각종 resin으로 정제하여 비흡착 분획에서 xylanase Ⅰ과 흡착 분획에서 xylanase Ⅱ를 분리정제하였고 SDS-Polyacrylamide gel electrophoresis에 의하여 분자량을 측정하였던바 xylanase Ⅰ은 23,000, xylanase Ⅱ는 47,000으로 추정되었다. Xylanase Ⅰ 및 Ⅱ는 최적온도, 최적 pH, 열 안정성, pH 안정성, 기질 친화력에서 차이가 있었으며 Cu^{++}, Ag^{+}, Fe^{++}, Hg^{++}에 의해 다같이 저해되었다. Xylanase Ⅰ은 xylan을 Xylobiose, xylotriose, xylotetraose로 분해시키며 그 분해율이 22%로 나타났다. 그리고 xylanase Ⅱ는 xylose, xylobiose, xylotriose로 분해시키며 그 분해율이 30%이었다.

酵素的 分析은 효소가 갖는 基質特異性으로 인해 多成分系의 분석재료로부터 측정한 성분만을 정량가능하므로 生体成分의 분석에 널리 활용되고 있다.

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