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# Purification and Characterization of a Xylanase from *Bacillus* sp. KK-1

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**Abstract:** A thermophilic *Bacillus* sp. strain KK-1 isolated from soil produced an extracellular xylanase. From the culture supernatant of *Bacillus* sp., the xylanase was purified to homogeneity by ammonium sulfate precipitation and DEAE-Sephadex A-50 chromatography. The molecular weight of the purified xylanase was estimated to be 45 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel permeation chromatography. The apparent $K_m$ values for xylanase, using oat spelt xylan and birchwood xylan as substrates, were 7.1 mg/ml and 3.2 mg/ml, and $V_{max}$ values were 27.0 μmol·min$^{-1}$·mg$^{-1}$ and 29.0 μmol·min$^{-1}$·mg$^{-1}$, respectively. The xylanase hydrolyzed oat spelt xylan to mostly xylobiose, xylotriose, and xylose. The amino acid composition indicated that the xylanase contained high amounts of amino acid residues of glutamic acid and glutamine (Glx) and aspartic acid and asparagine (Asx).

**Key words:** cellulase-free xylanase, thermophilic *Bacillus* sp..

Plant cell walls consist of three major polymeric constituents: cellulose, hemicellulose and lignin. As a major part of hemicellulose, xylan is composed of β-1,4-linked D-xylose units with various branches including the acetyl group, L-arabinofuranose and D-glucuronopyranose (Biely, 1985, Thomson, 1993). The enzymes that degrade xylan include xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), α-L-arabinofuranosidase and acetylxylan esterase (Greve *et al.*, 1984; Lee *et al.*, 1987). By the cooperative action of these enzymes, microorganisms can effectively use xylan as a carbon source (Deshpande *et al.*, 1986; Bachmann and McCarthy, 1991). Xylanase, which attacks internal xylosidic linkages on the xylan backbone to produce xylooligosaccharides, is the most important of these enzymes. The potential applications of xylanases include bioconversion of lignocellulose to fermentative products, clarification of juices, and improving the digestibility of animal feedstock (Wong *et al.*, 1988).

Recently, in connection with the problem of environmental pollution resulting from the bleaching process in the pulp industry, a new application of xylanase has appeared (Daneault *et al.*, 1994; Senior *et al.*, 1992). The removal of xylan and the xylan-lignin complex of pulp by xylanase improves the extractability of lignin to obtain higher degrees of brightness, and reduces the consumption of chemicals used in the bleaching process.

Xylanases with thermostable and alkalo-tolerant properties are needed for application in the pulp industry because the bio-bleaching involves processes of both high temperature and basic pH. Consequently, several thermostable and alkaline xylanases have been isolated and characterized from various microorganisms. Recently, Nakamura *et al.* (1993) reported the purfication of the alkaline xylanase of *Bacillus* sp. 45M-1. The xylanase has no remarkable cellulase activity. A *Bacillus stearothermophilus* (Khasin *et al.*, 1993) produced the thermostable xylanase T-6, which is stable for more than 10 h at 65℃ and pH 7.0. In addition, the xylanase T-6, which displays very low activity on carboxymethyl cellulose, has been investigated for the bio-bleaching of paper.

Recently, we have screened a thermophilic *Bacillus* sp. strain KK-1 which produces a thermostable cellulase-free xylanase (Kim *et al.*, 1994) comparable with that of *B. stearothermophilus*. In this paper, the purification and characterization of xylanase from thermophilic *Bacillus* sp. KK-1 are described.

## Materials and Methods

### Organism and culture conditions

The microorganism used in this study was *Bacillus*

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sp. KK-1 isolated from soil as described previously (Kim *et al.*, 1994). For production of the extracellular xylanase, 1 l of the complex medium (yeast extract, 5 g; bacto-tryptone, 5 g; beef extract, 5 g; polypeptone, 5 g; NaCl, 2 g; $K_2HPO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; water 1 l; pH 7.0) was inoculated with 10 ml of bacterial culture in logarithmic growth phase and cultured at 55℃ with shaking for 20 to 24 h.

**Purification of xylanase**

The culture supernatant (500 ml) was precipitated with 60% saturated $(NH_4)_2SO_4$ and the precipitate was dissolved in 40 ml. Then, the concentrate was dialyzed against 20 mM Tris·Cl (pH 8.3) for 24 h with several changes of buffer. The dialyzed solution (40 ml) was loaded into a DEAE- Sephadex A-50 column (2.0 by 15.0 cm) which had been equilibrated with 20 mM Tris·Cl (pH 8.3). The column was washed with 75 ml of additional equilibration buffer to remove unabsorbed protein. Elution was then carried out at a flow rate of 16 ml/h, with a linear gradient from 0.0 to 0.1 M NaCl in the same buffer. Fractions were collected every 4.0 ml and assayed for xylanase activity. Active fractions showing xylanase activity were pooled and dialyzed against 10 mM Na-phosphate buffer (pH 7.0).

**Xylanase assay**

Xylanase activity was measured by the 3,5-dinitrosalicylic acid method using xylan as a substrate (Miller, 1959). 0.1 ml of an appropriate dilution of the enzyme was mixed with 0.5 ml of 1.0% (w/v) oat spelt xylan suspended in distilled water and 0.4 ml of 100 mM Na-phosphate buffer (pH 7.0). The reaction mixture was incubated for 20 min at 65℃. The reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid reagent followed by immersion for 5 min in boiling water. The reducing sugar liberated was determined by measuring the absorbance at 540 nm, using D-xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme which released reducing sugars equivalent to 1 μmol of xylose per min.

**SDS-polyacrylamide gel electrophoresis**

By the method of Laemmli, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Laemmli, 1970). The protein samples, which included 12 mM Tris·Cl (pH 6.8), 5.0% glycerol, 0.4% SDS, 2.8 mM 2-mercaptoethanol, 0.2% bromophenol blue were boiled at 95℃ for 5 min and then loaded on to a 10% gel. The low-molecular weight calibration kit (Pharmacia, Uppsala, Sweden) was used for standard markers of molecular mass.

**Zymogram analysis**

For detecting and localizing the xylanase activity on SDS-polyacrylamide gel, an agar replica was prepared with 1.0% agarose, 0.5% xylan and 50 mM Na-phosphate (pH 7.0). After SDS-PAGE of xylanase, the gel was treated with 2.5% Nonidet P-40 to remove SDS and then washed several times with distilled water. The gel was then placed on the agar replica and incubated at 50℃ for 30 min. The agar replica was soaked in 0.2% Congo red solution for 1 h and washed with 1 M NaCl solution for 2 h at room temperature. The clear zones of the xylanase activity were visualized by soaking the agar replica in 0.5% acetic acid solution.

**Physicochemical analysis of enzyme**

Fast protein liquid chromatography (FPLC) was performed to determine the molecular weight of the native xylanase with a Superose 6 H/R 10/30 column (Phamacia). The buffer used was composed of 0.15 M NaCl and 0.05 M phosphate (pH 7.0). The flow rate was 0.4 ml/min and 1 ml fractions were collected. Thyroglobulin, ferritin, bovine serum albumin, ribonuclease A and glycyltyrosine were used as standards.

Amino acid analyses were performed on the amino acid analyzer (Beckmann Model 6300) according to the method of Spackman *et al.* (1958). Samples (5~10 nmol) were hydrolyzed in sealed, evacuated test tubes with 200 μl of twice-distilled HCl for 24 h at 110℃.

**Analysis of hydrolysis products**

The reaction mixture containing 2.0% (w/v) oat spelt xylan and 50 U of enzyme in 50 mM Na-phosphate buffer (pH 7.0) was incubated for 24 h at 65℃. Aliquots were periodically withdrawn and analyzed by high performance liquid chromatography (HPLC) using a carbohydrate analysis column (3.9 by 300 mm, Waters) at room temperature. The flow rate was 1.2 ml/min and the eluent was water. 10 μl samples from reaction mixtures were filtered and injected onto the column. Xylose, xylobiose, xylotriose, xylotetraose and xylopentose were used as standards.

## Results and Discussion

**Purification of xylanase**

The xylanase produced by *Bacillus* sp. strain KK-1 was fractionated by precipitaion with 60% saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 20 mM Tris·Cl (pH 8.3), and dialyzed. The dialyzed enzyme solution, which contained about 209 U of xylanase with a purification factor of

about 5.5, was applied to a column of DEAE-Sephadex A-50. Most of the xylanase was absorbed on the resin and eluted from the column at the gradient of approximately 30 mM NaCl (Fig. 1). The fractions showing the xylanase activity were pooled and dialyzed. When it was analyzed by SDS-PAGE, the pooled fraction gave a single band on the gel (Fig. 2B). Consequently, the xylanase of strain KK-1 was purified in a single step on the basis of the high selectivity of anion exchange chromatography. Typical results of this single-step purification are shown in Table 1. The total yield of xylanase was 13.1% with a purification factor of 66.0 with respect to the culture supernatant.

When zymogram analysis was performed to detect the xylanase activity, it was found that the active band of xylanase comigrated with the protein band by SDS-PAGE (Fig. 2A). Especially, zymogram analysis showed that both the culture supernatant of the organism and the ammonium sulfate precipitate had only one active band, corresponding to that of the purified xylanase. It has been reported that many microorganisms produced multiple xylanases to degrade xylan effectively (Wong *et al.*, 1988). It is worthwhile to note that *Bacillus* sp. strain KK-1 produces only one kind of xylanase.

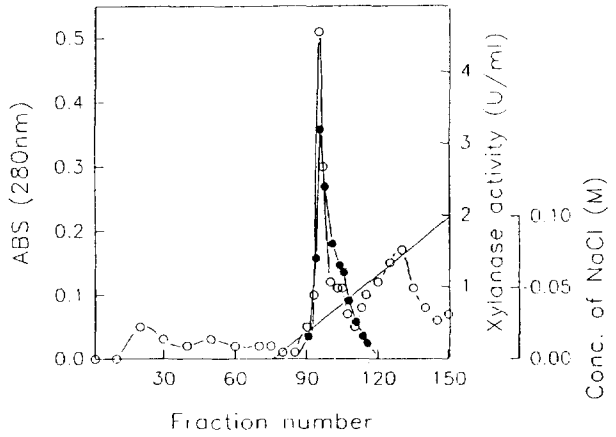


**Fig. 1.** Ion exchange chromatography of xylanase on DEAE-Sephadex A-50 column (2.0 by 15 cm). Elution was carried out with a linear gradient from 0.0 to 0.1 M NaCl in 20 mM Tris·Cl, pH 8.3. Fractions of 4 ml were collected and the flow rate was 16 ml/h. Absorbance at 280 nm (○–○), xylanase activity (●–●).

**Physicochemical properties of xylanase**

The molecular weight of the purified xylanase was 45 kDa by SDS-PAGE (Fig. 2B). The molecular weight of the native xylanase was also estimated to be 45 kDa by gel permeation of FPLC (Fig. 3). Therefore, it was confirmed that the xylanase of *Bacillus* sp. strain KK-1 was a single polypeptide in accord with most extracellular enzymes.

The amino acid composition of xylanase is shown Table 2. The enzyme had higher molar ratios of the charged residues of Asx and Glx, and lower molar ratios of threonine, serine, histidine and methionine residues. The cysteine residue could not be detected. The xylanase of strain KK-1 was identified to be comparable with that of *B. stearothermophilus* T-6 on the basis of amino acid composition.

In addition, both xylanases had similar molecular weights (45 kDa for this xylanase, 43 kDa for xylanase T-6) and showed highly similar characteristics in many aspects including the temperature and pH profiles of enzymatic reactions, thermostability and their reaction products (Khasin *et al.*, 1993; Kim *et al.*, 1994). The two xylanases, however, revealed apparent differences with respect to the effect of various metal ions. When 1 mM $HgCl_2$ was added, the residual activities for this xylanase and xylanase T-6 were 0.0% and 17.0%, re-

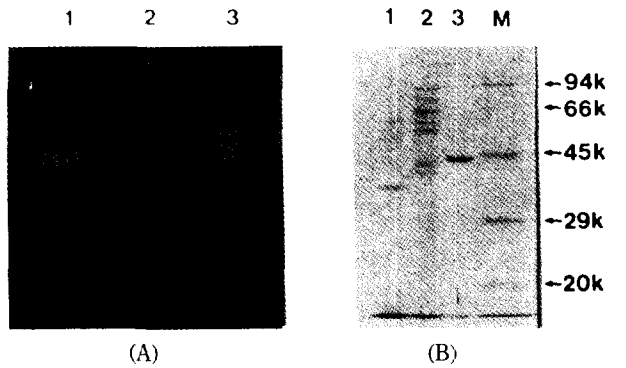


**Fig. 2.** SDS-polyacrylamide gel electrophoresis of the xylanase during purification steps. (A) Zymogram for detecting the xylanase activity, (B) Coomassie brilliant blue R250 staining of the gel. Lanes 1 : culture supernatant; lanes 2 : precipitate with 60% saturated $(NH_4)_2SO_4$; lanes 3 : purified xylanase from DEAE-Sephadex A-50 column. A mixture of standard protein subunits was run in lane M. The molecular size was indicated to the right side of the gel.

**Table 1.** Summary of the purification of xylanase

| Purification step | Vol (ml) | Activity (U) | Protein (mg) | Specific activity (U/mg) | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Culture supernatant | 500 | 583 | 3000 | 0.2 | 1.0 | 100 |
| 60% $(NH_4)_2SO_4$ fractionation | 40 | 209 | 191 | 1.1 | 5.5 | 35.9 |
| DEAE-Sephadex chromatography | 40 | 76.4 | 5.8 | 13.2 | 66.0 | 13.1 |

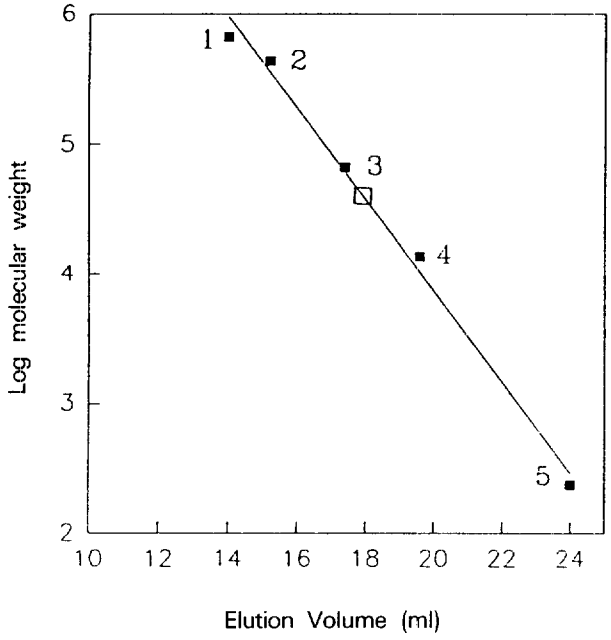


**Fig. 3.** Determination of the molecular weight of the native xylanase by gel filtration chromatography. The molecular size marker proteins are as follows: 1, thyroglobulin ($M_r$ 669,000); 2, ferritin ($M_r$ 440,000); 3, bovine serum albumin ($M_r$ 66,000); 4, ribonuclease A ($M_r$ 13,700); 5, glycyltyrosine ($M_r$ 238).

**Table 2.** Amino acid composition of xylanase

| Amino acid | Mol (%) |
|---|---|
| Aspartic acid (Asp+Asn) | 17.0 |
| Threonine | 3.0 |
| Serine | 2.0 |
| Glutamic acid (Glu+Gln) | 12.0 |
| Proline | 7.0 |
| Glycine | 6.0 |
| Alanine | 9.0 |
| Cysteine | 0.0 |
| Valine | 7.0 |
| Methionine | 1.0 |
| Isoleucine | 5.0 |
| Leucine | 6.0 |
| Tyrosine | 4.0 |
| Phenylalanine | 4.0 |
| Histidine | 2.0 |
| Lysine | 10.0 |
| Arginine | 4.0 |
| Tryptophan | ND |

ND: Not determined.

spectively. Also, the residual activities for this xylanase and xylanase T-6 were 0.0% and 104.0% by 1 mM $FeSO_4$, 90.0% and 38.0% by 1 mM $ZnCl_2$, and 121.0% and 54.0% by 1 mM $AlCl_3$, respectively. The analysis of the amino acid sequence of these enzymes will help to identify the residues of the active sites of the enzyme, the regions and residues responsible for effect of metal ions.

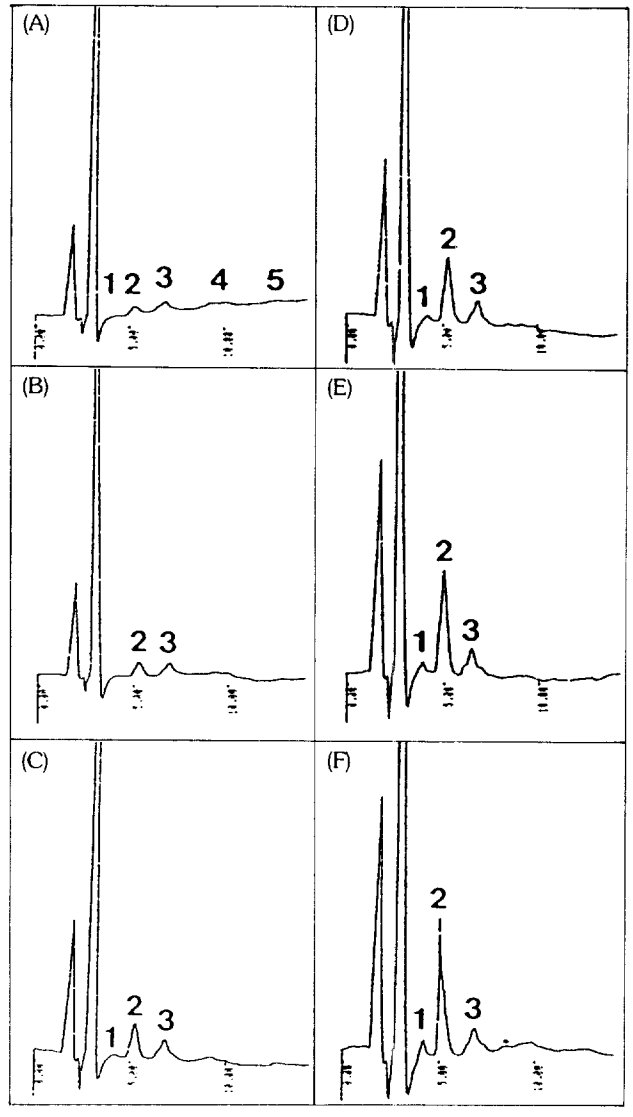


**Fig. 4.** HPLC analysis of xylan hydrolysis products by xylanase. Aliquots from reaction mixtures were taken after (A) 1 h, (B) 2 h, (C) 4 h, (D) 8 h, (E) 14 h and (F) 24 h and analyzed by HPLC. Symbols: 1, xylose; 2, xylobiose; 3, xylotriose; 4, xylotetraose; 5, xylopentose.

### Hydrolysis of xylan by xylanase

The hydrolysis products of oat spelt xylan by xylanase were analyzed by HPLC (Fig. 4). After incubation of 1 h, xylobiose and xylotriose were produced. Under prolonged incubation, the release of xylobiose increased. After incubation for 4 h, small amounts of xylose were detected and then increased with prolonged incubation. This indicates the xylanase is an endo-type xylanase that cleaves xylan at random.

While xylotriose was released as an initial product, its release showed little increase with time. In contrast,

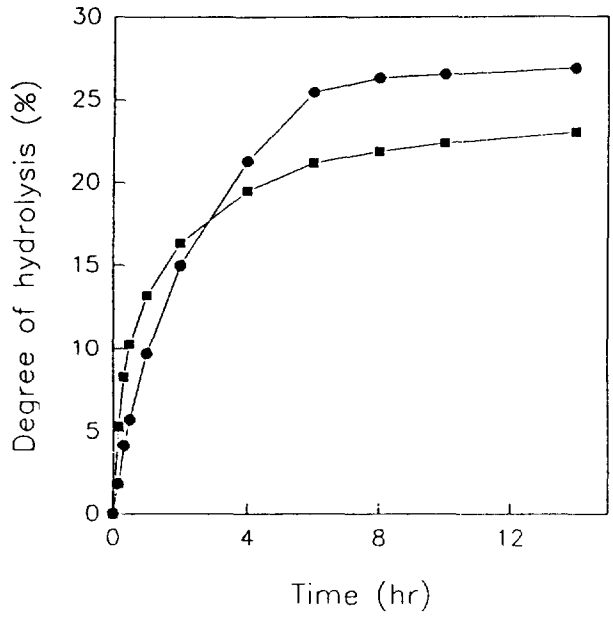


**Fig. 5.** Time course of hydrolysis of oat spelt xylan (■–■) and birchwood xylan (●–●) by xylanase.

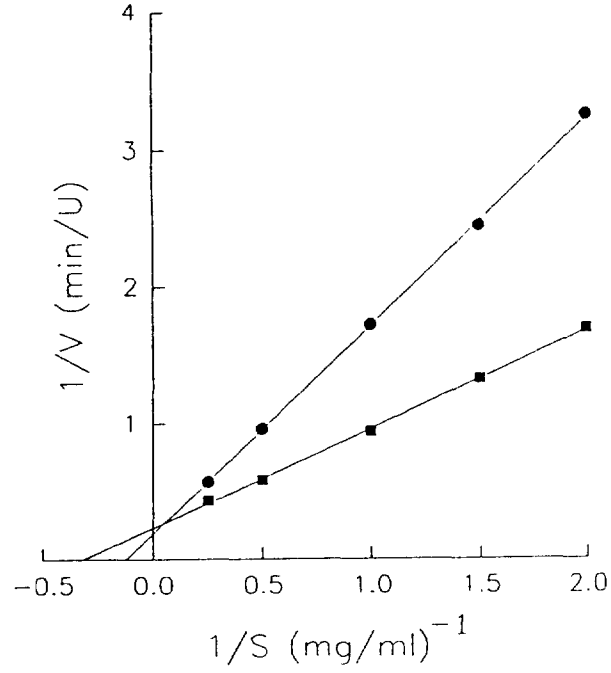


**Fig. 6.** Lineweaver-Burk plots of xylanase for oat spelt xylan (●–●) and birchwood xylan (■–■).

the release of xylose and xylobiose continuously increased with reaction time. Thus, it is assumed that xylotriose is converted to xylose and xylobiose by the enzymatic reaction. Consequently, the xylanase produced xylobiose, xylotriose and xylose as main products. The xylan-hydrolyzing pattern of the xylanase has also been observed to be similar to those of other xylanases (Takenishi and Tsujisaka, 1975; Brillouet, 1987; Morales *et al.*, 1993; Khasin *et al.*, 1993).

The time course of hydrolysis of oat spelt xylan and birchwood xylan by xylanase was shown in Fig. 5. The maximum degree of hydrolysis of oat spelt xylan and birchwood xylan were 23% and 26%, respectively.

### Kinetic parameters

The effect of substrate concentration on the activity of xylanase was determined and the rates were plotted as Lineweaver-Burk plots (Fig. 6). The apparent $K_m$ values for oat spelt xylan and birchwood xylan were 7.1 mg/ml and 3.2 mg/ml, respectively, indicating that the affinity of xylanase for birchwood xylan was twice as high as that for oat spelt xylan. The apparent $V_{max}$ values for oat spelt xylan and birchwood xylan were 27.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 29.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

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