Purification and Characterization of a Xylanase from Alkalophilic *Cephalosporium* sp. RYM-202

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Alkalophilic *Cephalosporium* sp. RYM-202 produced multiple xylanases extracellularly. One of these xylanases was purified to electrophoretical homogeneity by chromatography with DEAE-Sephadex A-50, Sephacryl S-200 HR and Superose 12 HR. The purified xylanase differed from most other microbial xylanases in that it had low-molecular weight and acidic isoelectric point. The molecular weight of the xylanase was 23 kDa by SDS-polyacrylamide electrophoresis and 24 kDa by gel permeation chromatography, and the isoelectric point was 4.3. The xylanase had the highest activity at pH 8.0 and 50°C. It was stable over a wide range of pH and retained more than 80% of its original activity after 24 h of incubation even at pH 12. The Km values of this enzyme on birchwood xylan and oat spelts xylan were 2.33 and 3.45 mg/ml, respectively. The complete inhibition of the enzyme by *n*-bromosuccinimide suggests the involvement of tryptophan in the active site. The xylanase lacked activity towards crystalline cellulose and carboxymethyl cellulose.

Key words: alkaline xylanase, alkalophilic *Cephalosporium* sp., enzyme purification, characterization

Xylan is one of the major components of hemicellulose present in plant cell wall. The hydrolysis of xylan involves xylanolytic enzyme systems that include β-1,4-endoxylanases (xylanases, 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 (8). Recently, the utilization of xylanases for the biopulping and biobleaching of kraft pulp has been considered as one of the most important new industrial application of enzymes (12, 21, 23). Since the pulp is normally processed at a highly alkaline pH, xylanases active at alkaline pH are preferable for pulp applications (19, 28).

Xylanases have been described in a wide variety of bacteria and fungi. However, the great majority of microbial xylanases usually exhibit acidic or neutral pH optima, which is inadequate for applications in pulp and paper industries. Some alkaline xylanases from several bacteria including *Bacillus* spp. (9, 19, 20) and *Streptomyces* sp. (22) have been purified and characterized, but there is a lack of published information relating to the purification of fungal xylanase that is active at alkaline pH.

Recently, we have reported the isolation of an alkalophilic fungal strain, *Cephalosporium* sp. RYM-202, that grows optimally at pH of 9.5-10.0 (10). This strain produced large amounts of multiple xylanases extracellularly and one major form of them exhibited high activity at alkaline pH. In this paper, we report the purification and characterization of the xylanase from *Cephalosporium* sp. RYM-202. Particularly, physicochemical properties such as molecular weight and isoelectric point of this enzyme are compared with those of xylanases from other microorganisms.

Materials and Methods

Microorganism and culture conditions

Alkalophilic *Cephalosporium* sp. RYM-202 was used in this study. The strain was isolated from the soil sample as described previously (10). Stock cultures were maintained at 4°C by periodical transfer on potato dextrose agar plates containing 0.5% sodium carbonate. For en-

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zyme preparation, batch cultures were carried out in a 5 L jar fermentor with a working volume of 2.5 L. The culture medium contained 2% wheat bran, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$. The initial pH of the medium was adjusted to 10.0 by adding 0.5% sodium carbonate. The inoculum of 1×10^{10} conidia was transferred to the medium and cultivated at 30℃ for 3 days. The air flow rate was 1.0 vvm and agitation speed was set at 400 rpm. Three batch-culture experiments were carried out to obtain a desired volume of cultures (7 L). After incubation, the culture broth was centrifuged at $10,000 \times g$ for 20 min and the clarified supernatant was used as the source of enzymes.

Enzyme assays

Xylanase activity was determined by measuring the reducing sugar by dinitrosalicylic acid method (17). 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 solution was incubated at 50℃ for 20 min. One unit of xylanase was defined as the amount of enzyme that liberated 1 μmol of xylose equivalent per min under the reaction conditions.

Purification of xylanase

Fractionation of the culture supernatant was carried out by salting out with ammonium sulfate (30~80%). The precipitate was then dissolved in 20 mM sodium phosphate buffer, pH 7.0 and dialyzed against the same buffer for 24 h with periodical change of the buffer. The resulting solution was concentrated 3-fold by using a 10,000-molecular weight cutoff membrane (Millipore). The concentrated enzyme solution was applied to a DEAE-Sephadex A-50 column (4.6×25 cm) previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with 400 ml of the buffer and then eluted with a linear gradient of KCl (0~0.6 M) in the same buffer. The fraction volume was 8 ml at a flow rate of 40 ml/h. In this study, the xylanase fractions that eluted at around 0.15 M KCl were pooled and concentrated by ultrafiltration through a Diaflo membrane PM 10. The concentrate was applied to a Sephacryl S-200 HR column (2.5 × 110 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and eluted with the same buffer. The active xylanase fractions from Sephacryl S-200 HR column were concentrated by ultrafiltration and subjected to fast protein liquid chromatography (FPLC) through a Superose 12 HR 10/30 column (Pharmacia). Eluent used was 20 mM sodium phosphate buffer, pH 7.0. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min, and those with high xylanase activity were pooled and concentrated. All purification procedures described above were carried out at 4℃.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (15) with bovine serum albumin as a standard or by measuring absorbance at 280 nm.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (13) with the acrylamide concentration of 11% (w/v). Protein bands in the gels were visualized by Coomassie brilliant blue R-250 staining. Molecular weight markers used were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20.1 kDa).

Isoelectric focusing and zymogram analysis

Analytical isoelectric focusing (IEF) was performed on PhastGel slab (pH 3-9, Pharmacia) using a PhastSystem (Pharmacia-LKB). IEF markers used were amyloglucosidase (3.6), soybean trypsin inhibitor (4.6), β-lactoglobulin A (5.1), bovine carbonic anhydrase II (5.4), bovine carbonic anhydrase II (5.9), and human carbonic anhydrase I (6.6). After focusing had been performed, proteins were detected by Coomassie brilliant blue R-250 staining and xylanase activity was detected by zymogram analysis (3). For zymogram analysis an agar replica gel containing 0.2% (W/V) xylan and 3% agar (Difco) in 50 mM sodium phosphate buffer (pH 7.0) was laid on the PhastGel slab. After incubation at 50℃ for 1 h, the replica gel was cooled at 4℃ for 10 min and then stained with 0.2% (W/V) congo red solution with gentle agitation for 2 h. The replica gel was washed with 1 M NaCl solution and then treated with 5% (V/V) acetic acid for clear observations.

Kinetic parameters

For the determination of K_m and V_{max} values of the purified xylanase for three types of substrates (birchwood xylan, larchwood xylan, and oat spelts xylan), the enzyme was incubated with different amounts of xylans (0.3~5.0 mg/ml) under the assay conditions described above. The K_m and V_{max} values were determined from Lineweaver-Burk plots.

Amino acid analysis

Amino acid analysis of the purified xylanase was done

on a Hitachi L-8500 amino acid analyzer equipped with a D-2850 chromato-integrator after hydrolysis in a sealed test tubes at 110℃ for 24 and 72 h with 6 N HCl.

Results and Discussion

Purification of xylanase

As previously noted with many highly xylanolytic microorganisms, *Cephalosporium* sp. RYM-202 produced multiple xylanases extracellularly when cultured in a submerged culture medium containing 2% wheat bran as a carbon source. The ion-exchange chromatography on DEAE-Sephadex A-50 revealed that the culture supernatant of the organism contained at least three major xylanases and several minor ones (data not shown). In this study, one main xylanase exibiting high activity at alkaline pH was purified by a series of chromatography techniques. A quantitative evaluation of the results obtained from the consecutive purification steps is given in Table 1. The specific activity of the purified xylanase was increased 24-fold over that of the culture supernatant with a 6.3% recovery of activity. The purified xylanase gave a single band on SDS-PAGE (Fig. 1), suggesting the homogeneity of the enzyme.

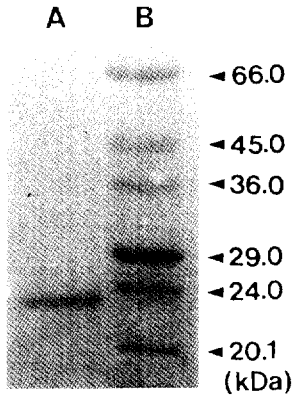

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified xylanase. Lane A, xylanase; lane B, molecular weight marker proteins.

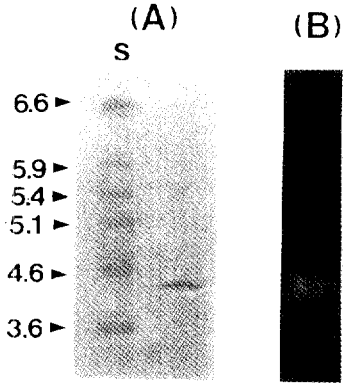

Fig. 2. Isoelectric focusing of purified xylanase using precast gels with pHs of 3 to 9. After focusing had been performed, gel plates were subjected to Coomassie brilliant blue staining (A) or xylanase zymogram analysis (B). S, IEF markers.

Physicochemical properties

The molecular weight of the purified xylanase was approximately 23 kDa as determined by SDS-PAGE. By gel permeation chromatography on a Superose 12 HR 10/30 column, the corresponding native molecular weight was estimated to be 24 kDa. These results indicate that the enzyme is monomeric, which is one of the known characteristics of xylanases (14, 31). The pI of the xylanase was determined to be 4.3 by IEF gels (Fig. 2). The molecular weight and pI of this xylanase was compared with those values reported for other microbial xylanases. Xylanases from *Bacillus* spp. can be divided into two major groups on the basis of their molecular weights and pI values (18, 31). One is basic (pI, 8.3 to 10.0) with a low-molecular weight (16 to 22 kDa), and the other is acidic (pI, 3.4 to 4.5) with a high-molecular weight (43 to 50 kDa). Earlier studies have shown that these groupings apply well in xylanases produced by other bacteria including *Streptomyces* spp. (5, 22, 26) and fungi, such as *Aspergillus niger* (7) and *Trichoderma* spp. (11, 24, 25, 27), in which low-molecular weight basic xylanases are relatively more common. In this study, however, the purified xylanase which had the apparent low-molecular weight with acidic pI value did not belong to either of the above two groups.

Table 1. Purification of xylanase from *Cephalosporium* RYM-202.

Purification steps	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (fold	Yield (%)
Culture supernatant	43,120	5,544	7.8	1	100
$(NH_4)_2SO_4$ fractionation	31,150	2,078	15.0	1.9	72
DEAE-Sephadex A-50	3,568	163	22.0	2.8	8.3
Sephacryl S-200 HR	3,153	41.0	76.9	9.8	7.3
Superose 12 HR 10/30	2,750	14.8	186.0	23.9	6.3

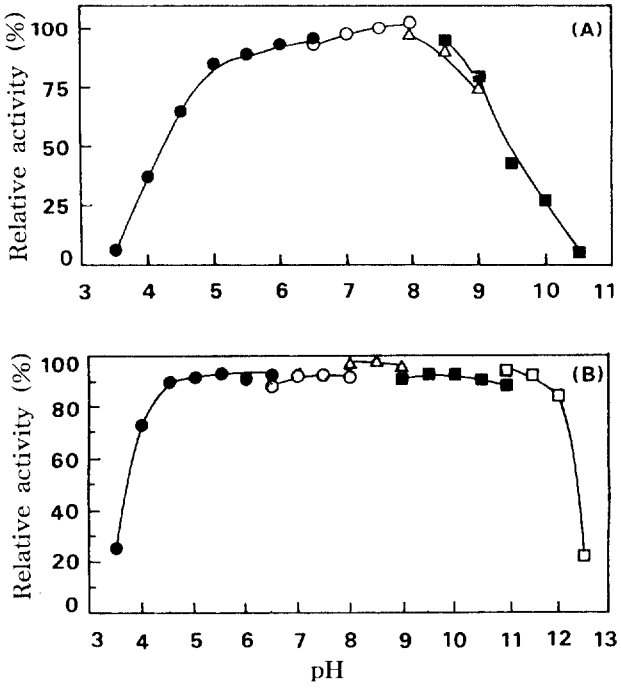

Fig. 3. Effects of pH on the activity (A) and stability (B) of xylanase. The following buffer systems were used: 100 mM MacIlvaine's (●, pH 3.5~6.5); 100 mM sodium phosphate (○, pH 6.5~8.0); 100 mM Tris-HCl (△, pH 8.0~9.0); 100 mM glycine-NaOH (■, pH 9.0~11.0); 100 mM KCl-NaOH (□, pH 11.0~12.5). The residual activities were measured by the standard assay procedure after incubation of the enzyme for 24 h at 4℃ in the buffers of various pH values.

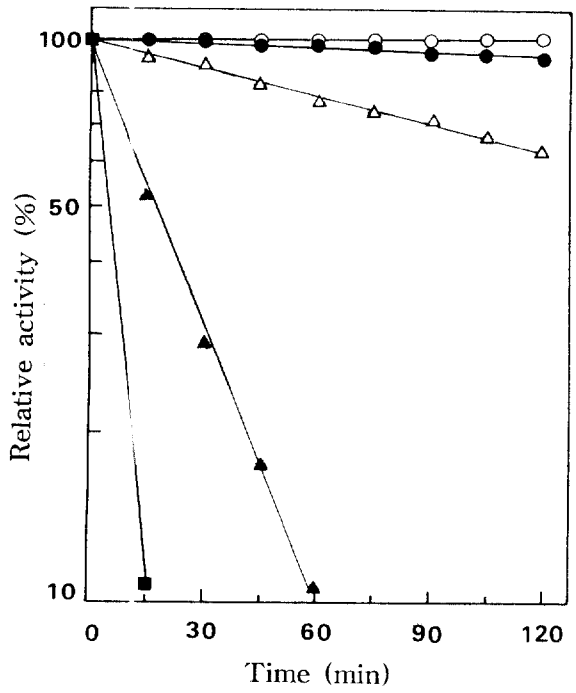

Fig. 4. Thermal stability of purified xylanase. Purified xylanase in 100 mM sodium phosphate buffer (pH 7.0) was preincubated at 40℃ (○), 50℃ (●), 55℃ (△), 60℃ (▲), or 70℃ (■), and aliquots were withdrawn over time for assay under the standard conditions.

Table 2. Kinetic parameter values of purified xylanase on three types of xylan substrates

Substrate	K_m (mg/ml)	V_{max} (units/mg protein)
Birchwood xylan	2.33	180.2
Larchwood xylan	2.78	170.6
Oat spelts xylan	3.45	142.7

Effects of pH on activity and stability

Fig. 3 shows the pH dependence of the purified xylanase. The maximum activity of this enzyme was observed at pH 8.0, and at least 80% of the maximum activity was attained from pH 5.0 to pH 9.0. Moreover, the xylanase was fairly stable over a wide range of pH and retained more than 80% of its original activity after preincubation even at pH 12 for 24 h. Although many xylanases have been known from various fungi, no fungal xylanase having high stability at a such alkaline pH has been reported. It is interesting that the first reported fungal xylanase active at alkaline pH was also from an alkalotolerant *Cephalosporium* sp. (2). When compared with it, however, the xylanase in this study was more stable at high alkaline pH.

Effects of temperature on activity and stability

The purified xylanase gave the highest activity at a temperature of 50℃ in 50 mM sodium phosphate buffer, pH 7.0. For the assessment of thermal stability of the xylanase, the enzyme was incubated at various temperatures in the absence of substrate, and then the residual activity was measured at time intervals (Fig. 4). The xylanase showed relatively higher stability at temperatures up to 55℃. After 2 h of incubation at 55℃, the enzyme retained 65% of its activity. This enzyme, however, was rapidly inactivated at 60℃, and it lost more than 85% of the initial activity after 1 h of incubation at this temperature.

Effects of various reagents on xylanase activity

The effects of various metal ions, *N*-bromosuccinimide (NBS), iodoacetamide, and diethylpyrocarbonate on xylanase activity were examined (data not shown). None of the metal ions tested seemed to stimulate the xylanase activity. On the other hand, the enzyme was completely inhibited by 1 mM of Hg^{2+}. Other metal ions such as Mn^{2+} and Cu^{2+} inhibited the enzyme activities by 36~64%. Iodoacetamide and diethylpyrocarbonate did not influence the activity, whereas the xylanase was completely inhibited by 1 mM NBS. These results indicate that the presence of tryptophan is essential for the activity of

this xylanase. The involvement of tryptophan residue(s) in the active site has already been reported for xylanases of different microbial strains (5, 19) and, as suggested by Deshpande *et al.* (4), it may be conserved among microbial xylanases in general.

Kinetic parameters

Table 2 summarizes the data obtained from Lineweaver-Burk plots for the purified xylanase on three types of substrates, birchwood xylan, larchwood xylan, and oat spelts xylan. The birchwood xylan is typically 0-acetyl-4-0-methylglucuronoxylan and the larchwood xylan is consisting of 99.2% of xylose and 0.8% of arabinose, whereas oat spelts xylan is typically arabinoxylan in which 10% of xylose units are substituted with arabinofuranose residues. The xylanase had higher affinities for less-branched xylans (birchwood xylan and larchwood xylan) than for the highly substituted oat spelts arabinoxylan. The Km values of this enzyme are low as compared to two xylanases from *Trichoderma reesei* (25) in which the Km values for three types of substrates (xylans) were 3.0~6.8 mg/ml and 14.8~22.3 mg/ml, respectively.

Substrate specificity

The purified xylanase was assayed for hydrolytic activity against a variety of natural and synthetic substrates (data not shown). After 1 h of incubation at 50℃, this enzyme could not release reducing sugars from carboxymethyl cellulose, avicel, polygalacturonic acid, starch, and laminarin. It also did not hydrolyze *p*-nitrophenyl (PNP)-*β*-D-xylopyranoside, PNP-*β*-D-glucopyranoside, and xylobiose. From these results it is apparent that the xylanase is typically free of cellulase activity. Some xylanases have been reported to have both xylanase and cellulase activities (6, 7, 11, 16, 32). However, cellulase activity is unwanted for pulp and paper applications because it degrades cellulose fibers and destroys pulp properties.

Amino acid composition

The amino acid composition of the purified xylanase is shown in Table 3. The xylanase contained high levels of Gly (14.78%), Asx (9.69%), and Glx (9.91%) but very little Met (0.14%). The amino acid composition of this enzyme was similar to that of xylanases from *Aureobasidium pullulans* (APX-II) (14), *T. harzianum* (30), *Humicola lanuginosa* (1), and *Bacillus* sp. (19) but distinctly different from that of *A. pullulans* xylanase, APX-I (14).

In conclusion, a xylanase active at alkaline pH from an alkalophilic fungus, *Cephalosporium* sp. RYM-202 was reported here for the first time. To our knowledge, there has been so far no report concerning the purification and characterization of xylanase produced by alkalophilic fungi. The present results show that the purified xylanase from *Cephalosporium* sp. RYM-202 has an exceptional characteristic in its molecular weight and pI value. The novel features of this enzyme may extend the knowledge of microbial xylanases. Moreover, this study provides the possibility of this organism as a candidate for the production of xylanase active and stable at alkaline pH, which makes this fungal strain worthy of further investigation.

Table 3. Amino acid composition of purified xylanase.

Amino acid	Composition (mol %)[a]		
	Xylanase	APX-II	Xylanase J
Ala	8.07	7.78	5.4
Arg	5.87	3.09	3.7
Asp+Asn	9.69	12.64	13.9
Cys	ND[b]	0	0
Glu+Gln	9.91	8.29	9.1
Gly	14.78	15.90	12.8
His	1.25	3.04	1.6
Ile	4.84	2.35	5.4
Leu	3.96	2.73	4.3
Lys	1.06	2.17	2.9
Met	0.14	0.34	2.0
Phe	4.85	3.11	4.1
Pro	5.36	2.40	2.8
Ser	6.68	10.78	8.8
Thr	7.63	12.07	9.5
Trp	ND	ND	1.9
Tyr	7.37	7.19	6.4
Val	8.53	6.42	5.4

[a] Data for APX-II and xylanase J are from references, Li *et al.* (14) and Nakamura *et al.* (19), respectively.
[b] ND, not determined.

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