Purification and Characterization of Two Xylanases from Alkalophilic *Cephalosporium* sp. Strain RYM-202

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Two different xylanases, CX-I and CX-II, from an alkalophilic fungus, *Cephalosporium* sp. strain RYM-202, have been purified to homogeneity. The enzymes had similar pH (7.5 to 8.0) and temperature (50°C) optima and were stable over a wide pH range of 5.5 to 12.0. Both enzymes were shown to be cellulase-free endoxylanases with transglycosidation activity.

In addition to a possible application in recovering fermentable sugars from hemicellulose (11), the use of xylanases (β -1,4-endoxylanases [EC 3.2.1.8]) for the prebleaching of kraft pulp has been considered one of the most important new industrial applications of the enzymes (17, 23). Since many bleaching processes are performed at a highly alkaline pH, xylanases active at alkaline pH are preferable for pulp applications (13). Several bacteria, including *Bacillus* spp. (8, 15, 16) and a *Streptomyces* sp. (18), are known to produce alkaline xylanases. A few fungal xylanases active at alkaline pH from a *Cephalosporium* sp. (2) and *Aspergillus fischeri* (4) were reported recently, but none of these enzymes have been purified, and thus their enzymatic properties have not been extensively investigated.

Cephalosporium sp. strain RYM-202 is an alkalophilic fungal strain that grows optimally at pH 9.5 to 10.0 (9). In addition to having multiple carboxymethyl cellulases (10), this organism produces multiple xylanases extracellularly when wheat bran is used as a carbon source. In this paper, we report on the purification and characterization of two xylanases from *Cephalosporium* sp. strain RYM-202, CX-I and CX-II, which exhibit high activity at alkaline pH.

For enzyme preparation, batch cultures were carried out in a 5-liter jar fermentor at 30°C for 3 days with 2% wheat bran medium (10). The initial pH of the medium was adjusted to 10.0 by adding 0.5% sodium carbonate. The culture filtrate was fractionated by salting out with ammonium sulfate (30 to 80% saturation), dialyzed, and concentrated by ultrafiltration. The enzyme solution was purified by a series of column chromatographies. A quantitative evaluation of the results obtained from the consecutive purification steps is given in Table 1. The homogeneity of the final enzyme preparations of CX-I and CX-II was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), during which each of the xylanases gave a single protein band (Fig. 1).

The molecular masses of CX-I and CX-II as determined by SDS-PAGE were approximately 35 and 24 kDa, respectively (Fig. 1). By gel permeation chromatography on a Superose 12 HR 10/30 column, the corresponding native molecular masses were estimated to be 33 and 23 kDa. These results indicate that the purified enzymes have monomeric structures, which is consistent with the known characteristics of microbial xylanases (25). The isoelectric point (pI) values of CX-I and CX-II were

determined to be 6.3 and 4.4, respectively, by analytical isoelectric focusing on PhastGel slabs (pH 3 to 9). It is known that xylanases from *Bacillus* spp. can be divided into two major groups on the basis of their molecular masses and pIs (25). One is a basic group with alkaline pIs (8.3 to 10.0) and low molecular masses (16 to 22 kDa), and the other is an acidic group with acidic pIs (3.4 to 4.5) and high molecular masses (43 to 50 kDa). These groupings apply well to xylanases produced by other microorganisms, including *Streptomyces* spp. (6, 18, 21), *Aspergillus niger* (7), and *Trichoderma* spp. (19, 22, 24). In this study, however, CX-I, which has an apparent intermediate molecular mass and pI, did not fall into either of the two groups discussed above. Similarly, CX-II, which has a low molecular mass and acidic pI, was also very exceptional and did not fit into either of the two groups.

The overall pH-activity profiles of the two xylanases appeared quite similar, with optimal pHs of 7.5 for CX-I and 8.0 for CX-II (Fig. 2A). Both enzymes showed at least 80% of their maximum activities over a pH range from pH 5.0 to 8.5. The xylanases were fairly stable over a wide pH range (5.5 to 12.0) and retained more than 95% of their original activities after a 1-h preincubation, even at pH 12 and 50°C (Fig. 2B). These properties make these xylanases attractive in view of their applications for the pulp and paper industries. Although many fungal xylanases have been isolated and characterized, no fungal xylanase with such high stability at such high levels of alkaline pH has been reported to date. There have been only two reports so far on fungal xylanases exhibiting activity and stability at alkaline pH (the enzymes were from an alkalotolerant *Cephalosporium* sp. [2] and *A. fischeri* [4]). However,

TABLE 1. Purification of xylanases from *Cephalosporium* sp. strain RYM-202

Step and xylanase	Activity (U)	Protein (mg)	Sp act (U/mg of protein)	Yield (%)	Fold purification
Culture supernatant	43,120	5,544	7.8	100	1
(NH ₄) ₂ SO ₄ fractionation	31,150	2,078	15.0	72.2	1.9
DEAE-Sephadex A-50					
CX-I	6,845	327	21.0	16.0	2.7
CX-II	9,350	294	31.8	22.0	4.1
Sephacryl S-200 HR					
CX-I	5,390	65.2	82.0	13.0	10.5
CX-II	7,580	73.0	103.8	18.0	13.3
Superose 12 HR 10/30					
CX-I	4,260	31.5	135.2	9.9	17.3
CX-II	6,380	35.7	178.7	15.0	22.9

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FIG. 1. SDS-PAGE of the purified xylanases. Lane 1, CX-I (4.5 µg); lane 2, CX-II (6.0 µg); lane S, molecular mass marker proteins.

these enzymes were examined only in an unpurified state and were less stable than CX-I and CX-II at alkaline pHs over 10.

Both xylanases showed essentially identical temperature-activity profiles and gave the highest activity at 50°C. They showed relatively high stabilities at temperatures up to 50°C. After 2 h of incubation at 55°C, the enzymes retained 54% (CX-I) and 65% (CX-II) of their original activities. CX-I and CX-II were completely inhibited by 1 mM Hg^{2+} but were partially inactivated by 1 mM Mn^{2+} and Cu^{2+} . Other metal ions, such as Co^{2+} , Pb^{2+} , and Fe^{2+} , showed slight inhibitory or stimulatory effects. Iodoacetamide and diethylpyrocarbonate did not influence the activity of any enzyme, suggesting that cysteine and histidine residues are not involved in the active sites. On the other hand, both xylanases were totally inhibited by 1 mM *N*-bromosuccinimide, which supports the view that the presence of a tryptophan residue in the active site is a characteristic property which may be conserved among many different microbial xylanases (5, 15, 21).

The xylanases showed higher affinities for less-branched xylans, such as birchwood and larchwood xylans, than for highly substituted oat speltis arabinoxylan (Table 2). CX-II, in particular, showed higher affinities for all the substrates than did CX-I, exceeding those of the latter by 25 to 45%. Neither of the two enzymes could attack carboxymethyl cellulose, Avicel, polygalacturonic acid, starch, laminarin, *p*-nitrophenyl- β -D-glucopyranoside, or *p*-nitrophenyl- β -D-xylopyranoside. On the basis of these results, it is apparent that the xylanases are wholly free of cellulase activity as well as β -xylosidase activity. Some xylanases have been reported to possess cellulase activities (3, 7, 14). Because cellulase activity degrades cellulose

FIG. 2. Effects of pH on the activity (A) and stability (B) of the purified xylanases. The buffer systems used were McIlvaine's buffer (pH 3.5 to 6.5), 100 mM sodium phosphate (pH 6.5 to 8.0), 100 mM Tris-HCl (pH 8.0 to 9.0), 100 mM glycine-NaOH (pH 9.0 to 11.0), and 100 mM KCl-NaOH (pH 11.0 to 13.0). CX-I, ●; CX-II, ○. (A) The xylanase activities were assayed at 50°C in the reaction mixtures with different pH values (pH 3.5 to 10.5). (B) The xylanases were preincubated in the buffers with different pH values (pH 3.5 to 13.0) in the absence of substrate for 1 h at 50°C, and the residual activities were assayed in the standard reaction mixtures at pH 7.5 and 50°C.

TABLE 2. Kinetic parameter values for two purified xylanases

Substrate	K_m (mg/ml)		V_{max} (U/mg of protein)	
	CX-I	CX-II	CX-I	CX-II
Birchwood xylan	3.18	2.20	140.2	175.6
Larchwood xylan	3.63	2.56	156.2	172.1
Oat speltis xylan	5.26	4.16	118.4	145.2

fibers and destroys pulp properties, an important criterion in the use of xylanases for pulp and paper applications is that they must be cellulase free (13).

Both enzymes were shown to be endo-type xylanases, producing xylobiose (X_2) as a predominant end product from xylan along with X_3 and higher xylooligosaccharides as intermediates. The hydrolysis patterns of CX-I (Fig. 3) and CX-II (data not shown) were quite similar to each other but significantly different from those of the xylanases from *Trichoderma viride* (22) and *Trichoderma harzianum* (19), in which X_1 and X_2 were the main hydrolysis products. Neither of the two enzymes was active on xylobiose. On the other hand, the enzymes readily attacked X_3 and finally accumulated X_2 as a major product and X_1 as a minor product. Throughout the reaction, the amount of X_2 was much larger than that of X_1 . The most plausible explanation for this pattern of degradation is that a donor X_3 molecule was joined to an acceptor X_3 by the transglycosidation activity of the enzymes to yield xylohexaose (X_6), which was quite rapidly hydrolyzed into three X_2 molecules or into X_2 and X_4 . The main hydrolysis products from X_4 were X_2 and X_3 , and after a prolonged reaction, the latter was further hydrolyzed into X_2 without any accumulation of X_1 . The results confirm that X_4 molecules were mainly split into X_2 molecules and that the majority of X_3 molecules were released from X_6 molecules previously formed from X_2 and X_4 by transglycosidation. It is thus probable that both the enzymes are endo-type xylanases possessing transglycosidase activity. It is also noteworthy that the amount of higher xylooligosaccharide corresponding to xylopentaose (X_5) increased throughout the hydrolysis of X_3 and X_4 . It is possible that the accumulation of X_5 in the hydrolysates could be due to the low activities of CX-I and CX-II against this xylooligosaccharide. Xylanases from *Trichoderma reesei* were also reported to be incapable of hydrolyzing X_5 (20).

There were no remarkable differences in the amino acid compositions of CX-I and CX-II. The xylanases contained

FIG. 3. Thin-layer chromatography analysis of hydrolysates formed by the action of CX-I from birchwood xylan and xylooligosaccharides. One hundred microliters of 5% (wt/vol) birchwood xylan (A), xylooligosaccharides (B), and xylooligosaccharides (C) in 50 mM sodium phosphate buffer (pH 7.5) was incubated with 6.3 U of CX-I 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) h. The xylooligosaccharide standards (lane S) included xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylooligosaccharide (X_4), xylopentaose (X_5), and xylohexaose (X_6).

relatively high levels of Gly, Asx, and Glx but very little Met. The amino acid compositions of these enzymes were somewhat similar to those of the xylanases from *Aureobasidium pullulans* (APX-II) (12), *T. harzianum* (24), *Humicola lanuginosa* (1), and a *Bacillus* sp. (15).

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