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Purification and Characterization of Two Endoxylanases from an Alkaliphilic *Bacillus halodurans* C-1

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Abstract Two endoxylanases from an alkaliphilic bacterium, *Bacillus halodurans* C-1, were purified 3.8- and 7.9- fold with specific activities of 9.4 and 19.8 U/mg protein, respectively. The molecular masses of both purified enzymes were 23 and 47 kDa, respectively, and 23 kDa xylanase I (Xyl I) exhibited an optimum pH at 7.0, whereas 47 kDa xylanase II (Xyl II) showed a broad pH range of 5.0 to 9.0. The temperature optima of both xylanases were 60°C and 70°C, respectively. Both were stable in the pH range of 6.0 to 9.0 and 5.0 to 10.0, respectively, and they were stable up to 60°C and 70°C, respectively. The K_m and V_{max} of Xyl I were 4.33 mg/ml and 63.5 μmol/min/mg, respectively, whereas Xyl II had a K_m value of 0.30 mg/ml and V_{max} of 210 μmol/min/mg. Both xylanases hydrolyzed xylans from birchwood, oat spelt, and larchwood. However, they showed different modes of action; a series of xylooligosaccharides larger than xylotriose were released as the major products by Xyl I, whereas xylobiose and xylotriose were the main products by Xyl II. The maximum synergistic action of the two enzymes on hydrolysis of xylan was 2.16 with the ratio of Xyl I to Xyl II at 1:9.

Key words: Alkaliphilic bacterium, *Bacillus halodurans* C-1, enzyme purification, synergism, endoxylanases

Xylan, a major constituent of hemicellulose, is composed of a backbone containing β-1,4-linked-D-xylose residues, which are substituted with glucuronic acid, arabinose, and acetate residues [5]. Endoxylanases (EC 3.2.1.8) randomly cleave internal β-1,4-glycosidic bonds of the backbone and show high activity toward highly polymerized xylan. Because of its complex structure, complete hydrolysis of xylan requires synergistic action or cooperation of multiple xylan-degrading enzymes [24]. The plant cell wall consists of a mixture of polysaccharides, cellulose, and hemicellulose. The hydrolysis of plant cell wall polysaccharides into soluble sugars is potentially important for the production of fermentable sugars, chemicals, and liquid fuel.

Xylooligosaccharides, the hydrolysis products of xylan, have been used as thickeners, fat substitutes, and antifreeze food additives in the food industry. Moreover, they are suitable as an agent for direct tableting in combination with other components used in the pharmaceutical industry [12]. Much research has also been performed to find the production and application of microbial protease [17, 20].

Recently, xylanases are being used in the pulp and paper industry, and the interest in many fields of applications is increasing [2, 12, 21]. Many microorganisms such as bacteria and fungi produce xylanases, and most of them show optimal activity at an acidic pH range and low activity at alkaline conditions [11, 12, 14]. However, some xylanases from alkaliphilic microorganisms can be active in alkaline conditions, and various alkaline xylanases from alkaliphilic bacteria have been studied [9, 16]. The endoxylanase expressd in *S. cerevisiae* [8] and surface immobilization on silica of endoxylanase [10] have been reported.

In this paper, we describe the purification and characterization of two endoxylanases produced by an alkaliphilic *B. halodurans* C-1 and their synergistic action on hydrolysis of xylan.

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MATERIALS AND METHODS

Identification of the Bacterium

A bacterium, *B. halodurans* C-1, was isolated from a wastewater treatment plant of Siam Cellulose Co., Ltd. in Ratchaburi Province, Thailand. Morphological properties and taxonomic characteristics of the bacterium were examined according to the methods in *Bergey's Manual of Systematic Bacteriology* [6]. The bacterium was also identified by 16S rRNA sequencing analysis [23], and the nucleotide sequence data were compared with 16S rDNA sequences of other *Bacillus* strains on BLAST of the National Center of Biotechnology Information databases (NCBI databases).

Culture Conditions

The bacterium was grown in Berg's mineral salt medium [3] containing 0.5% oat spelt xylan (Sigma, Saint Louis, MO, U.S.A.). The pH was initially adjusted to 10.0 with 1% Na_2CO_3 after autoclaving. The culture was incubated in a rotary shaker at 200 rpm and 37°C for 2 days and harvested by centrifugation at 10,000 rpm for 10 min. The culture supernatant was used as crude enzyme.

Assays

The xylanase activity was assayed by determining the reducing sugars released from oat spelt xylan (Sigma Chemical Co., St. Louis, MO, U.S.A.). The reaction mixture (0.6 ml) contained 0.5% xylan in 50 mM sodium phosphate buffer, pH 7.0, and enzyme. After incubation for 10 min at 50°C, the increase of reducing sugar was determined by the Somogyi-Nelson method [19]. One unit of the enzyme activity was defined as the amount of enzyme to release 1 μmole of reducing sugar in 1 min under the above condition. The cellulase activity was measured under the same condition as described above, using carboxymethylcellulose (Sigma Chemical Co., St. Louis, MO, U.S.A.) as a substrate.

β-Xylosidase, arabinofuranosidase, β-glucosidase, and acetyl esterase activities were assayed as previously described by Ratanakhanokchai *et al.* [18]. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard. The protein content of eluate from ion-exchange columns was measured at 280 nm.

Gel Electrophoresis and Zymogram Analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [13]. Samples were dissolved in a sample application buffer and heated in a boiling-water bath for 3 min. After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250. The molecular weight markers used were the prestained low molecular weight calibration kit (Bio-Rad, Hercules, CA, U.S.A.). Zymogram analysis for xylanase activity was performed on SDS-12% polyacrylamide gel containing 0.1% soluble xylan, as described previously by Ratanakhanokchai *et al.* [18].

Kinetic Determinations

Initial rates of hydrolysis of soluble oat spelt xylan by both purified xylanases (0.042 mg protein/ml) were determined at various concentrations of substrate (0.25–10.00 mg/ml) in 0.05 M phosphate buffer at pH 7.0 and 60°C for 5 min. The kinetic constants, K_m and V_{max}, were estimated by the method of Lineweaver and Burk [15].

Hydrolysis of Xylans

Soluble oat spelt, birchwood, and larchwood xylans were prepared by the method of Ghangas *et al.* [7]. Each of them (0.5% dry weight) in 0.05 M sodium phosphate buffer (pH 7.0) was hydrolyzed with both xylanases (23 μg protein) at 50°C. After 5 min of incubation, the hydrolysis products were taken, and the amounts of reducing sugars produced were determined by the Somogyi-Nelson method [19].

Analysis of Xylan Hydrolysis Products

The hydrolysis products of soluble birchwood xylan were determined by thin layer chromatography (TLC). Approximately 10 μg of each protein sample in 0.05 M sodium phosphate buffer (pH 7.0) was spotted on TLC plates (aluminum sheets silica gel 60 F_{254}, Merck), and the plates were resolved with a solvent system of isopropanol-acetone-0.1 M lactic acid (4:4:2). The sugar spots were detected by heating the plates to over 100°C after spraying them with a reagent of aniline-α-diphenylamine-acetone-80% H_3PO_4 (4 ml:4 g:20 ml:30 ml). Xylose and xylobiose were used as standards.

Synergism

The combination of Xyl I and Xyl II was tested for synergism at 50°C in 0.05 M phosphate buffer (pH 7.0) containing 0.5% soluble oat spelt xylan. After incubation for 10 min, reducing sugars produced were determined by the Somogyi-Nelson method [19]. Control experiments were also conducted with each enzyme alone, and the hydrolysis products were analyzed for reducing sugar. The degree of synergism for the combination of both enzymes was calculated as the reducing sugars released by the combination of both enzymes divided by the sum of reducing sugars released by each enzyme alone.

RESULTS AND DISCUSSION

Identification of the Bacterium

The bacterium strain C-1 was aerobic, spore-forming, Gram-positive, motile, and rod-shaped, and produced catalase. Thus, this bacterium was identified as a member

Table 1. Enzymatic activity of crude enzyme of *B. halodurans* C-1.

Enzyme	Specific activity (U/mg protein)
Xylanase	2.5
Arabinofuranosidase	0.09
β-Xylosidase	0.03
Acetylesterase	-
CMCase	-
β-Glucosidase	-

-, could not detect under the assay condition.

of the genus *Bacillus* according to *Bergey's Manual of Systematic Bacteriology* [6]. Moreover, we identified species, based on the comparison of the 16S rDNA sequence of this strain with those of other *Bacillus* spp. The 16S rDNA sequence of *Bacillus* sp. C-1 had 1,538 base pairs and 99% similarity with *B. halodurans* [9]. Therefore, it was tentatively identified as *B. halodurans*.

When *B. halodurans* C-1 was grown in an alkaline medium at pH 10.0 containing xylan as a sole source of carbon for 48 h, the extracellular xylanase was detected at 2.5 U/mg of protein. Low activities of arabinofuranosidase and β-xylosidase were detected at 0.09 and 0.03 U/mg of protein; however, there were no acetylesterase, CMCase, and β-glucosidase in the crude enzyme (Table 1).

Enzyme Purification

Xylanases were purified from the crude *B. halodurans* C-1 preparation. All purification steps were carried out at 4°C. The crude enzyme was precipitated with $(NH_4)_2SO_4$ at 90% saturation. The precipitate was collected by centrifugation and dissolved in 0.05 M Tris-HCl buffer (pH 8.0). After dialysis, the concentrated enzyme was applied to a DEAE-Toyopearl 650 M column (2.0×20 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0), and the column was eluted with the same buffer containing 1 M NaCl, at a flow rate of 0.5 ml/min. At this step, a large amount of protein with low xylanase activity did not bind to the DEAE-Toyopearl 650 M column, and only one protein peak was detected. The combined fractions of that peak were concentrated, and the concentrate was dialyzed and applied to a 16/10 DEAE fast-flow column (1.6×10.0 cm) (FPLC system, Amersham Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.), equilibrated with the same buffer as described above. The column was eluted with the same buffer, containing a 0 to 0.5 M NaCl linear gradient, at the flow rate of 1 ml/min. In this purification step, three peaks with xylanase activity were eluted, and peak I and peak II contained low xylanase activity. However, the active peak III was resolved into two protein bands on SDS-PAGE and the zymogram showed xylanase activity at 23 and 47 kDa regions (data not shown). These two xylanase bands were finally separated on a Mono-Q HR 5/5 column (0.5×5.0 cm; FPLC system, Pharmacia LKB Biotechnology) equilibrated with the above buffer by elution with a linear gradient of NaCl (0.25–0.5 M) in the same buffer at the flow rate of

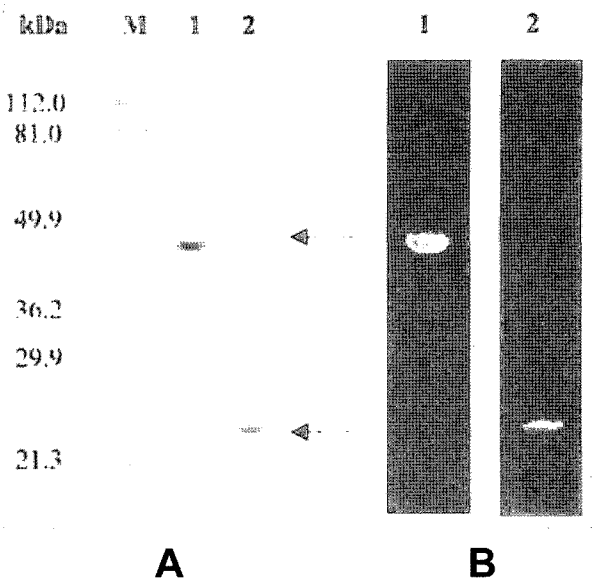

Fig. 1. SDS-PAGE and zymogram of purified xylanases from *B. halodurans* C-1.
Xylanases purified from *B. halodurans* C-1 were analyzed by SDS-PAGE (**A**) and zymography (**B**). The two purified xylanases, Xyl I and Xyl II, were shown as molecular masses of 23 kDa (lane 2A, 2B) and 47 kDa (lane 1A, 1B), respectively. The molecular weight markers used were the prestained low molecular weight calibration kit (Bio-Rad, Hercules, CA, U.S.A.). Lane M: Prestained molecular weight markers: phosphorylase B (112 kDa), bovine serum albumin (81 kDa), ovalbumin (49.9 kDa), carbonic anhydrase (36.2 kDa), soybean trypsin inhibitor (29.9 kDa), and lysozyme (21.3 kDa); Lanes 1A)and 1B: purified Xyl II; Lanes 2A and 2B: purified Xyl I.

Table 2. Purification of the xylanases produced by *B. halodurans* C-1.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	486.0	1,198.0	2.5	1.0	100.0
Ammonium sulfate precipitation	332.1	998.0	3.0	1.2	83.3
DEAE-Toyopearl column chromatography	102.0	444.0	4.4	1.8	37.1
DEAE-HiPrep FF 16/10 column chromatography	18.6	115.2	6.2	2.5	9.6
Mono-Q HR 5/5 column chromatography					
(Xyl I)	0.9	8.5	9.4	3.8	0.7
(Xyl II)	2.4	47.6	19.8	7.9	4.0

Table 3. Characterization of xylanases purified from *B. halodurans* C-1.

Properties	Xyl I	Xyl II
Molecular weight (kDa)	23	47
pH optimum	7.0	5.0–9.0
pH stability	6.0–9.0	5.0–10.0
Temperature optimum	60°C	70°C
Temperature stability	Up to 60°C	Up to 70°C
K_m (mg/ml)	4.33	0.30
V_{max} (μmol/min/mg)	63.5	210.0
V_{max}/K_m [(μmol min/mg protein)/(mg xylan/ml)]	14.7	700
The main hydrolysis products	Xylooligosaccharides	Xylobiose and xylotriose

1 ml/min. The two active xylanase bands were analyzed by SDS-PAGE and zymography and showed molecular masses of 47 kDa (Xyl II) (Fig. 1, lanes 1A and 1B) and 23 kDa (Xyl I) (Fig. 1, lanes 2A and 2B). The purification steps of both xylanases are summarized in Table 2. Honda *et al.* [9] reported that the two purified xylanases, xylanase N and xylanase A of *B. halodurans* C-125 (formerly known as *Bacillus* sp. C-125), had the molecular masses of 16 and 43 kDa, respectively, which are different from the sizes of the Xyl I and Xyl II of *B. halodurans* C-1 described in this work. Characteristics of the purified xylanases are shown in Table 3. Xyl I and Xyl II were purified 3.8- and 7.9-fold, respectively, when compared with crude enzyme preparation. The low magnitudes of purification obtained seem to be due to separation of synergistic enzymes during purification, as described by Sunna *et al.* [22].

pH and Temperature Optima and Stability

Optimum pH values were determined by using oat spelt xylan as the substrate at 60°C. The pHs of the reaction mixture were adjusted between 4.0 and 11.0 with 0.05 M various buffer systems: acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and Na_2CO_3-$NaHCO_3$ buffer (pH 9.0 to 11.0). The pH stabilities of enzymes were determined by preincubating the enzyme at 50°C for 1 h at different pHs between 4.0 and 11.0 (0.025 M), and the residual activities were measured by the standard assay method. As shown in Table 2, the optimum pH of Xyl I was 7.0, whereas Xyl II showed broad optimum pH and stability. The optimum pH of Xyl I was similar to that of other *Bacillus* xylanases, whereas the board optimum pH range of Xyl II was different from previously reported values [1, 16]. Both Xyl I and Xyl II were stable in alkaline pH condition. Moreover, both enzymes appeared to be more thermostable than xylanase N and xylanase A from *B. holodurans* C-125 [9].

Optimum temperatures for both xylanases were determined by varying the reaction temperatures, ranging from 40 to 90°C, at pH 7.0. The thermal stabilities of the enzymes were determined by preincubating the enzymes at pH 7.0 for 30 min at different temperatures ranging from 40 to 90°C, and the residual activities were determined by the standard assay method. The results are shown in Table 3. Xyl I retained 30% of original activity after incubation at 70°C, whereas Xyl II had 22% and 16% of the original activity retained after incubation at 80 and 90°C, respectively (data not shown).

Kinetic Parameters

The kinetic determinations of Xyl I and Xyl II at 60°C with various concentrations of xylan gave K_m values of 4.33 and 0.30 mg/ml and V_{max} values of 63.5 and 210 μmol/min/mg protein, respectively: They are similar to K_m and V_{max} values of other *Bacillus* xylanases (0.24–9.40 mg/ml and 19.5–288 μmol min/mg, respectively) [1, 2]. However, xylanase from *Bacillus* sp. 41M-1 [16] had a V_{max} value higher than those of Xyl I and Xyl II. V_{max}/K_m of Xyl II was approximately 48-fold higher than that of Xyl I (Table 3). The kinetic parameters indicate that Xyl II has a higher efficiency to hydrolyze soluble oat spelt xylan than Xyl I.

Substrate Specificity of Xyl I and Xyl II

In order to study the substrate specificity, both xylanases were assayed with various substrates (0.5%). Both xylanases had hydrolytic activity toward xylan, but no activity toward *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-acetate, or carboxymethylcellulose.

The initial hydrolysis of soluble xylans from oat spelt, birchwood, and larchwood by the purified Xyl I and Xyl II was conducted. Figure 2 shows the initial hydrolysis of soluble xylans of birchwood, oat spelt, and larchwood by Xyl I and Xyl II: The amounts of hydrolysis are shown as reducing sugar (μg/ml). Xyl I and Xyl II showed the highest hydrolytic activity toward soluble birchwood xylan, followed by soluble oat spelt and larchwood xylans. The hydrolytic activities of Xyl I and Xyl II on soluble birchwood were 116.8 μg/ml and 223.5 μg/ml, respectively. The hydrolysis rate of soluble birchwood by Xyl II was 1.91 times higher than that of Xyl I. Furthermore, all the hydrolysis rates of xylans used (birchwood, oat spelt, and larchwood) by Xyl II were higher than those of Xyl I. It may be due to different substituent groups of xylan [6] as well as different modes of enzyme action on hydrolysis.

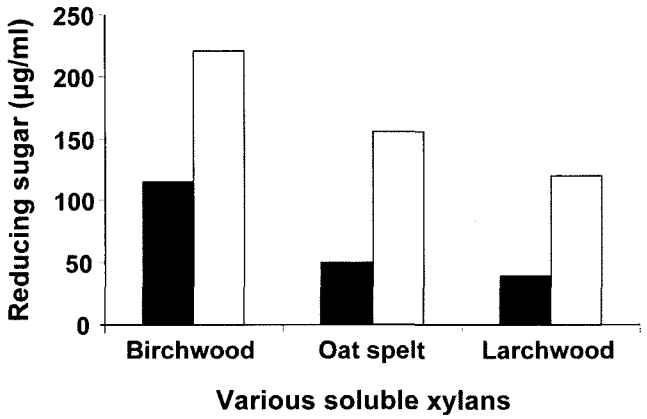

Fig. 2. Hydrolysis of soluble xylans by Xyl I and Xyl II.
The graph shows an initial hydrolysis of soluble xylans of oat spelt, birchwood, and larchwood by Xyl I and Xyl II. The hydrolysis amount shows a reducing sugar (μg/ml). Black and white bars indicate the hydrolysis rates of Xyl I and Xyl II, respectively. This graph represents data from similar results of three independent experiments.

Mode of Action of Xyl I and Xyl II

The hydrolysis products of soluble birchwood xylan by Xyl I and Xyl II were analyzed by TLC. As shown in Fig. 3, Xyl I liberated a series of xylooligosaccharides larger than xylotriose as the major products, whereas Xyl II liberated xylobiose and xylotriose as the major products, but a series of larger xylooligosaccharides as the minor products. These results indicate that both enzymes are endoxylanases. The prolonged incubation of the reaction mixture also showed similar product patterns (data not shown). Although both Xyl I and Xyl II are endoxylanases, their modes of action were clearly different. The hydrolysis patterns of both enzymes were also quite different from those of xylanases N and A from *B. halodurans* C-125 [9] and other endoxylanases [16, 18, 22] that showed a series of xylobiose and xylooligosaccharides. Most xylanolytic microorganisms are known to synthesize a range of xylanase isoenzymes with overlapping but different specificities for efficient hydrolysis of xylan [24].

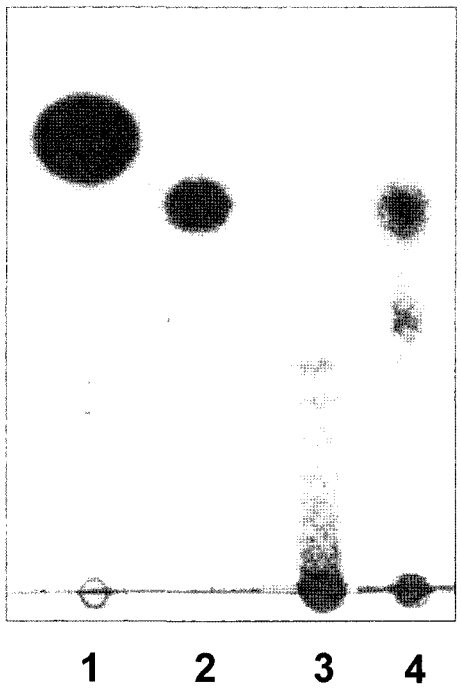

Fig. 3. TLC of hydrolysis products of soluble birchwood xylan by purified Xyl I and Xyl II.
The photograph shows the action mode of hydrolysis products of soluble birchwood xylan by Xyl I and Xyl II. The samples were spotted on thin-layer chromatography (TLC) silica gel 60 F_{254} (Merck), and were analyzed as described in Materials and Methods. Lane 1: xylose; Lane 2: xylobiose; Lanes 3 and 4: products of Xyl I and Xyl II, respectively.

Synergistic Action of Xyl I and Xyl II

The initial hydrolysis of soluble oat spelt xylan by the combination of Xyl I and Xyl II was conducted. Each of them was adjusted to contain equal amount of protein content (23 μg) and both were combined in different proportions to maintain a constant sum of individual protein content. Xyl I and Xyl II were also individually tested as controls. The amount of reducing sugars released by the combination of both enzymes was significantly higher than the sum of the amount of the reducing sugars

Table 4. Synergism of two xylanases on hydrolysis of soluble oat spelt xylan.

Enzyme ratios Xyl I:Xyl II	Sum of reducing sugar released by individual enzyme (μg/ml)	Reducing sugar released by combination of both enzymes (μg/ml)	Synergism
10:0	12	12	1.00
0:10	342	342	1.00
1:9	354	765	2.16
2:8	354	613	1.73
3:7	354	604	1.71
4:6	354	587	1.66
5:5	354	556	1.57
6:4	354	535	1.51
7:3	354	489	1.38
8:2	354	452	1.28
9:1	354	374	1.06

released by the individual enzymes, indicating a synergistic interaction between the two xylanases. The maximum synergism was found to be 2.16 with the ratio of Xyl I to Xyl II at 1:9 (Table 4). The second higher synergism was 1.73 with the ratio of Xyl I to Xyl II at 2:8. The synergism was decreased by 19.9%, compared with the maximum synergistic effect at the ratio of 1:9. The synergistic action of two xylanases with different modes of action is important for efficient hydrolysis of xylan. The synergistic effect at 1:9 ratio is 2.16 times higher than the sum of reducing sugar released by the individual enzyme, and this would be very useful in the application of pulp pre-bleaching and paper industrial purposes.

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