J. Microbiol. Biotechnol. (2004), **14**(5), 1014–1021

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of Two Thermostable Xylanases from *Paenibacillus* sp. DG-22

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Received: May 11, 2004
Accepted: August 10, 2004

Abstract Two thermostable xylanases, designated XynA and XynB, were purified to homogeneity from the culture supernatant of *Paenibacillus* sp. DG-22 by ion-exchange and gel-filtration chromatography. The molecular masses of xylanases A and B were 20 and 30 kDa, respectively, as determined by SDS-PAGE, and their isoelectric points were 9.1 and 8.9, respectively. Both enzymes had similar pH and temperature optima (pH 5.0–6.5 and 70°C), but their stability at various temperatures differed. Xylanase B was comparatively more stable than xylanase A at higher temperatures. Xylanases A and B differed in their K_m and V_{max} values. XynA had a K_m of 2.0 mg/ml and a V_{max} of 2,553 U/mg, whereas XynB had a K_m of 1.2 mg/ml and a V_{max} of 754 U/mg. Both enzymes were endo-acting, as revealed by their hydrolysis product profiles on birchwood xylan, but showed different modes of action. Xylotriose was the major product of XynA activity, whereas XynB produced mainly xylobiose. These enzymes utilized small oligosaccharides such as xylotriose and xylotetraose as substrates, but did not hydrolyzed xylobiose. The amino terminal sequences of XynA and XynB were determined. Xylanase A showed high similarity with low molecular mass xylanases of family 11.

Key words: Thermostable xylanases, purification, characterization, *Paenibacillus* sp.

Xylan is the major component of plant hemicellulose and the second most abundant renewable polysaccharide after cellulose [12]. It is a heteropolysaccharide with a backbone of β-1,4-linked xylopyranose residues, substituted with acetyl, arabinosyl, and glucuronysyl residues [7]. Due to its heterogeneity, complete hydrolysis of xylan requires the cooperative action of several enzymes such as endoxylanase, β-xylosidase, α-arabinosidase, and acetyl esterase. Among them, endo-β-1,4-xylanases (EC 3.2.1.8) are crucial enzymes since they initiate the degradation of xylan into short xylooligosaccharides. Based on amino acid sequence homologies and hydrophobic cluster analysis, xylanases can be classified into two major families of glycosyl hydrolases: family 10 and family 11 [15]. Many microorganisms are known to produce multiple forms of xylanase with different physical and kinetic properties [5, 12, 35]. The activity of different xylanases with subtle differences in substrate specificity and mode of action should improve degradation of heterogenous xylan. Fungal xylanases are usually acidic to neutral in nature [5, 19, 25, 32]. On the other hand, bacterial xylanases range from acidic to alkaline and from moderate to extremely thermostable depending on the type of organism [5, 13, 32].

Microbial xylanases have attracted considerable research interest due to their potential industrial applications including biomass conversion and biopulping [5, 32, 34]. The application of xylanases in the pulp and paper industry has received great attention recently because of environmental concerns. The cellulase-free xylanases with high thermostability are of particular interest for bleaching of kraft pulp to decrease the consumption of chlorine chemicals [5, 17, 30, 34].

In a previous report, the isolation of a moderately thermophilic *Paenibacillus* sp. strain DG-22 from a timber yard soil in Kyungju, Korea has been described [23]. This strain produced thermostable xylanases extracellularly without cellulase activity. In this paper, we describe the purification and characterization of two xylanases from the culture supernatant of *Paenibacillus* sp. DG-22.

Materials and Methods

Microorganism and Culture Conditions

Moderately thermophilic *Paenibacillus* sp. DG-22 [23] was used for enzyme production. *Paenibacillus* sp. DG-22 was grown in an M9 minimal medium [31] supplemented

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with 0.2% (w/v) birchwood xylan (Sigma, St. Louis, U.S.A.) and 0.05% yeast extract (w/v) followed by incubation on a rotatory shaker (150 rpm). After 24 h of growth at 45°C, the culture was harvested by centrifugation at 5,000 ×*g* for 20 min. This culture supernatant was used as the source of enzymes to carry out the purification reported in this study.

Purification of Xylanases

All purification steps were carried out at room temperature. The culture supernatant of *Paenibacillus* sp. DG-22 was concentrated approximately 20-fold by ultrafiltration using an Amicon system (Amicon Corp., Lexington, MA, U.S.A.) with a 10 kDa cutoff membrane (YM10). The concentrated supernatant was applied to ion-exchange chromatography on CM-Sepharose (Pharmacia, Uppsala, Sweden) column (2.5×20 cm) previously equilibrated with 10 mM sodium acetate buffer (pH 5.5) and washed with the same buffer. The bound proteins were eluted by a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 1.2 ml/min. Fractions were collected and analyzed for enzyme activity. Two major xylanase peaks were obtained which were named XynA and XynB, respectively. These active fractions were pooled and concentrated by ultrafiltration (YM10 membrane, Amicon). The partially purified xylanase samples were then subjected to gel filtration on Sephacryl S-200 HR (Pharmacia) column (1.5×70 cm) for further purification. The column was pre-equilibrated and run in 10 mM sodium acetate buffer (pH 5.5) containing 0.2 M NaCl at a flow rate of 0.5 ml/min. Fractions were again analyzed for enzyme activity. The active fractions were pooled, concentrated, and used as the purified enzyme preparations.

Enzyme and Protein Assay

The xylanase activity was assayed by measuring the amount of reducing sugars liberated from 1% (w/v) birchwood xylan according to the method of Bailey *et al.* [3]. The reaction mixture (2 ml) contained 1.8 ml of 1% (w/v) birchwood xylan and 0.2 ml of enzyme suitably diluted in 50 mM sodium acetate buffer (pH 5.5). Incubation was done at 65°C for 10 min and the reaction stopped by adding 3 ml dinitrosalicylic acid (DNS) [24]. The mixture was boiled for 5 min and the absorbance was determined at 540 nm. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol of xylose equivalent per minute from xylan.

The protein content was determined by the Bradford method [8] with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using bovine serum albumin as the standard.

Gel Electrophoresis and Determination of Molecular Weight

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [21], using 15% gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. The molecular mass markers used were the broad range SDS-PAGE standards (Bio-Rad, Richmond, CA, U.S.A.). Isoelectric focusing (IEF) gel electrophoresis was carried out using the Mini IEF cell system (Bio-Rad) with ampholytes covering the pH range 3-10 according to the manufacturer's instructions. IEF standards (*p*I 4.45 to 9.6) (Bio-Rad) was used as isoelectric point (*p*I) markers.

To determine the molecular mass of native xylanases by gel filtration, a Sephacryl S-200 column (1.5×70 cm) was used. The purified enzymes and standard proteins were passed through the column in 10 mM sodium phosphate buffer (pH 7.0) with 0.2 M NaCl at a flow rate of 18 ml/h. Protein peaks were monitored at 280 nm. Molecular mass standards used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Enzyme Properties

The effect of pH on the enzyme activity was estimated using xylanase assay in 100 mM citric acid-Na_2HPO_4 (McIlvaine) buffer (pH 3.0-7.5), Tris-HCl buffer (pH 7.5-9.0), and glycine-NaOH buffer (pH 9.0-10.0) at 65°C. The actual pH in the assay mixture was determined at the reaction temperature. The effect of temperature on the activity was assessed by incubating the purified enzymes with 1% (w/v) birchwood xylan in McIlvaine buffer (pH 6.0) at different temperatures in the range of 30°C to 95°C. The thermostabilities of the purified xylanases were monitored by preincubating the enzymes in the absence of substrate at 60°C and 65°C for xylanase A, and 75°C and 80°C for xylanase B. After various times, aliquots were withdrawn and the residual activities were measured under standard assay conditions. Kinetic parameters were determined by incubating the purified enzymes with different amounts of soluble birchwood xylan (1 to 8 mg/ml) in McIlvaine buffer (pH 6.0) at 65°C. For each assay, eight different substrate concentrations were used in three independent experiments. The values of the Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Lineweaver-Burk plots.

Hydrolysis of Xylan and Xylooligosaccharides

The hydrolysis products from birchwood xylan and xylooligosaccharides by purified xylanases were analyzed by thin-layer chromatography (TLC). The hydrolysis was carried out with 1 U of the purified xylanases and 10 mg of birchwood xylan or xylooligosaccharides in 1 ml of 100 mM McIlvaine buffer (pH 6.0) at 60°C for 2 h. Equal amounts of the aliquots was removed periodically and the reaction was stopped by placing the mixture in boiling water for 5 min. A 3 μl portion of each sample was spotted onto a silica gel plate 60 F_{254} (Merck, Darmstadt, Germany) and chromatographed in a solvent system containing n-

Table 1. Purification of xylanases from the culture supernatant of *Paenibacillus* sp. DG-22.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture supernatant	12,110	15.45	784	100
Ultrafiltration	8,998	10.98	819	74.3
CM-Sepharose				
XynA	6,976	0.58	8,944	57.6
XynB	1,334	2.29	883	11.0
Sephacryl S-200				
XynA	4,677	0.39	11,992	38.6
XynB	819	0.90	910	6.8

butanol:ethanol:water (7:1:2, v/v/v) at room temperature. The sugars on the plate were visualized by spraying the plate with 5% sulfuric acid in ethanol, followed by heating at 110°C for 5 min.

Sequencing of N-Terminal Amino Acids

The N-terminal amino acid sequencing of the purified enzymes was done by the Korea Basic Science Institute (Seoul, Korea) using Procise 491 HT protein sequencer (Applied Biosystem). The determined amino acid sequences were compared with protein sequence data available at National Center for Biotechnology Information (NCBI) databases using the online BLAST network service [1].

Results and Discussion

Purification of Xylanases

Paenibacillus sp. DG-22 has been demonstrated to be a potential producer of thermostable xylanases [23]. Extracellular xylanase activity was found to be present in the growth media of *Paenibacillus* sp. DG-22 grown in the presence of xylan. After 24 h of cultivation in the presence of birchwood xylan (0.2%, w/v), the extracellular xylanase activity was detected at 784 U/mg of protein in the culture supernatant (Table 1). The culture supernatant was concentrated by ultrafiltration, and the concentrated fraction was used as a crude enzyme for purification of xylanases. This crude enzyme preparation was taken through the two-step purification of ion-exchange chromatography and gel filtration. Ion-exchange chromatography on CM-Sepharose resulted in two major protein peaks containing the xylanase activity (Fig. 1A). The two forms of xylanase were designated xylanase A (XynA) and xylanase B (XynB). The first active protein component (XynA) on CM-Sepharose was eluted at 0.2 M NaCl using a 0–0.5 M NaCl gradient in 10 mM sodium acetate buffer (pH 5.5) and constituted 84% of the total activity. This fraction had the highest specific and total xylanase activity. The second xylanase (XynB) activity was eluted at 0.32 M NaCl concentration. These two enzyme fractions were further purified to homogeneity by gel filtration on a Sephacryl S-200 HR. The results of these two-step purification are summarized in Table 1. Final purification of the xylanases increased

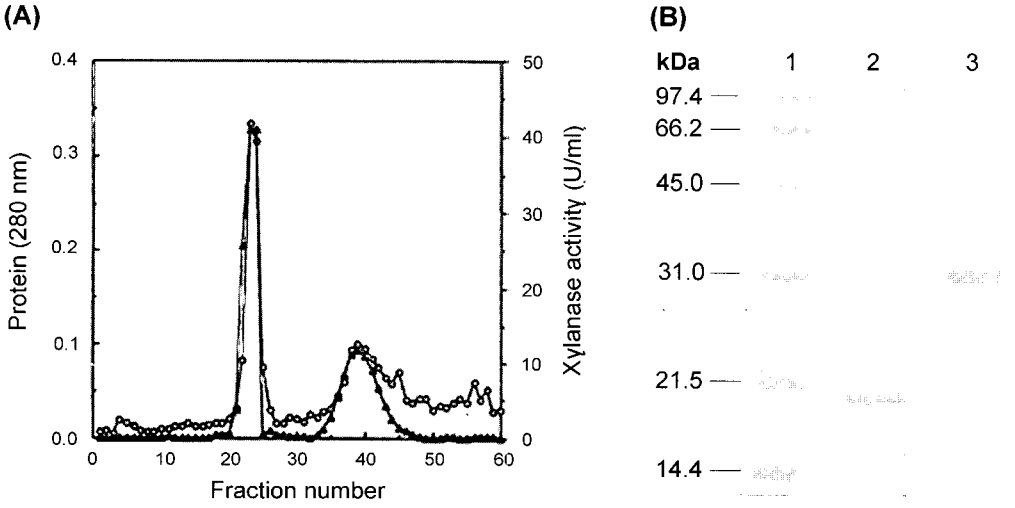

Fig. 1. (A) Elution profile of xylanases from CM-Sepharose column. The column was eluted with a linear gradient of 0.5 M NaCl in 10 mM sodium acetate (pH 5.5). (○) protein, 280 nm; (▲) xylanase activity (U/ml). (B) SDS-PAGE of purified xylanases from *Paenibacillus* sp. DG-22.

Lane 1, molecular mass markers; lane 2, purified xylanase A; lane 3, xylanase B. Molecular mass of marker proteins in kDa is shown on the left.

their specific activities considerably. In comparison, xylanase A from *Paenibacillus* sp. DG-22 showed a significantly higher specific activity of 11,992 U/mg, while xylanase B had a comparatively lower specific activity of 910 U/mg of protein.

Molecular Weights of Xylanases

Purified enzymes were analyzed by SDS-PAGE and gel filtration to estimate the molecular mass. The purity and molecular mass of two purified xylanases were examined by SDS-PAGE (Fig. 1B). The purified enzymes appeared to be homogeneous since two purified xylanase proteins appeared as single bands on SDS-PAGE. The molecular weights as determined by SDS-PAGE were approximately 20 and 30 kDa for xylanases A and B, respectively. The low apparent molecular weight of these enzymes could be advantageous for their pulp application, as such enzymes would have greater access to the xylan component of the wood matrix [30].

Attempt to determine the molecular weights of xylanases by gel filtration was unsuccessful. The native molecular masses of purified enzymes were much lower than those calculated from SDS-PAGE (data not shown). The anomalously low molecular mass estimated by the gel filtration method might be due to interaction between the enzymes and the gel matrix. Such abnormal behavior of xylanases on gel filtration columns have been reported in the case of several low molecular weight xylanases [4, 6, 9, 14, 18].

Properties of Purified Xylanases

The activities of purified xylanases were measured in three different buffers with pH values ranging from 3.0–10.0, and the other experimental conditions were the same as those for the standard assay. Both xylanases showed enzyme activity over a broad pH range of 4.5 to 7.5 at 65°C. The overall pH-activity profiles of the two xylanases appeared quite similar, with an optimum at pH 5.0 to 6.5 (Figs. 2A, 2B). Most bacterial xylanases known so far have their optimum pH around the acid-neutral range [5, 32]. The pH profiles of the purified enzymes indicated that the xylanase activities of both proteins remained considerable in the alkaline pH range. The xylanase B was slightly more alkaline resistant than xylanase A. The xylanase B showed a good level of activity at alkaline pH, exhibiting 80% of its maximum activity at pH 8.5 and over 48% of activity at pH 9.0. These properties may be advantageous in the application of prebleaching of kraft pulps. The optimum temperatures of the purified enzymes were determined by varying the reaction temperature at pH 6.0. As shown in

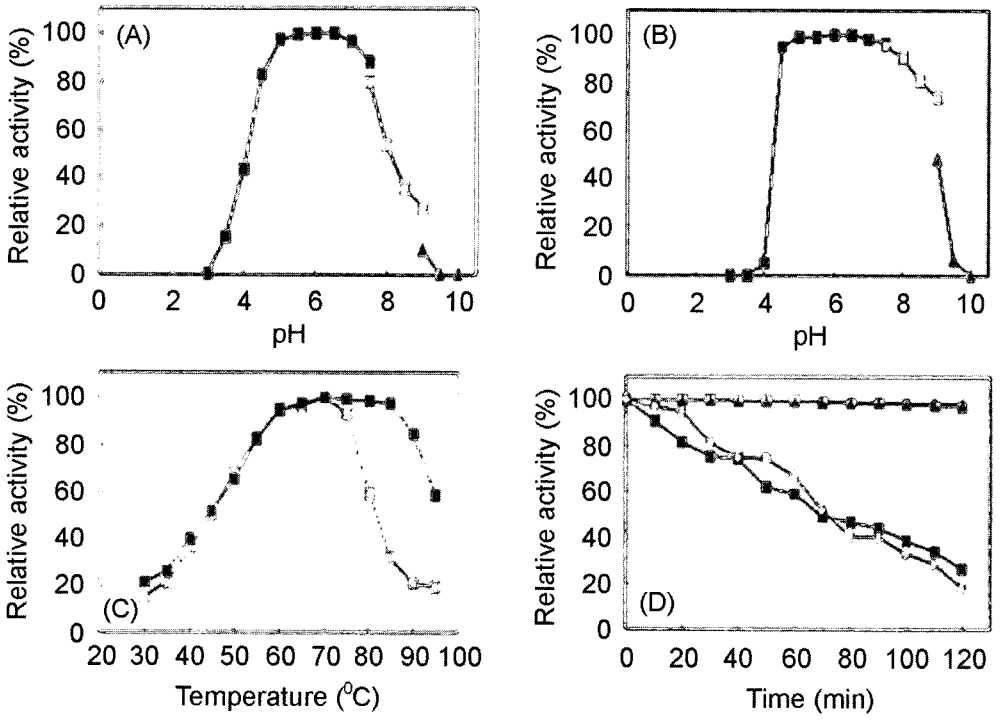

Fig. 2. Effects of pH and temperature on the activities of purified xylanases.

Effect of reaction pH on the activity of purified XynA (A) and XynB (B). Buffers (100 mM) used include citric acid-Na_2HPO_4 (McIlvaine) buffer (■), Tris-HCl buffer (□), and glycine-NaOH buffer (▲). (C) Effect of temperature on the activity of purified XynA (□) and XynB (■). (D) Thermostability of purified xylanases A and B at pH 6.0 in the absence of xylan. Residual activity was monitored at various times after incubation at 60°C (□) and 65°C (■) for XynA, and 75°C (▲) and 80°C (○) for XynB.

Table 2. Properties of purified xylanases A and B from *Paenibacillis* sp. DG-22.

Property	Xylanase A	Xylanase B
Molecular mass (by SDS-PAGE)	20 kDa	30 kDa
Isoelectric point (*p*I)	9.1	8.9
Optimum pH	5.0-6.5	5.0-6.5
Optimum temperature	70°C	70°C
Thermal stability	Up to 60°C	Up to 75°C
Half-life at 65°C	~70 min	ND*
Half-life at 80°C	ND	~70 min
K_m (for birchwood xylan)	2.0 mg/ml	1.2 mg/ml
V_{max}	2,553 U/mg	754 U/mg
Mode of action	Endo-type	Endo-type
Final hydrolysis products (from birchwood xylan)	Xylotriose, xylobiose	Xylotriose, xylobiose, xylose

*ND=Not Determined.

Fig. 2C, xylanases A and B had identical optimum temperatures of 70°C, but XynB was more thermoresistant than XynA with higher relative activity remained at higher temperatures. The optimum temperatures of the two xylanases are higher than those reported for xylanases purified from other moderately thermophilic bacteria [2, 4, 9]. Most bacterial xylanases are optimally active below 65°C, except xylanases from hyperthermophiles [5, 32]. Thermostability experiments for xylanases A and B were performed by incubating the purified enzymes at different temperatures and determining the residual activities at various times. As shown in Fig. 2D, xylanase B was more thermostable than xylanase A. Exposure of the XynA and XynB for 2 h at 60°C and 75°C, respectively, did not affect the activity. At 65°C, the half-life of XynA was about 70 min. The XynB showed a half-life of 70 min at 80°C. The half-lives of the two purified xylanases from *Paenibacillus* sp. DG-22 were greater than those reported for other thermostable xylanases from *Bacillus* spp. For example, the xylanase from *B. licheniformis* [2], although stable at 60°C for 8 h, was highly unstable at temperatures above 70°C. Similarly, the xylanase from *B. amyloliquefaciens* [9] lost nearly all activity at 75°C. The xylanase from thermophile *B. stearothermophilus* [7], which was optimally active at 75°C, demonstrated a half-life of 20 min at this temperature. The properties of these two xylanases may be advantageous in the application of biobleaching of kraft pulps. A suitable enzyme for industrial pulp bleaching must have a high specific activity, low molecular weight, a broad pH range, and high thermostability [30]. Although the xylanases of the strain DG-22 have a low molecular weight, high specific activity and thermostability, it would be interesting to improve their activities at high pH.

Purified xylanases were homogeneous when further examined by isoelectrofocusing. The 20 kDa xylanase A had a *p*I value of 9.1 whereas the 30 kDa xylanase B had a *p*I value of 8.9 (data not shown). Bacterial xylanases are generally classified into two types, the low molecular weight basic proteins and the high molecular weight acidic proteins [12, 35]. The xylanases from *Paenibacillus* sp. DG-22 fall into the former category. The kinetic parameters of purified xylanases were determined at 65°C and pH 6.0 by using soluble birchwood xylan as the substrate. Xylanases A and B differed in their K_m and V_{max} values. The apparent K_m values of purified XynA and XynB were 2.0 mg/ml and 1.2 mg/ml, respectively. The V_{max} values of purified XynA and XynB were calculated to be 2,553 U/mg of protein and 754 U/mg of protein, respectively. The properties of these two xylanases are summarized in Table 2.

Hydrolysis of Xylan

In order to better understand the mode of action of the xylanases from *Paenibacillus* sp. DG-22, the products of the time course of hydrolysis of birchwood xylan by the purified xylanases were analyzed by thin-layer chromatography (Fig. 3). Hydrolysis was carried out under identical conditions of enzyme concentration, substrate, and time. These enzymes degraded xylan at random, and the major end products released were xylobiose, xylotriose, and higher oligosaccharides. However, the TLC profiles of the two enzymes showed some differences in the hydrolysis pattern. The predominant products of hydrolysis by xylanase A appeared to be xylotriose and a small amount of xylobiose was detected (Fig. 3A). In comparison, xylanase B produced comparatively larger amounts of xylobiose and a small amount of xylotriose. Under prolonged incubation, a small amount of xylose was also detected (Fig. 3B). These data indicated that these two xylanases were both endoxylanases that randomly cleave xylan as a substrate. The action of xylanases A and B on xylooligosaccharides was also investigated (Fig. 4). These enzymes had no activity toward xylobiose. Xylanase A cleaved xylotriose and xylotetraose to xylobiose and xylotriose, while xylanase B cleaved xylotriose and xylotetraose to xylobiose and xylose, indicating the mode of action of the two xylanases to be very different although endo in action. Thus, these xylanases required at least three xylose residues for catalytic activity. It should be noted that trace amounts of

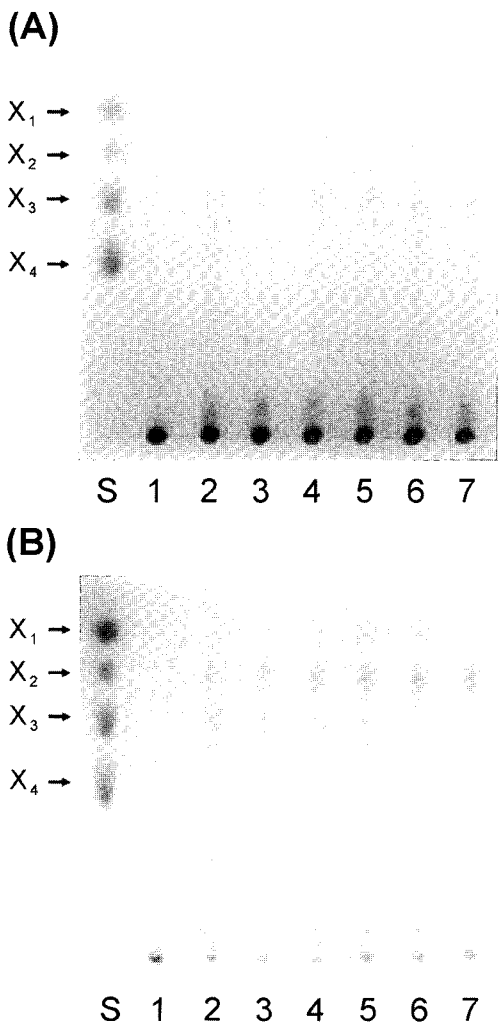

Fig. 3. Thin-layer chromatogram of xylan hydrolysis products catalyzed by the purified XynA (A) and XynB (B).
The purified enzymes were incubated with 1% (w/v) birchwood xylan in 100 mM McIlvaine buffer (pH 6.0) at 60°C for 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), 80 min (lane 5), 100 min (lane 6), and 120 min (lane 7), respectively. The standards (lane S) used were xylose (X_1), xylobiose (X_2), xylotriose (X_3), and xylotetraose (X_4).

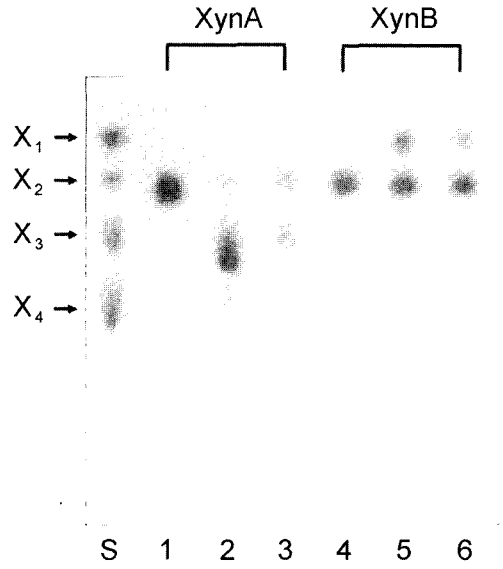

Fig. 4. TLC analysis of the hydrolysis products of xylooligosaccharides by purified xylanases A and B.
The purified enzymes (XynA or XynB) and substrates (X_2: lanes 1, 4; X_3: lanes 2, 5; or X_4: lanes 3, 6) were mixed and incubated at 60°C for 2 h. The standards (lane S) used were X_1, X_2, X_3, and X_4.

xylotetraose and xylopentaose were formed when xylotriose and xylotetraose were used for xylanase A as the substrates. This result suggests that xylanase A has transxylosidase activity, as found with several xylanases from other *Bacillus* spp. [16, 26, 28]. Evidence for the transferase reaction during the hydrolysis of xylooligosaccharides has been also obtained with fungal endoxylanases [10, 29].

Analysis of N-Terminal Sequences

The N-terminal amino acid sequences of the purified xylanases were determined up to 12 residues and compared with protein sequence data available at NCBI databases using the online BLAST network service. [1] (Fig. 5). Xylanases, based on their amino acid sequence and on hydrophobic cluster analysis, have been classified in two families: family 10 and family 11 [15]. Family 11 xylanases seem to be attractive in improving pulp bleaching, as shown by comparing enzymes belonging to both families [11]. Xylanase A from *Paenibacillus* sp. DG-22 can probably be assigned to family 11 based on its high homology to several xylanases of this family (Fig. 5A). The highest homology was found to five of these enzymes: Xylanase A showed 91% (10 of 11 positions) identity with the xylanase I from *Aeromonas caviae* ME-1 [20] and XynA from *Paenibacillus* sp. KCTC 8848P [22], and 82% (9 of 11 positions) identity with xylanases from *Bacillus subtilis* [27], *B. circulans* [36], and *B. halodurans* C-125 [33]. On the other hand, xylanase B from *Paenibacillus* sp. DG-22 did not reveal significant homology to those from other published xylanase sequences. The only xylanase which showed significant homology with xylanase B was XynB from *Paenibacillus* sp. KCTC 8848P [22] (Fig. 5B). No family assignment can be made for xylanase B at this point.

Xylanases are usually secreted to the extracellular medium and, accordingly, have a signal peptide. Comparison of the N-terminal amino acid sequences of the xylanases from the strain DG-22 with those deduced from the DNA sequences of other xylanases suggested that xylanases from *Paenibacillus* sp. DG-22 would have signal peptides. The lack of homology in the amino terminal sequences of these two xylanases strongly suggests that these enzymes are different proteins, products of separate genes. Definite proof of this hypothesis will be obtained by isolation and

Species	Sequence	NCBI Accession number
(A)		
Paenibacillus sp. DG-22	1 A T D Y W Q Y W T D G 11	This study
Aeromonas caviae ME-1	29 A T D Y W Q N W T D G 39	BAA06837
Paenibacillus sp. KCTC 8848P	29 A T D Y W Q N W T D G 39	AAG23526
Bacillus subtilis	30 S T D Y W Q N W T D G 40	I40569
Bacillus circulans	30 S T D Y W Q N W T D G 40	P09850
Bacillus halodurans C-125	28 A N T Y W Q Y W T D G 38	NP-241765
(B)		
Paenibacillus sp. DG-22	1 G L A R - S K F L G N V I 12	This study
Paenibacillus sp. KCTC 8848P	31 G L A N G S K F L G N I I 43	AAG23527

Fig. 5. The N-terminal sequences of the purified xylanases compared with those of other xylanases.
The partial sequences of the xylanases were aligned relatively to XynA (A) and XynB (B) of *Paenibacillus* sp. DG-22 to indicate identical amino acid residues. The numerical numbers show the positions of amino acid residues in their entire sequences. Amino acid sequences that are not identical are in gray.

sequencing of their genes. The cloning of these xylanases is underway in order to fully understand their characteristics and to provide some information at the molecular level.

Acknowledgments

This work was supported by the Dongguk University Research Fund. We thank Mr. Tae-Hyung Lee and Ms. In-Soon Chung for their technical assistance.

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