Cloning and Characterization of a Multidomain GH10 Xylanase from Paenibacillus sp. DG-22

Sun Hwa Lee and Yong-Eok Lee*

Department of Biotechnology, Dongguk University, Gyeongju 780-714, Republic of Korea

The xynC gene, which encodes high molecular weight xylanase from Paenibacillus sp. DG-22, was cloned and expressed in Escherichia coli, and its nucleotide sequence was determined. The xynC gene comprised a 4,419 bp open reading frame encoding 1,472 amino acid residues, including a 27 amino acid signal sequence. Sequence analysis indicated that XynC is a multidomain enzyme composed of two family 4_9 carbohydrate-binding modules (CBMs), a catalytic domain of family 10 glycosyl hydrolases, a family 9 CBM, and three S-layer homologous domains. Recombinant XynC was purified to homogeneity by heat treatment, followed by Avicel affinity chromatography. SDS-PAGE and zymogram analysis of the purified enzyme identified three active truncated xylanase species. Protein sequencing of these truncated proteins showed that all had identical N-terminal sequences. In the protein characterization, recombinant XynC exhibited optimal activity at pH 6.5 and 65°C and remained stable at neutral to alkaline pH (pH 6.0–10.0). The xylanase activity of recombinant XynC was strongly inhibited by 1 mM Cu²⁺ and Hg²⁺, whereas it was noticeably enhanced by 10 mM dithiothreitol. The enzyme exhibited strong activity towards xylans, including beechwood xylan and arabinoxylan, whereas it showed no cellulase activity. The hydrolyzed product patterns of birchwood xylan and xylooligosaccharides by thin-layer chromatography confirmed XynC as an endoxylanase.

Keywords: Xylanase, Paenibacillus, multidomain, cloning and expression

Introduction

Xylan is a major constituent of hemicellulose and represents the second most abundant renewable polysaccharide in plant cell walls. It consists of a linear backbone of β-1,4-linked D-xylopyranose residues, which are substituted by acetyl, glucuronosyl, and arabinofuranosyl residues [5, 20]. Complete enzymatic degradation of xylan requires the cooperative activities of several enzymes, including endoxylanase, β-xylosidase, acetyl xylan esterase, and α-L-arabinofuranosidase [4, 30]. Among these enzymes, endo-β-1,4-D-xylanes (E.C. 3.2.1.8) act as the main xylanolytic enzymes by depolymerizing the xylan backbone into short xylooligosaccharides. Several microorganisms produce multiple xylanases for the effective utilization of xylan. This multiplicity is the result of multiple genes encoding different xylanases with specialized functions for the degradation of xylan [10, 35].

Many xylanases from bacteria and fungi have been cloned and characterized [20, 31]. Based on hydrophobic cluster analyses examining amino acid sequence similarity among their catalytic domains, xylanases are mainly confined to glycoside hydrolase (GH) families 10 and 11 [15]. The general properties of GH10 xylanases include a relatively high molecular weight with low pI values as well as an (α/β)8 barrel fold, which is typical of this family [10, 17, 28]. Although numerous reports have examined GH10 xylanases in terms of gene cloning and enzymatic characterization, there are only a few publications on xylanases containing multiple domains [11, 18, 33, 34, 37]. These multidomain GH10 xylanases have been shown to have similar modular architectures; two to three carbohydrate-binding modules (CBM_4_9 or CBM22), a GH10 catalytic domain, one or two CBM9 modules, and up to three S-layer
homology (SLH) domains, in order from the N-terminus. The role of the CBMs is to potentiate the catalytic activity of carbohydrate-active enzymes by binding to soluble sugars or polysaccharides [1, 11, 13]. SLH domains promote the binding of extracellular enzymes to the cell surface and facilitate the efficient uptake of hydrolysis products by cells on the surface of the substrate [11, 19, 24].

Xylanases have attracted considerable research interest owing to their potential industrial applications in the food, animal feed, and paper and pulp industries [4, 30]. Bleaching paper pulps with xylanases is the principal commercial application of these enzymes. In this respect, thermostable and cellulase-free xylanases are especially preferred since the biomeleaching process is carried out at high temperature [4, 20].

*Paenibacillus* sp. DG-22 is a moderately thermophilic bacterium that grows actively on xylan as a sole carbon source and does not have cellulase activity. This bacterium possesses a multiple xylanase system, two of which (XynA and XynB) have been purified and characterized [23], and a gene encoding low molecular weight xylanase (20 kDa, XynA) was cloned and expressed in *Escherichia coli* [22]. Therefore, to understand the entire function of the xylanolytic system of *Paenibacillus* sp. DG-22, investigations into xylanases and their genes are necessary. In this paper, we report the cloning, sequencing, and expression of the *xynC* gene encoding a multidomain xylanase from *Paenibacillus* sp. DG-22.

**Materials and Methods**

**Strains, Plasmid, and Chemicals**

*Paenibacillus* sp. DG-22 (KEMB 9007-001) was grown as previously described [23] and was used as the source of genomic DNA. *E. coli* DH5α and the plasmid pUC19 were used for genomic library construction. Transformants were grown in LB medium consisting of 1% peptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0) supplemented with 50 µg/ml of ampicillin. Beechwood xylan, birchwood xylan, oat spelt xylan, carboxymethylcellulose (CMC), D(+)-cellobiose, and xylose were purchased from Sigma (St. Louis, USA), whereas crystalline cellulose Avicel PH101 was a product of Fluka. Wheat arabinoxylan and thin-layer chromatography (TLC) standards xylobiose, xylotriolose, and xylotetraose were obtained from Megazyme (Wicklow, Ireland). All other chemicals were of analytical grade.

**Screening, Sequencing, and Sequence Analysis of Xylanase-Positive Clone**

The genomic library of *Paenibacillus* sp. DG-22 was constructed as described previously [22]. Screening of the xylanase-positive clone was carried out on 0.5% birchwood xylan-LB agar plates by Congo red plate assay [36]. The colonies harboring xylanase activity showed clear zones on the plates. Recombinant plasmids were isolated from the xylanase-positive clones, and DNA sequencing was conducted at the Genotech DNA sequencing facility (Daejeon, Korea) by automated sequencing using the dideoxynucleotide chain termination method. The nucleotide sequence was analyzed using the National Center for Biotechnology Information (NCBI) Open Reading Frame (ORF) Finder tool. The signal peptide in the deduced amino acid sequence was predicted by the SignalP 4.0 server [25]. Homology searches in the GenBank database were carried out using the BLAST program [2]. Multiple sequence alignments were carried out by the Clustal Omega program [27].

**Purification of Recombinant Xylanase**

Recombinant enzyme was purified from the xylanase-positive clone harboring the *xynC* gene. The recombinant strain was grown in 500 ml of LB medium containing ampicillin (50 µg/ml) at 37°C. The cells were harvested by centrifugation (4,000 × g for 30 min at 4°C) and resuspended in 20 ml of 50 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM phenylmethanesulfonyl fluoride as a protease inhibitor. The cell suspension was disrupted by sonication using a SONIFIER 450 (Branson, Danbury, USA) on ice, and cell debris was removed by centrifugation (7,000 × g for 30 min at 4°C). The cell extract was heat-treated at 60°C for 10 min in a water bath and then chilled on ice. The denatured proteins were removed by centrifugation (7,000 × g for 30 min at 4°C). The resulting supernatants were loaded onto a 10 ml Avicel affinity column [33], washed with wash buffer (50 mM sodium phosphate, 1 M NaCl, pH 6.5), and eluted with elution buffer (50 mM sodium phosphate, 1% cellobiose, pH 6.5). The active fractions were collected and desalted by dialysis against 50 mM sodium phosphate buffer (pH 6.5). The purified enzyme was used for the analysis of enzymatic properties.

**Enzyme Assay**

Xylanase activity was determined by measuring the amount of reducing sugars liberated from xylan. The reaction mixture (1 ml) contained 0.2% (w/v) birchwood xylan, 50 mM sodium phosphate buffer (pH 6.5), and appropriately diluted enzyme solution. Incubation was carried out at 65°C for 10 min, and the reaction was stopped by adding 4 ml of dinitrosaliclycic acid. The mixture was boiled for 10 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 [7] with Protein Assay reagent (Bio-Rad, Hercules, USA) using bovine serum albumin as the standard.

**Gel Electrophoresis, Zymogram Analysis, and Sequencing of N-Terminal Amino Acids**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% running gel [21], and
Biochemical Characterization of Recombinant Enzyme

The effect of pH on the activity of recombinant xylanase was investigated at various pH values ranging from pH 4.0 to 10.5 at 65°C. The following buffers (each at 50 mM) were used: sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 7.5), Tris-HCl (pH 7.5 to 9.0), and glycine-NaOH (pH 9.0 to 10.5). The pH stability was determined by pre-incubating purified enzyme in the absence of substrate at pH values ranging from pH 4.0 to 10.0 at 45°C for 2 h, and the remaining activity was measured under standard conditions. The effect of temperature on activity was estimated by incubating the purified enzyme at different temperatures ranging from 30°C to 75°C at pH 6.5. The thermostability of the purified enzyme was monitored by pre-incubating the enzyme in the absence of substrate at 50°C, 55°C, and 60°C. After various times, aliquots were withdrawn and the residual activity was measured under standard assay conditions.

The substrate specificity of purified XynC was determined by measuring its enzyme activity under standard assay conditions after replacing birchwood xylan with beechwood xylan, oat spelt xylan, wheat arabinoxylan, Avicel, CMC, and cellulose. The effects of metal ions and some chemicals on recombinant enzyme activity were assessed in the presence of 1 and 10 mM (final concentration) test compounds under standard conditions. Enzyme activities were expressed as a percentage of the activity obtained in the absence of compound. For determination of kinetic parameters, purified enzyme was incubated with birchwood xylan, beechwood xylan, and wheat arabinoxylan at various concentrations under optimal conditions. The $K_m$ and $V_{max}$ values were calculated from a Lineweaver-Burk plot of the Michaelis-Menten equation. All experiments were performed in triplicate, and reported values are the means of three experiments.

Analysis of Xylan Hydrolysis Products

The hydrolysis products from xylooligosaccharides and birchwood xylan by purified xylanase were analyzed by TLC on a silica gel 60 F$_254$ plate (Merck, Darmstadt, Germany) with a mixture of n-butanol:acetic acid:water (6:3:2) as the solvent system. Equal amounts of aliquots were removed periodically, and the reaction was stopped by placing the mixture in boiling water for 5 min. The sugars on the plate were visualized by spraying the plate with reagent consisting of 4 g of α-diphenylamine, 4 ml of aniline, 200 ml of acetone, and 30 ml of 80% phosphoric acid [16], followed by heating at 110°C for 5 min. A xyloooligosaccharide mixture consisting of xylose, xylobiose, xylotriose, and xylotetraose was used as the standard.

Nucleotide Sequence Accession Number

The nucleotide sequence of xynC was deposited in the GenBank database under the accession number KF373081.

Results

Cloning and Nucleotide Sequence Analysis of Xylanase Gene

To elucidate the xylanolytic system of Paenibacillus sp. DG-22, we constructed a genomic library of Paenibacillus sp. DG-22 using E. coli DH5α and pUC19 as a cloning vector. Screening of transformants by Congo red plate assay led to the isolation of three xylanase-positive clones. Recombinant plasmids were isolated from these positive clones, and the nucleotide sequences of their insert DNAs were determined. Two of them contained the same gene (xynA), which has been previously cloned and reported [22]. The other recombinant plasmid, designated as pXC1, contained a 5.5 kb insert DNA consisting of one large open reading frame (ORF) of 4,419 bp. This ORF was shown to encode a hypothetical protein of 1,472 amino acids with a calculated molecular mass of 159.5 kDa and a pl of 4.77. The ORF was shown to have an ATG initiation codon and TGA termination codon, and a signal peptide of 27 amino acid residues was predicted based on SignalP 4.0 analysis.

Upstream of the coding region, putative -35 (TTGATG) and -10 (TAAACT) sequences were confirmed with 18 bp spacing. A putative ribosome binding sequence, GAAGGA, was found 6 bp upstream of the potential ATG initiation codon. Palindromic sequences were localized downstream of the stop codon. The N-terminal amino acid region deduced from this ORF was different from those of XynA and XynB of Paenibacillus sp. DG-22, which were reported in a previous paper [23]. This result indicates that the predicted protein is a third Paenibacillus sp. DG-22 xylanase, and this ORF was thus designated as xynC. BLAST analysis of the deduced amino acid sequence of XynC showed that it had significant similarity with GH10 xylanases. The
A similarity search of the GenBank database using the BLAST program confirmed that XynC is a multidomain enzyme composed of a signal peptide and seven discrete domains; that is, two consecutive CBM_4_9s, GH10 catalytic domain, CBM9, and three consecutive SLH domains in order from the N-terminus (Fig. 1). An identical domain organization has been detected in a xylanase gene, xyn-h39, which was directly cloned from genomic DNA of an alkaline wastewater sludge [37]. Similar domain structures can be found in several xylanases classified in GH family 10, in which two CBM_4_9s are replaced by two or three CBM22, followed by a GH10 catalytic domain and then one or two CBM9 modules, and the C-terminal region is sometimes composed of up to three SLH domains [18, 29, 33]. SignalP analysis of the deduced amino acid sequence confirmed the presence of an N-terminal signal peptide of 27 amino acids, having characteristics of a typical gram-positive bacteria signal peptide. A potential cleavage site was predicted between Ala<sup>27</sup> and Ala<sup>28</sup>, which is a typical Ala-X-Ala motif for signal peptidase I [32]. Removal of the signal peptide would result in a mature protein of 156.7 kDa.

XynC contained three carbohydrate-binding modules (two CBM_4_9s and one CBM9). CBMs are found as discrete domains within carbohydrate-active enzymes and are essential for carbohydrate binding as well as insoluble substrate hydrolysis [1, 6]. The two CBM_4_9 repeats of XynC (CBM_4_9-1 and CBM_4_9-2, extending from positions 33 to 171 and 201 to 339, respectively) shared 27% sequence identity with each other. CBM_4_9-1 showed the highest identities of 61% and 60% with CBM_4_9-1 of xylanases from *Paenibacillus* sp. Y412MC10 (YP_003241008) and *Paenibacillus* sp. HGF5 (WP_009591126), respectively. CBM_4_9-2 showed 56% identity with CBM_4_9-2 from *Paenibacillus* sp. Y412MC10 and *Paenibacillus* sp. HGF5. These two CBM_4_9s in XynC were shown to have about 25% to 46% sequence identity with CBM_4_9s from *Paenibacillus* sp. JDR-2 XynA [29], *Paenibacillus curdlanolyticus* B-6 Xyn10A [33], and *Paenibacillus* sp. β-glucanase [9]. The GH10 catalytic domain of XynC, extending from positions 361 to 701, showed considerable sequence similarity with the catalytic domains of other xylanases in GH family 10 (Fig. 2); for example, 79% sequence identity with endoxylanase from *Paenibacillus* sp. HGF5 (WP_009591126), 62% identity with endoxylanase from *Paenibacillus curdlanolyticus* YK9 (WP_006040335), 45% identity with *Geobacillus stearothermophilus* T-6 XynA [28], and 43% identity with *Thermotoga maritima* MSB8 XynB [17]. CBM9 of XynC, extending from positions 869 to 1054, showed high sequence similarity with CBM9s of other family 10 xylanases; for example, 74% sequence identity with *Paenibacillus* sp. Y412MC10 and endoxylanase from *Paenibacillus curdlanolyticus* YK9 (WP_006040335), and 45% identity with *Thermotoga maritima* XynA [34]. Finally, the C-terminus of XynC included triplicated SLH domains, which are predicted to function in cell surface anchoring [19, 24]. The SLH domains of XynC, ranging from positions 1294 to 1463, showed 56% to 75% sequence identity with those of *Paenibacillus* sp. JDR-2 XynA, *Paenibacillus* sp. Y412MC10 endoxylanase, and *Paenibacillus* sp. HGF5 xylanase.

**Purification of Recombinant XynC**

The xynC gene cloned into vector pUC19 was expressed in *E. coli* DH5α. It has been shown that the CBM9 of several GH10 xylanases, such as *T. maritima* Xyn10A, *Clostridium stercorarium* Xyn10B, and *Paenibacillus* sp. W-61 Xyn5, has the ability to bind to crystalline cellulose and Avicel PH-101 [1, 18, 34]. Recombinant XynC (rXynC) also showed significant binding to Avicel PH-101. For elution of the bound enzyme, several mono- or disaccharides as well as soluble xylans were tested as eluents. Only cellobiose successfully eluted the bound enzyme (data not shown).

![Fig. 1. Schematic diagram of *Paenibacillus* sp. DG-22 XynC domain structure.](image-url)

The numbers indicate the amino acid position in XynC: 1–27 signal peptide (SP); 33–171 and 201–339 CBM_4_9s; 361–701 GH10 catalytic domain (CD); 869–1054 CBM9; 1294–1337, 1335–1398, and 1420–1463 SLH domains.
rXynC was purified by heat treatment and Avicel affinity chromatography. Heat treatment of the cell extract at 60°C for 10 min was a very efficient purification step for the xylanase from *E. coli* (pXC1), since most of the thermolabile *E. coli* proteins could be removed by this procedure. After heat treatment, the specific activity increased 3.3-fold, with a recovery yield of 90%. Final purification was performed by affinity chromatography with Avicel PH-101, which increased the specific activity of rXynC 6.7-fold, with a recovery yield of 49.5% (Table 1). The purified rXynC was then analyzed by SDS-PAGE and zymography (Fig. 3). SDS-PAGE of the purified enzyme resulted in three protein bands with approximate molecular masses of 128 kDa (XynC1), 100 kDa (XynC2), and 82 kDa (XynC3). Zymogram
analysis demonstrated that these three protein bands have xylanase activity. The molecular masses of these fragments were all smaller than the predicted molecular mass of mature XynC (156.7 kDa), suggesting that rXynC was truncated by proteolytic cleavage. N-Terminal amino acid sequencing of these truncated proteins found that all three had identical amino acid sequences, AAPQIGDVIL, which coincided precisely with residues Ala$_{28}$ to Leu$_{37}$ of the deduced amino acid sequence of XynC. These results indicate that rXynC was truncated at its C-terminal region by host proteases to yield a smaller molecular species and that the N-terminal sequence of 27 amino acids was functional as a signal peptide in _E. coli_. Judging from the molecular masses of these proteins, all three truncated xylanases appear to contain the CBM_4_9 and GH10 domains. In addition to CBM_4_9 and GH10, XynC$_1$ had an entire CBM9 domain, whereas XynC$_2$ had 60% of CBM9. The entire CBM9 domain was deleted in XynC$_3$.

**Biochemical Characterization**

The purified enzyme contained three truncated forms of XynC. We used this heterogeneous mixture for biochemical characterization. The effects of pH and temperature on the activity and stability of purified rXynC were determined. The optimum pH of rXynC was determined in four different buffers ranging from pH 4 to 10.5 at 65°C, using birchwood xylan as the substrate. The purified enzyme showed an optimum pH of 6.5 and retained greater than 60% of its activity at pH 9.5 (Fig. 4A). Purified rXynC was fairly stable over an alkaline pH range, as greater than 85% of its activity was retained at pH 10.0 when treated at 45°C for 2 h (Fig. 4B). The activity of rXynC was also measured at temperatures from 30°C to 75°C. After reaction for 10 min, the optimum temperature was 65°C (Fig. 4C), while enzyme activity rapidly decreased at 75°C. To examine the thermostability of rXynC, the purified enzyme was incubated at 50°C, 55°C, and 60°C without substrate for up to 4 h, after which residual activities were measured. rXynC was stable at 50°C and retained 65% of its initial activity after 4 h of pre-incubation at 55°C, but its activity was gradually decreased at 60°C. The half-life of rXynC was about 1 h at 60°C (Fig. 4D).

The effects of various metal ions and chemical reagents at concentrations of 1 and 10 mM on rXynC activity were measured in the absence of metal ions or chemicals was taken as 100%. Values presented are the averages and standard deviations of three independent experiments.

### Table 1. Purification of rXynC from _E. coli_ DH5α harboring pXC1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>176</td>
<td>11,934</td>
<td>67.8</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>48</td>
<td>10,747</td>
<td>223.9</td>
<td>3.3</td>
<td>90.0</td>
</tr>
<tr>
<td>Avicel PH-101</td>
<td>13</td>
<td>5,907</td>
<td>454.4</td>
<td>6.7</td>
<td>49.5</td>
</tr>
</tbody>
</table>

### Table 2. Effects of metal ions and chemicals on rXynC activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>1 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 0.1</td>
<td>100 ± 0.1</td>
</tr>
<tr>
<td>AgCl</td>
<td>96.1 ± 3.8</td>
<td>95.2 ± 1.4</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>106.9 ± 2.7</td>
<td>98.7 ± 4.6</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>101.7 ± 3.2</td>
<td>69.3 ± 2.3</td>
</tr>
<tr>
<td>CuCl$_2$</td>
<td>4.8 ± 0.7</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>FeCl$_2$</td>
<td>104.5 ± 4.5</td>
<td>46.7 ± 1.0</td>
</tr>
<tr>
<td>HgCl$_2$</td>
<td>4.2 ± 0.7</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>KCl</td>
<td>100.4 ± 3.2</td>
<td>95.9 ± 2.2</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>103.9 ± 4.4</td>
<td>95.7 ± 2.0</td>
</tr>
<tr>
<td>NiSO$_4$</td>
<td>51.0 ± 3.5</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>85.5 ± 3.0</td>
<td>66.1 ± 4.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>73.3 ± 2.9</td>
<td>70.9 ± 3.1</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>101.8 ± 4.6</td>
<td>92.9 ± 0.7</td>
</tr>
<tr>
<td>DTT</td>
<td>100.6 ± 4.7</td>
<td>131.7 ± 2.5</td>
</tr>
<tr>
<td>SDS</td>
<td>97.3 ± 2.1</td>
<td>14.0 ± 0.1</td>
</tr>
</tbody>
</table>

Enzyme activity assayed in the absence of metal ions or chemicals was taken as 100%. Values presented are the averages and standard deviations of three independent experiments.

**Fig. 3.** SDS-PAGE and zymography of purified rXynC. Lane M, molecular weight marker; lane 1, cell extract; lane 2, proteins after heat treatment; lane 3, purified rXynC after Avicel affinity chromatography; lane 4, zymography of purified rXynC.
measured (Table 2). rXynC activity was strongly inhibited by 1 mM Cu$^{2+}$ and Hg$^{2+}$ as well as 10 mM Ni$^{2+}$. The enzyme was moderately inhibited by 10 mM Co$^{2+}$, Fe$^{2+}$, and Zn$^{2+}$. No effect was detected with Ag$^+$, Ca$^{2+}$, K$^+$, and Mg$^{2+}$. rXynC was activated by 10 mM dithiothreitol (DTT), but β-mercaptoethanol had no effect on enzyme activity. The chelating agent EDTA reduced enzyme activity to 73% at a concentration of 1 mM, and the enzyme activity was markedly inhibited in the presence of 10 mM SDS.

Substrate Specificity and Kinetic Analysis
The hydrolytic activities of purified rXynC on various substrates were determined (Table 3). The enzyme was highly active on xylans from hardwood (beechwood and birchwood) and cereals (oat spelt and wheat arabinoxylan). Based on the rXynC activity towards birchwood xylan

**Table 3. Substrate specificity of purified rXynC.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylan from birchwood</td>
<td>100</td>
</tr>
<tr>
<td>Xylan from beechwood</td>
<td>129.4</td>
</tr>
<tr>
<td>Xylan from oat spelt</td>
<td>53.4</td>
</tr>
<tr>
<td>Wheat arabinoxylan</td>
<td>115.9</td>
</tr>
<tr>
<td>Avicel</td>
<td>Not detected</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>Not detected</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Enzyme activity assayed with birchwood xylan was taken as 100%. Values presented are the means of three independent experiments.
being defined as 100%, the enzyme exhibited high activity on beechwood xylan (129%), followed by wheat arabinoxylan (116%) and oat spelt xylan (53%). There was no detectable activity on Avicel, carboxymethylcellulose (CMC), or cellulose.

The kinetic parameters of purified rXynC were determined for birchwood xylan, beechwood xylan, and wheat arabinoxylan at 65°C and pH 6.5. The calculated $K_m$ and $V_{max}$ values were 21.28 mg/ml and 358.49 U/mg protein for birchwood xylan, 3.49 mg/ml and 201.34 U/mg for beechwood xylan, and 12.23 mg/ml and 396.71 U/mg for wheat arabinoxylan, respectively.

Hydrolysis Product Analysis

The hydrolysis products of birchwood xylan by purified recombinant XynC were analyzed by TLC (Fig. 5). rXynC released xylooligosaccharides of various length at the initial stage of xylan hydrolysis, confirming rXynC as a typical endoxylanase. After 2 h of incubation, the major products of hydrolysis were xylobiose, xylotriose, xylotetraose, and short-chain xylooligosaccharides. The ability of purified rXynC to hydrolyze shorter xylooligosaccharides was also investigated. rXynC showed no detectable activity on xylobiose, whereas xylotriose and xylotetraose were hydrolyzed to xylobiose and xylose (Fig. 5). Thus, rXynC required at least three xylose residues for catalytic activity. Generally, GH family 10 xylanases generate xylobiose and xylotriose as prominent products during the depolymerization of xylan [10, 16, 18, 33].

Discussion

*Paenibacillus* sp. DG-22 produces at least three extracellular xylanases based on zymogram analysis [23]. This multiplicity of xylanases is common in microorganisms. To deal with the heterogeneous structure of xylan, microbes produce several specialized xylanases, all of which have different enzymatic properties and substrate specificities [35]. In previous papers, we purified and characterized two xylanases (XynA and XynB) as well as cloned the *xynA* gene from *Paenibacillus* sp. DG-22 [22, 23]. To investigate the xylanolytic system of this bacterium, we cloned and expressed the *xynC* gene in *E. coli* and studied its enzymatic properties.

Homology searches revealed that XynC from *Paenibacillus* sp. DG-22 is a multidomain enzyme classified as an endo-1,4-β-xylanase of GH family 10. XynC is one of the largest xylanases from bacteria and is composed of an N-terminal signal peptide and seven domains. After cleavage of the signal peptide comprising 27 amino acids, mature XynC was shown to consist of 1,445 amino acid residues. Although XynC had no cellulolytic activity, it showed significant binding ability toward Avicel. When purified xylanase was analyzed by SDS-PAGE, three truncated proteins having molecular masses lower than that of mature XynC (156.7 kDa) were detected. Zymography showed that all of these truncated proteins had xylanase activities (Fig. 3). The N-terminal amino acid sequences of these three truncated xylanases were identical, indicating that these proteins were produced by C-terminal proteolytic cleavage of full-length XynC. Proteolytic cleavage of cloned enzymes in *E. coli* has also been reported for *Clostridium thermocellum* XynX [26] and *Clostridium josui* Xyn10A [11].

Purification of the truncated enzymes by Avicel affinity chromatography showed that the cellulose-binding ability of these proteins was conferred by CBMs. CBMs are essential for carbohydrate binding, and enhance enzyme efficiency by increasing the accessibility of insoluble substrates to the enzyme catalytic module [1, 11, 13]. XynC has three carbohydrate-binding modules (two CBM_{4,9}s and one CBM9). The CBM_{4,9} motif is found in numerous
glycosylases such as cellulase, xylanase, and glucanase and is located either at the N- or C-terminus, either as a single copy or as repeats. Members of this family are thought to be capable of binding to a variety of polysaccharides as well as increasing the efficiency of substrate degradation [9, 16, 37]. The CBM9s of Clostridium stercorarium Xyn10B [1], Clostridium thermocellum XynX [26], and Thermotoga maritima Xyn10A [6] were shown to bind to cellulose and insoluble xylan. rXynC₁ bound to Avicel as a result of the presence of two CBM_4.9s and CBM9, judging from its size. Unexpectedly, rXynC₂, which was truncated within its CBM9, and rXynC₃, which totally lacked CBM9, also bound to Avicel. A similar result has been reported for Clostridium josui Xyn10A, in which an 85 kDa protein truncated within CBM9 was shown to bind to cellulose [11]. Since rXynC₂ and rXynC₃ do not contain a functional CBM9, it is possible that the CBM_4.9 repeats of XynC are responsible for binding to cellulose. Cheng et al. [9] demonstrated that binding of endo-β-1,3-glucanase from Paenibacillus sp. to Avicel could be attributed to CBM_4.9. Therefore, binding of rXynC to Avicel might also be due to the presence of CBM_4.9 repeats. However, the function of CBM_4.9 remains controversial, and the binding specificities of CBM_4.9 and CBM9 remain to be addressed. It has been suggested that the presence of CBMs in xylanases promotes binding to cellulose, resulting in a higher concentration of xylan, which coexists with cellulose in plant cell walls [11, 18, 26]. Although the precise function of CBMs in xylan degradation remains unclear, the high level of conservation of these modules in xylanases across diverse bacteria suggests that these modules are integral to xylan degradation [14].

The general property of family 10 xylanases is their relatively high molecular weight, low pI values, and an (α/β)₉ barrel fold [10]. The crystal structures of several GH10 xylanases have been reported, and two glutamates were identified as catalytic residues [17, 28]. Two catalytic Glu residues (Glu⁴⁹⁵ and Glu⁶⁰¹), which are considered to be involved in general acid-base catalysis, are well conserved in XynC from Paenibacillus sp. DG-22 (Fig. 2). The C-terminal region of XynC includes three SLH domains, which are predicted to function in cell surface anchoring [19, 24]. SLH domains are found in several surface layer proteins and extracellular enzymes acting on polysaccharides. It has been suggested that secreted enzymes attach to the cell surface via SLH domains, which allows the cells to attach to the surface of the substrate [11]. Therefore, XynC from Paenibacillus sp. DG-22 might be a cell-surface-anchored modular xylanase possessing cellulose-binding domains. The multiple modular structure of XynC could combine bacterial cells with cellulose, leading to efficient hydrolysis of neighboring xylans by XynC as well as efficient uptake of hydrolysis products on the surface of plant cell walls, as demonstrated in Xyn5 from Paenibacillus sp. strain W-61 [18].

Some metal ions and reagents are known to affect xylanase activities. As a common trend, the enzyme activity of many xylanases is inhibited by sulfydryl oxidant heavy metals, such as Cu²⁺ and Hg²⁺ [3, 8]. In the present study, rXynC activity was almost completely inhibited by Cu²⁺, Hg²⁺, and Ni²⁺ as well as moderately inhibited by Co²⁺, Fe²⁺, and Zn²⁺. rXynC was activated by 10 mM DTT (Table 2). Enhancement of xylanase activity by the reducing agent DTT was also reported for xylanases from B. amylobacter [8] and Bacillus sp. SPS-0 [3]. Although rXynC activity was moderately inhibited by EDTA, enhancement of xylanase activity in the presence of metal ions was not detected. These results suggest that Paenibacillus sp. DG-22 XynC may not require metal ions for enzyme activity.

Application of xylanase for the purpose of pulp bleaching requires strong activity and stability at high temperature under alkaline conditions [4, 10]. The XynC produced by Paenibacillus sp. DG-22 showed high activity against several xylans, but no cellulase activity. At pH 10.0, rXynC retained greater than 85% of its maximum activity. These properties of rXynC suggest it can be used in biotechnological applications, especially in kraft pulp bleaching in the paper industry.

Acknowledgments

This work was supported by the Dongguk University Research Fund of 2014.

References

within the multidomain 120 kDa xylanase XynA of the hyperthermophilic bacterium Thermotoga maritima. Mol. Microbiol. 15: 431-444.

