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Biocatalytic Properties and Substrate-binding Ability of a Modular GH10 β-1,4-Xylanase from an Insect-symbiotic Bacterium, *Streptomyces mexicanus* HY-14

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The gene (1350-bp) encoding a modular β-1,4-xylanase (XylU), which consists of an N-terminal catalytic GH10 domain and a C-terminal carbohydrate-binding module 2 (CBM 2), from *Streptomyces mexicanus* HY-14 was cloned and functionally characterized. The purified His-tagged recombinant enzyme (rXylU, 44.0 kDa) was capable of efficiently hydrolyze diverse xylosidic compounds, *p*-nitrophenyl-cellobioside, and *p*-nitrophenyl-xylopyranoside when incubated at pH 5.5 and 65°C. Especially, the specific activities (649.8 U/mg and 587.0 U/mg, respectively) of rXylU toward oat spelts xylan and beechwood xylan were relatively higher than those (<500.0 U/mg) of many other GH10 homologs toward the same substrates. The results of enzymatic degradation of birchwood xylan and xylooligosaccharides (xylotriose to xylohexaose) revealed that rXylU preferentially hydrolyzed the substrates to xylobiose (>75%) as the primary degradation product. Moreover, a small amount (4%<) of xylose was detected as the degradation product of the evaluated xylosidic substrates, indicating that rXylU was a peculiar GH10 β-1,4-xylanase with substrate specificity, which was different from its retaining homologs. A significant reduction of the binding ability of rXylU caused by deletion of the C-terminal CBM 2 to various insoluble substrates strongly suggested that the additional domain might considerably contribute to the enzyme-substrate interaction.

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Introduction

Lignocellulosic biomass, which is made mainly of cellulose, hemicelluloses such as β-1,4-xylan and β-1,4-mannan, and lignin, has attracted much industrial attention as eco-freindly renewable resources for the preparation of ultimately various biological compounds. In environments, complete mineralization of plant biomass is generally accomplished by cooperative action of diverse lignocellulose-degrading enzymes (Wackett, 2008).

β-1,4-Xylanases are representative glycoside hydrolase (GH) enzymes, which play a critical role in the degradation of β-1,4-xylan polysaccharides and their oligosaccharides by the random cleavage of β-1,4-xylosidic bonds in the backbone (Shallom and Shoham, 2003). These GH enzymes are generally categorized into six GH families (5, 8, 10, 11, 30, and 43) based on their primary structure (http://www.cazy.org/Glycoside-Hydrolases.html) but recently, a retaining β-1,4-xylanase from *Cellulosimicrobium* sp. strain HY-13, which is a structural homolog of inverting GH6 cellobiohydrolases, has also been identified (Kim *et al.*, 2012). However, most of the identified β-1,4-xylanases are affiliated with the following two GH families (10 and 11). GH10 β-1,4-xylanases are more active against a wide variety of branched or glucose-derived substrates when compared to GH11 β-1,4-xylanases, while β-1,4-xylanases of GH family 11 generally display higher biocatalytic activities against xylosidic compounds (Ducros *et al.*, 2000; Pell *et al.*, 2004). Moreover, many GH10 β-1,4-xylanases are multi-domain enzymes, which are composed of two or more substrate-binding modules, such as xylan-binding domain, cellulose-binding domain, carbohydrate-binding module (CBM), and ricin domain, combined to the N- and/or C-terminus region(s) of their catalytic GH10 domain (http://www.cazy.org/Glycoside-Hydrolases.html).

In recent, gut microorganisms of herbivorous insects have drawn a great deal of industrial attention because some of them have diverse GH enzymes with unique biocatalytic functions that are able to efficiently deconstruct plant biomass to valuable compounds such as prebiotics and fermentable saccharides for bioenergy production (Warnecke *et al.*, 2007; Juturu and Wu, 2012). Similar to other herbivorous insects (Brenann *et al.*, 2004; Heo *et al.*, 2006; Oh *et al.*, 2008), a recent study has shown that mole crickets also

contain various hemicellulosic biomass-degrading prokaryotic microbiota in their digestive tracts, which may participate in the digestion of plant roots and seeds. Until now, however, only two non-modular GH10 enzymes, *Cellulosimicrobium* sp. HY-12 β-1,4-xylanase (XylA) (Oh *et al.*, 2008) and *Microbacterium trichothecenolyticum* HY-17 β-1,4-xylanase (XylH) (Kim *et al.*, 2014), which are hemicellulolytic bacteria in the gut of the mole cricket *Gryllotalpa orientalis*, have been identified and characterized. Thus, more intensive study on various mole cricket-symbiotic gut bacteria and their glycoside hydrolases will encourage the discovery and industrial application of novel plant biomass-degrading enzymes.

Here, we describe the molecular and biochemical characteristics of a modular GH10 β-1,4-xylanase (XylU) from the strain HY-14, a gastrointestinal bacterial isolate of *G. orientalis*, which digestes plant biomass such as plant roots and seeds. Moreover, the binding affinity of its CBM 2 to diverse insoluble polymers is reported.

Materials and Methods

Chemicals

β-1,4-D-Xylooligosaccharides of xylobiose to xylohexaose, azo-xylan, xyloglucan, wheat arabinoxylan, and ivory nut mannan were obtained from Megazyme International Ireland Ltd. (Ireland) and pectin and chitosan were provided by USB Co. (USA). Poly(3-hydroxybutyrate) granules were extracted from glucose-grown cells of *Wautersia eutropha* KHB-8862 (Chung *et al.*, 2001). All other compounds including *p*-nitrophenyl (*p*NP)-sugar derivatives, xylose, and xylans from birchwood, beechwood, and oat spelt were purchased from Sigma Chemical Co. (USA).

Isolation and identification of a xylan-degrading microorganism

A xylanolytic Gram-positive bacterium, strain HY-14 [previously designated as strain RU-31 in Fig. 1 of our recent paper (Kim *et al.*, 2014)] was isolated and identified from the digestive tract of the mole cricket *G. orientalis.* The 16S rRNA gene sequence (GenBank accession number: JQ943651) of strain HY-14 was aligned with those of strains belonging to the genus *Streptomyces* using the CLUSTAL_X program.

Molecular cloning of the XylU gene

To amplify a partial sequence of the XylU gene from the genomic DNA, degenerate PCR primers were designed based on sequence regions (WDVVNE and ITELDI) shared by some GH10 β-1,4-xylanases (Fig. 1 and http://www.cazy.org/Glycoside-Hydrolases.html). The upstream primer (GF) was 5′-TGGGACGTCSTCAACGAG-3′ and the downstream primer (GR) was 5′-GATGTCGAGCTCSGTGAT-3′, which produced a 351 bp DNA fragment. The full XylU gene was cloned by repeated genomic walking and nested PCR protocols using a DNA Walking *SpeedUp*™ Premix Kit (Seegene Inc., Korea), according to the manufacturer's instructions.

Overproduction and purification of His-tagged rXylU

To overproduce XylU, its encoding gene was cloned into the *Nde*I/*Hin*dIII sites of a pET-28a(+) vector (Novagen, Germany). For this, the full XylU gene with *Nde*I and *Hin*dIII sites in the N-terminus and the C-terminus, respectively, was amplified by PCR using the genomic DNA of *S. mexicanus* HY-14 as a template, according to the method described by Kim *et al.* (2010a). The primer sequences with the *Nde*I and *Hin*dIII sites, respectively, are as follows: the upstream primer (F-GN) was 5′-CATATGGCCGACACGCTGGGCTC-3′ and the downstream primer (R-GH) was 5′-AAGCTTTCACGACGCCGTGCAGG-3′. Similarly, to overproduce recombinant XylUΔCBM 2, a partial sequence containing the catalytic GH10 domain (Ala31 to Gly339) of XylU without a signal sequence was amplified using the oligonucleotides F-GN1 (5′-CATATGGCCGACACGCTGGGCTC-3′) and R-GH1 (5′-AAGCTTTCAGGCGGCACCGGTGT-3′). The purified PCR products were cloned into a pGEM-T easy vector (Promega, USA), and the pGEM-T easy/*xylU* vectors were digested with *Nde*I and *Hin*dIII to generate the *xylU* fragments with the corresponding sticky ends. To overexpress and purify the rXylU, the generated *xylU* fragments were ligated into a pET-28a(+) vector with the same ends, followed by transformation of the resulting pET-28a (+)/*xylU* into *E. coli* BL21. Overproduction of the rXylU was then conducted by cultivating the recombinant *E. coli* BL21 cells harboring pET-28a(+)/*xylU*, as described elsewhere (Kim *et al.*, 2010b). Lysis of the harvested cells was conducted by sonication, after which the soluble fraction containing the rXylU proteins was collected and then directly used as the crude enzyme source. In this study, enzyme purification was conducted using a HisTrap™ HP (GE Healthcare, Sweden) (5 × 5 ml) column attached to a fast-protein liquid chromatography system (FPLC) system (Amersham Pharmacia Biotech, Sweden), as described elsewhere (Kim *et al.*, 2014). The relative molecular mass of the denatured rXylU and rXylUΔCBM 2 was determined by SDS-PAGE on a 12% gel. The separated proteins were stained with Coomassie brilliant blue R-250. The protein concentrations were determined using the Bradford reagent (Bio-Rad Korea Ltd., Korea).

Enzyme assays

β-1,4-Xylanase activity was assayed by measuring the amount of reducing sugars released from birchwood xylan by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose as a standard. The standard assay mixture (0.5 ml) consisted of 1.0% birchwood xylan and suitably diluted enzyme solution (0.05 ml) in a 50 mM sodium phosphate buffer (pH 5.5) and the enzyme reaction was conducted at 60°C for 15 min. One unit (U) of β-1,4-xylanase activity for xylans was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min under standard assay conditions. Sugars (5 mM) substituted with *p*-nitrophenol (*p*NP) were employed to assay other degradation activities of the purified enzyme. The activity assay of rXylU toward *p*NP-sugar derivatives was conducted under the same conditions used in the standard assay of xylanolytic activity. One unit (U) of cleavage activity for *p*NP-sugar derivatives

Table 1. Effects of metal ions (1 mM) and chemical reagents (5 mM) on rXylU activity

Compound	Relative activity (%)
None	100.0
$HgCl_2$	0.0
$CaCl_2$	100.0
$NiSO_4$	100.1
$CuCl_2$	87.5
$ZnSO_4$	86.3
$MgSO_4$	94.3
$MnCl_2$	109.6
$SnCl_2$	91.5
$BaCl_2$	88.7
$CoCl_2$	89.5
$FeSO_4$	105.2
N-Bromosuccinimide	1.4
Iodoacetamide	93.9
Sodium azide	109.2
N-Ethylmaleimide	117.4
EDTA	95.9
Tween 80 (0.5%)	130.7
Triton X-100 (0.5%)	129.1

was defined as the amount of enzyme required to produce 1 μmol of *p*NP per min under standard assay conditions.

Effects of pH, temperature, and chemicals on the enzyme activity

The optimal pH of the purified rXylU was evaluated at a range of 4.0 to 10.5 using the following buffers (50 mM) at 60°C for 15 min: sodium citrate (pH 4.0 to 5.5), sodium phosphate (pH 5.5 to 7.5), Tris-HCl (pH 7.5 to 9.0), and glycine-NaOH (pH 9.0 to 10.5). The effect of temperature on the maximum activity of rXylU was determined at 40, 45, 50, 55, 60, 65, 70, and 75°C under the standard assay conditions. To evaluate the thermostability of rXylU, it was pre-incubated at the corresponding temperature for 1 h in 50 mM sodium phosphate buffer (pH 5.5), after which the enzyme reaction was initiated by adding the substrate to the reaction mixture. To assess the effects on the β-1,4-xylanase activity of metal ions (1 mM) and chemical reagents (5 mM or 0.5%), rXylU was pre-incubated at room temperature for 10 min in 50 mM sodium phosphate buffer (pH 5.5) containing the compound of interest at the indicated concentration (Table 1). The enzyme reaction was then started by adding the substrate to the reaction mixture at 60°C for 15 min.

Identification of the hydrolysis products

To identify the hydrolysis products, enzymatic degradation of birchwood xylan (10 mg) was conducted using the purified rXylU (2 μg) in 0.5 ml of 50 mM sodium phosphate buffer (pH 5.5) for 12 h at 30°C, during which time the enzyme remained fairly stable. The reaction was then heated to 100°C for 5 min to stop the enzyme reaction. Similarly, xylooligosaccharides (xylobiose to xylohexaose, each 1 mg) were digested under the same hydrolytic conditions for 3 h. The hydrolysis products were then analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS), as demonstrated in detail elsewhere (Kim *et al.*, 2014).

Binding assay

Insoluble polymers such as mannan from ivory nut, xylan from oat spelts, curdlan from *Alcaligenes faecalis*, chitosan from crab shells, chitin from crab shells, Avicel PH-101, lignin, and poly(3HB) granules were employed as candidate substrates to evaluate the binding ability of rXylU and rXylUΔCBM 2. Prior to the binding assay, the insoluble substrates were washed five times in 50 mM sodium phosphate buffer (pH 5.5) and the binding capacity of the enzyme to hydrophobic materials was investigated. Specifically, the suitably diluted enzyme preparation (approximately 5.0 U/ml) was incubated in 1.5 ml Eppendorf tubes on ice for 2 h with an equal volume of substrate polymer and stirred every 5 min. After centrifugation (12,000 × *g*) of the reaction mixtures, the supernatant was carefully recovered and applied directly for analysis of the remaining β-1,4-xylanase activity and protein concentration.

Results and Discussion

Identification of a xylanolytic isolate

A xylan-degading bacterium, strain HY-14 was isolated from the gut of a mole cricket, *G. orientalis*, according to the method described in our recent paper (Kim *et al.*, 2014). Phylogenetic analysis of the nucleotide sequence of the strain HY-14 16S rRNA gene revealed that it was closely related to *Streptomyces mexicanus* CH-M-1035^T, with a sequence similarity of 100.0%. Based on this result, the isolated strain HY-14 was identified as *S. mexicanus* and deposited in the Korean Collection for Type Cultures under code no. *S. mexicanus* HY-14 KCTC 12531BP.

Genetic characterization of XylU gene

The XylU gene (GenBank accession no.: KM027334) isolated in this study contained a 1350-bp open reading frame (ORF) that encodes a protein of 449 amino acids (Fig. 1). A deduced molecular mass (M_r) and a calculated pI of XylU were estimated to be 46,774 Da and 6.24, respectively. It was predicted that the signal peptide cleavage site of premature XylU was between Ala30 and Ala31, which may generate a mature XylU of 419 amino acids with a deduced M_r of 43,838 Da and a calculated pI of 5.82. When the XylU sequence was aligned with other β-1,4-xylanases available in the NCBI database, it was found to possess the molecular features of an β-1,4-xylanase with a sequence similarity similar to that of the GH10 β-1,4-xylanases. Moreover, the sequence similarity levels of XylU with the β-1,4-xylanases in GH family 10, which have not yet been biochemically characterized, were relatively high. Specifically, the highest sequence similarity was 91%, and this was obtained when XylU was compared to the primary structure of *Streptomyces* sp. Ame12xE9 β-1,4-xylanase (accession no.: WP_019983605). In addition, XylU was also 84, 82, and 77% identical to *Streptomyces lividans* TK24 β-1,4-xylanase (WP_

003978188), *Streptomyces pristinaespiralis* ATCC 25486 β-1,4-xylanase (WP_005310297), and *Streptomyces megasporus* β-1,4-xylanase (ADZ99362), respectively. The results of a protein BLAST survey revealed that XylU consisted of an N-terminal GH10 domain (from Tyr44 to Leu332) and a C-terminal CBM 2 domain (from Cys351 to Ser449) that was most similar to the domain structure of GH family 10 (pfam00331). In XylU, the Glu157 (acid/base catalyst) and Glu265 (catalytic nucleophile) residues, which likely participate in the double-displacement of retaining glycoside hydrolases (MacLeod *et al.*, 1994), were found in highly conserved regions of the catalytic GH10 domain (Fig. 1).

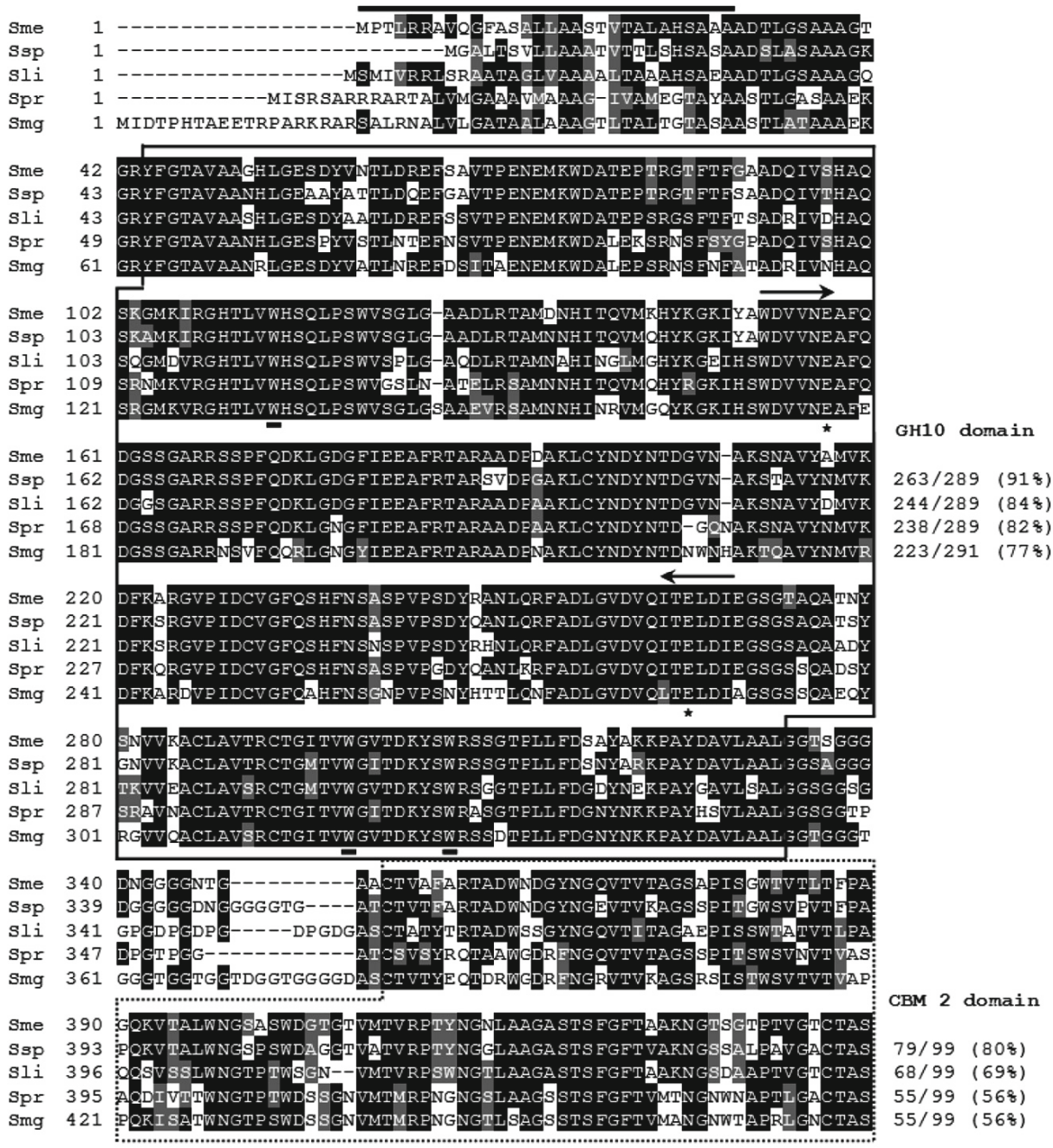

Fig. 1. Primary sequence alignment of *S. mexicanus* HY-14 GH10 β-1,4-xylanase and its structural homologs. Sequences (accession numbers): Sme, *S. mexicanus* HY-14 β-1,4-xylanase (KM027334); Ssp, *Streptomyces* sp. Ame12xE9 β-1,4-xylanase (WP_019983605); Sli, *Streptomyces lividans* TK24 β-1,4-xylanase (WP_003978188); Spr, *Streptomyces pristinaespiralis* ATCC 25486 β-1,4-xylanase (WP_005310297); Smg, *Streptomyces megasporus* β-1,4-xylanase (ADZ99362). The identical and similar amino acids are shown by black and gray boxes, respectively. The predicted signal peptide is indicated by a black bar. The internal peptide sequences used in the design of degenerate oligonucleotides for PCR are marked by arrows. Strictly conserved amino acid residues that play an essential role in the biocatalytic reaction are indicated by asterisks. Highly conserved Trp residues in the active site that play a crucial role in enzyme-substrate interaction are underlined. GH10 and CBM 2 domains are outlined by solid and dashed lines, respectively.

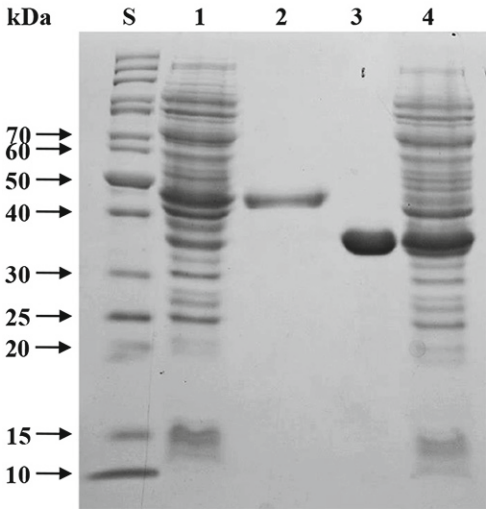

Fig. 2. SDS-PAGE of the purified rXylU and rXylUΔCBM 2. Lanes: S, standard marker proteins; 1, the soluble cell lysate of *E. coli* BL21 horboring pET-28a(+)/*xylU* after IPTG induction; 2, purified rXylU after affinity chromatography on HisTrap™ HP; 3, purified rXylUΔCBM 2 after affinity chromatography on HisTrap™ HP; 4, the soluble cell lysate of *E. coli* BL21 horboring pET-28a(+)/*xylUΔCBM 2* after IPTG induction.

Purification and molecular mass of rXylU and rXylUΔCBM 2

In this study, we purified the recombinant proteins, rXylU and rXylUΔCBM 2, to electrophoretic homogeneity by an affinity chromatography, as described in 'Materials and Methods'. The recombinant enzymes were released from the HisTrap™ HP column when imidazole was applied at concentrations of 200 to 250 mM. The molecular masses of the rXylU and rXylUΔCBM 2 proteins were estimated to be approximately 44.0 kDa and 33.0 kDa, respectively, as determined by SDS-PAGE (Fig. 2).

Enzymatic characterization of rXylU

The highest biocatalytic activity of rXylU toward beechwood xylan were measured at pH 5.5 (Fig. 3A) and 65°C (Fig. 3B), and the enzyme displayed over 80% of its maximum activity at a pH range of 5.0–7.5 (Fig. 3A). However, the hydrolytic ability of rXylU for the same substrate was remarkably reduced when the enzyme reactions were conducted at pHs < pH 4.5 or > pH 9.0. The enzyme maintained over 80% of its maximum β-1,4-xylanase activity after pre-incubation of 1 h at pH 4.5–10.0 (Fig. 3C) and retained over 70% of its original activity at temperatures below 50°C for at least 1 h but its activity was greatly reduced when the enzyme was exposed to temperatures above 55°C for 1 h (Fig. 3D).

In this study, the stimulatory or inhibitory effect of rXylU by various divalent cations and chemical reagents was extensively investigated as shown below (Table 1). Similar to most of the known GH10 enzymes (Oh *et al.*, 2008; Kim *et al.*, 2010a, 2010b, 2014), the rXylU almost completely lost its hydrolytic activity when preincubated in the presence of 5 mM *N*-bromosuccinimide and 1 mM Hg^{2+} for 10 min at

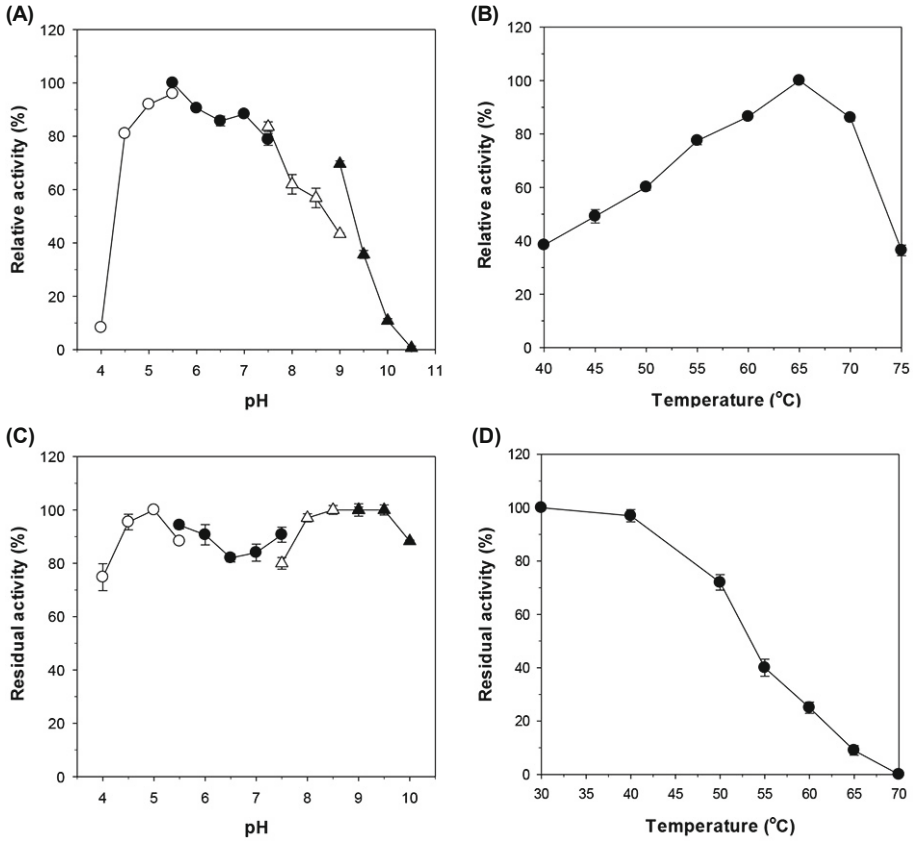

Fig. 3. Effects of pH and temperature on the β-1,4-xylanase activity and stability of rXylU. (A) Effect of pH on rXylU activity, (B) Effect of temperature on rXylU activity, (C) The pH stability of rXylU, (D) The thermal stability of rXylU. The optimal pH of the enzyme was determined using the following buffers (50 mM): sodium citrate (○), sodium phosphate (●), Tris-HCl (△), and glycine-NaOH (▲).

Table 2. Hydrolysis activity of rXylU for different substrates

Substrate	Specific activity (U/mg)[a]
Xylan from birchwood	441.4 ± 4.6
Xylan from beechwood	587.0 ± 8.9
Xylan from oat spelts	649.8 ± 10.1
Arabinoxylan from wheat	161.7 ± 0.1
Xyloglucan	ND[b]
Soluble starch	ND
Pectin	ND
Locust bean gum	ND
Carboxymethylcellulose	ND
*p*NP-cellobioside	328.1 ± 11.1
*p*NP-glucopyranoside	ND
*p*NP-xylopyranoside	9.0 ± 0.2
*p*NP-mannopyranoside	ND
*p*NP-galactopyranoside	ND

[a] Specific activity was obtained from the three repeated experiments.
[b] Not detected.

room temperature, indicative of its high sensitivity to these Trp-directed modifiers. These results suggested that highly conserved three Trp residues (Trp114, Trp298, and Trp306) in the active site might play a crucial role in enzyme-substrate interaction. Conversely, the two β-1,4-xylanases produced by *Bacillus* sp. NG-27 (Gupta *et al.*, 1992) and *Bacillus halodurans* S7 (Mamo *et al.*, 2006) were relatively insensitive to Hg^{2+} ions. In this study, the enzyme was nearly insensitive to EDTA (5 mM) and divalent cations (each 1 mM) such as Ca^{2+}, Ni^{2+}, Mg^{2+}. Moreover, the stimulatory or inhibitory effect of rXylU by chemical compounds such as iodoacetamide, sodium azide, Cu^{2+}, Zn^{2+}, Mn^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, and Fe^{2+} was also observed to be only marginal (Table 1). The catalytic activity of rXylU increased by approximately 1.3-fold when the enzyme assay was conducted in the presence of non-ionic detergents (0.5%) such as Tween 80 and Triton X-100.

When compared to xylans (≥ 90% xylose) from beechwood and birchwood, oat spelt xylan, which is composed of xylose (≥ 70%), glucose (≤ 15%), and arabinose (≤ 10%), was most efficiently depolymerized by the rXylU (Table 2). In this case, the specific activity of the enzyme for oat spelt xylan was determined to be approximately 649.8 U/mg, which was higher than that of birchwood xylan (441.4 U/mg), beechwood xylan (587.0 U/mg) and wheat arabinoxylan (161.7 U/mg). Especially, the biocatalytic activity (649.8 U/mg) of rXylU toward oat spelts xylan was evaluated to be relatively higher than those of other known GH10 β-1,4-xylanases except for that (885.3 U/mg) of a β-1,4-xylanase (STXF10) from *Streptomyces thermonitrificans* (Cheng *et al.*, 2009) toward the same substrate, which have been biocatalytically characterized (Table 3). Based on the results, the susceptibilities of xylosic polysaccharides to the rXylU were determined to be as follows: oat spelt xylan > birchwood xylan > beechwood xylan > wheat arabinoxylan. Conversely, glucose-based polysaccharides such as avicel, carboxymethylcellulose (CMC), or soluble starch were not susceptible to rXylU. It should be noted that the rXylU exhibited high activity (328.1 U/mg) toward *p*NP-cellobioside (Table 2). In addition, the enzyme was able to cleave *p*NP-xylopyranoside but other *p*NP-sugar derivatives were apparently insensitive to the enzyme.

Table 4. LC analysis of the hydrolysis products of xylosidic materials by rXylU

Substrate	Composition (%) of products formed by hydrolysis reaction				
	X_1	X_2	X_3	X_4	X_5
X_2	0.4	99.6			
X_3	3.8	76.2	20.0		
X_4	3.8	82.4	13.5	0.3	
X_5	3.3	80.0	16.4	0.3	
X_6	2.8	76.1	20.7	0.4	
Birchwood xylan	2.6	83.9	11.0	2.3	0.2

Hydrolysis products of xylosidic materials

The results of the high performance liquid chromatography (HPLC) analyses revealed that rXylU was capable of hydrolyzing xylobiose (X_2) to xylose (X_1) molecules, even though the catalytic activity of the enzyme toward X_2 was significantly low (Table 4). However, it was found that rXylU could readily degrade birchwood xylan and a series of oligomers of xylotriose (X_3) to xylohexaose (X_6). Specifically, it decomposed birchwood xylan to X_1 (2.6%), X_2 (83.9%), X_3 (11.0%), X_4 (2.3%), and X_5 (0.2%) without forming detectable amounts of xylooligomers (≥ X_6). Similar to birchwood xylan, enzymatic hydrolysis of xylooligomers (X_3 – X_6) by rXylU resulted in the production of X_2 (> 76.0%) as the primary end product together with X_1 (< 4.0%), X_3 (< 21.0%), and X_4 (< 0.5%) as the minor hydrolysis products. The production of X_1 by enzymatic degradation of xylosidic

Table 3. Enzymatic features of bacterial GH10 β-1,4-xylanases active toward xylan polymers

Strain	Enzyme	M_r (kDa)	Opt. pH	Opt. temp. (°C)	Specific activity (U/mg)		Reference
S. thermonitrificans	STXF10[a]	26	6.0	50	885.2[c]	725.8[d]	Cheng *et al.* (2009)
S. mexicanus HY-14	rXylU[b]	46	5.5	65	649.8[c]	441.4[d]	This study
Streptomyces sp. S9	XynAS9[b]	46	6.5	60	453.7[c]	ND[e]	Li *et al.* (2008)
A. cellulolyticus 11B	Xyn10A[b]	43	6.0	90	350.0[c]	ND[e]	Barabote *et al.* (2010)
B. halodurans S7	Xyn10A[a]	43	10.0	70	290.7[c]	342.0[d]	Mamo *et al.* (2006)
C. acetobutylicum	Xyn10A[b]	34	6.3	60	213.0[c]	266.0[d]	Ali *et al.* (2005)

[a] Wild type GH10 β-1,4-xylanase.
[b] Recombinant GH10 β-1,4-xylanase produced in *E. coli*.
[c] Specific activity toward oat spelts xylan.
[d] Specific activity toward birchwood xylan.
[e] Not determined.

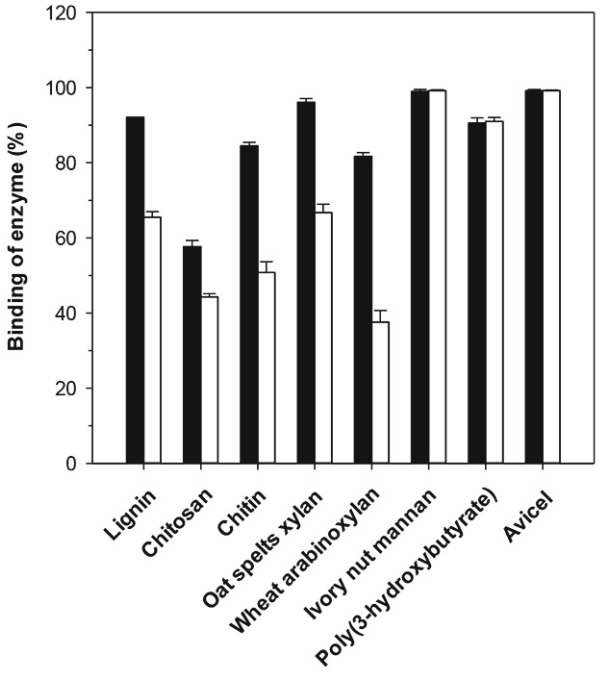

Fig. 4. Binding of rXylU (■) and rXylUΔCBM 2 (□) to insoluble polymers.

compounds has been also shown in some GH10 β-1,4-xylanases. For example, Ali *et al.* (2005) reported that a thermostable β-1,4-xylanase from *Clostridium acetobutylicum* ATCC 824 exclusively produces X_1 from birchwood xylan and xylooligomers (X_2 to X_6). In addition, the prominent production of X_1 as the hydrolysis product was observed when birchwood xylan was decomposed by a cold-active β-1,4-xylanase identified from the goat rumen content (Wang *et al.*, 2011) and a β-1,4-xylanase from *Streptomyces* sp. S9 (Li *et al.*, 2008). However, these degradation patterns of the xylosidic compounds by rXylU were comparable to those of the same compounds by many retaining GH10 β-1,4-xylanases (Gallardo *et al.*, 2003; Kim *et al.*, 2009, 2010b, 2014), which did not produce X_1 molecules from biocatalytic degradation of the same substrates due to their transglycosylation activity.

Binding ability of rXylU and rXylUΔCBM 2 to hydrophobic polymers

Because rXylU contains a CBM 2 domain in the C-terminus (Fig. 1), which has been known to be responsible for the considerable roles in enzyme-substrate interaction (Moreira and Filho, 2008), the enzyme is expected to bind to various hydrophobic sugar-based polymers. Thus, we extensively investigated the substrate-binding abilities of intact rXylU and its C-terminal CBM 2 domain-truncated form using various hydrophobic materials with different microstructures including polysaccharides, poly(3HB) granules, and lignin. In this study, when determined by analysis of the remaining β-1,4-xylanase activity in the supernatants recovered after incubation of rXylU with the respective substrate polymers for 2 h on ice, the enzyme was found to be strongly attached to the surface of oat spelts xylan, lignin, Avicel, and ivory nut mannan, but showed relatively weak binding affinity to chitosan, chitin, arabinoxylan, or poly(3HB) granules. The binding of rXylU to the insoluble polymers was also confirmed by determining the concentration of proteins in the respective supernatants recovered after centrifugation of the reaction mixtures (Fig. 4). It is assumed that the weak binding affinities of rXylU and rXylUΔCBM 2 to the polymers might be due to their hydrophobic nature that enables the enzymes to weakly bind to various surfaces of hydrophobic materials. However, deletion of the CBM 2 domain in the C-terminus of rXylU gave rise to a significant decrease of binding capacity of rXylU to some hydrophobic materials such as lignin, chitin, oat spelts xylan, and wheat arabinoxylan. Based on the above results, it is proposed that the CBM 2 domain of rXylU plays a considerable role in binding of rXylU to various hydrophobic polymers. It is also interesting to note that rXylU and its C-terminally truncated version could be tightly attached to Avicel and ivory nut mannan, regardless of the presence or absence of CBM 2. These results indicate that the catalytic GH10 domain of rXylU displays a strong binding affinity to the hydrophobic polymers.

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