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Characterization and Application of Carbohydrate-binding Modules of β-1,3-xylanase XYL4

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β-1,3-Xylanase from *Vibrio* sp. strain AX-4 (XYL4) is a modular enzyme composed of an N-terminal catalytic module belonging to glycoside hydrolase family 26 and two putative carbohydrate-binding modules (CBMs) belonging to family 31 in the C-terminal region. To investigate the functions of these three modules, five deletion mutants lacking individual modules were constructed. The binding assay of these mutants showed that a repeating unit of the CBM was a non-catalytic β-1,3-xylan-binding module, while the catalytic module *per se* was not likely to contribute to the binding activity when insoluble β-1,3-xylan was used for the assay. The repeating CBMs were found to specifically bind to insoluble β-1,3-xylan, but not to β-1,4-xylan, Avicel, β-1,4-mannan, curdlan, chitin or soluble glycol-β-1,3-xylan. Both the enzyme and the binding activities for insoluble β-1,3-xylan but not soluble glycol-β-1,3-xylan were enhanced by NaCl in a concentration-dependent manner, indicating that the CBMs of XYL4 bound to β-1,3-xylan through hydrophobic interaction. This property of the CBMs was successfully applied to the purification of a recombinant XYL4 from the cell extracts of *Escherichia coli* transformed with the *xyl4* gene and the detection of β-1,3-xylan-binding proteins including β-1,3-xylanase from the extract of a turban shell, *Turbo cornutus*.

Key words: β-1,3-xylan, β-1,3-xylanase, carbohydrate-binding module, deletion mutant, glycoside hydrolase.

Abbreviations: AlcCBM, CBM of *Alcaligenes* sp. strain XY-234; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; CBM(s), carbohydrate-binding module(s); CM, catalytic module; GH, glycoside hydrolase; IPTG, isopropyl β-D-thiogalactoside; PCR, polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electophoresis; TBS, Tris-buffered saline; T-TBS, TBS containing 0.02% Tween 20; TLC, thin-layer chromatography; XYL4, β-1,3-xylanase from *Vibrio* sp. strain AX-4.

In general, plant polysaccharide-degrading glycoside hydrolases (GHs) such as cellulases (β-1,4-glucanases) and β-1,4-xylanases possess a modular structure, consisting of a catalytic module and one or several non-catalytic carbohydrate-binding modules (CBMs) (*1*). The general role of CBMs appears to promote the association of the catalytic module on the substrate, resulting in an increase in hydrolysis of the substrate. Many CBMs have now been identified experimentally and classified into families based on amino acid sequence similarity, the same as the catalytic modules of GHs (*2*). At present, CBMs are divided into 53 families in the CAZy database (http://www.cazy.org/) and the information accumulated on their structure and ligand specificity is useful for elucidating the mechanisms of protein–carbohydrate recognition and interaction (*3–6*).

β-1,3-Xylanases (EC3.2.1.32) are endo-type enzymes that specifically act on the internal β-1,3-xylosidic linkages in β-1,3-xylan, which is an insoluble polysaccharide existing mainly as a component of algal cell walls (*7*). The enzymes have a modular architecture similar to other plant cell wall-degrading enzymes. β-1,3-Xylanase from a marine bacterium, *Alcaligenes* sp. strain XY-234, was found to consist of an N-terminal catalytic module belonging to family 26 (GH26) and a C-terminal CBM belonging to family 31 (CBM31) (*8*). We previously reported that β-1,3-xylanase from *Vibrio* sp. strain AX-4 (XYL4) is also a modular enzyme composed of a catalytic module in the N-terminal region and a putative CBM module belonging to family 31 at the C-terminus. In contrast to the *Alcaligenes* enzyme, however, the putative CBM module of XYL4 is composed of two CBMs, which show 55% identity to each other (*9*).

In the present article, we describe the significance of the repeating CBMs of XYL4 for binding to a substrate and the catalytic activity of the enzyme. Moreover, we report a new method of purifying recombinant XYL4 using the specific interaction between the CBMs of the enzyme and β-1,3-xylan, and show the usefulness of this method to detect β-1,3-xylan-binding proteins including β-1,3-xylanase in natural sources.

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MATERIALS AND METHODS

Materials—Pyrobest DNA polymerase was purchased from TAKARA BIO INC. (Shiga, Japan). The restriction endonucleases and T4 DNA ligase were obtained from Wako Pure Chemical Industries (Osaka, Japan). Silica Gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). β-1,3-Xylan and β-1,4-mannan were prepared from the green alga *Caulerpa racemosa* var. *laete-virens* according to the method of Iriki *et al*. (*7*) and *Codium fragile* according to the method of Love and Percival (*10*), respectively. Avicel and β-1,4-xylan were purchased from Sigma (St. Lousis, MO, USA). Chitin 500 and Chitin 1000 were obtained from SEIKAGAKU CORPORATION (Tokyo, Japan). Glycol-β-1,3-xylan was prepared according to the method of Yamaura *et al*. (*11*). Turban shells, *Turbo cornutus* (6–7 cm in shell length), were purchased from a local market in Fukuoka city, Fukuoka prefecture, Japan. All other reagents were of the highest purity available.

Bacterial Strains and Plasmids—*Escherichia coli* strains BL21(λDE3) and BL21(λDE3)pLysS were purchased from TAKARA BIO INC. Plasmids pET23a(+) and pET22b(+) were obtained from Novagen (Darmstadt, Germany). The expression vector pCBM containing CBM of *Alcaligenes* sp. strain XY-234 was prepared as described in (*8*).

Sequence Analysis—The nucleotide and amino acid sequences were analysed using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The homology search of amino acid sequences was performed with BLAST (*12*). The alignment of amino acid sequences was performed with CLUSTAL W (*13*).

Construction of Deletion Mutants of XYL4—General cloning techniques were essentially performed as described by Sambrook *et al*. (*14*). For the construction of deletion mutants, AETFLU, AXG-L (*9*) and the following oligonucleotide primers were used for polymearase chain reaction (PCR); 22CBM-1s (5′-GGG ATC CGA CAA ACC CAC CAG AGC CTT-3′), 22CBM-2s (5′-GGG ATC CGA CCG ATC CCA GTC AAT GTA-3′), AXD-L1 (5′-GTA AGC TTA ATG ATG ATG ATG ATG ATG AGT TGA GCC ATA GCC TAA CG-3′) and AXD-L2 (5′-GTA AGC TTA ATG ATG ATG ATG ATG ATG AGG TGT GCC AGT AGA GCC AC-3′). 22CBM-1s and 22CBM-2s contained a BamHI site (underlined). AXD-L1 and AXD-L2 contained a HindIII site (double underlined) following the histidine tag-encoding sequence. For construction of the deletion mutants containing a catalytic module, PCR was performed in a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA, USA) for 30 cycles (each consisting of denaturation at 94°C for 20 s, annealing at 52°C for 1 min and extension at 72°C for 1.5 min) using Pyrobest DNA polymerase, pETXYL4 (*9*) as a template, and the following sets of primers: AETFLU/AXD-L1 for the construction of pET23CM and AETFLU/AXD-L2 for the construction of pET23CM-CBM-1. PCR for construction of the deletion mutants lacking a catalytic module was conducted under the same conditions as described earlier, but the annealing temperature and extension time were changed to 61°C and 45 s, respectively. The following sets of primers were used for PCR: 22CBM-1s/AXG-L for the construction of pET22CBM-1-2, 22CBM-1s/AXD-L2 for the construction of pET22CBM-1 and 22CBM-2s/AXG-L for the construction of pET22CBM-2. After gel purification, the products amplified using the primer sets AETFLU/AXD-L1 and AETFLU/AXD-L2 were digested with BamHI/HindIII and cloned into a pET23a(+) vector digested with the same restriction enzymes. The amplified products obtained using 22CBM-1s/AXG-L, 22CBM-1s/AXD-L2 and 22CBM-2s/AXG-L were digested with BamHI/HindIII and cloned into pET22b(+) digested with the same restriction enzymes. Then the expression plasmids were sequenced by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and a DNA sequencer (model 377A, PE Biosystems), and their sequences were confirmed.

Expression of XYL4 and its Deletion Mutants—The expression of XYL4 and its deletion mutants possessing a catalytic module (pET23CM and pET23CM-CBM-1) was the same as described in our previous report (*9*). The deletion mutants lacking a catalytic module were generated as follows: *E. coli* strain BL21(λDE3) cells transformed with each expression vector (pET22CBM-1-2, pET22CBM-1 or pET22CBM-2) were grown at 25°C for 12 h in 20 ml of Luria-Bertani medium supplemented with 100 μg/ml of ampicillin with shaking. The culture was then transferred to 400 ml of fresh medium with the same constituents and incubated until the A_{600} reached approximately 0.6. Then isopropyl β-D-thiogalactoside (IPTG) was added to the culture at a final concentration of 1 mM to cause transcription, and cultivation was continued for an additional 3 h at 25°C. After cultivation, cells were harvested by centrifugation (8,000*g* for 10 min), and suspended in a solution for extraction (deionized water containing 5 μg/ml each of leupeptine and pepstatin A).

Purification of the Recombinant Proteins—The cell suspension of transformed *E. coli* was sonicated and cell debris was then removed by centrifugation (8,000*g* for 10 min). The supernatant obtained was loaded onto a HiTrap Chelating HP column (GE Healthcare, Buckinghamshire, UK), which was chelated with Ni^{2+}, and then the column was washed with 20 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl and 25 mM imidazole. The adsorbed proteins were eluted from the column with 20 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl and 250 mM imidazole. The fraction eluted was subjected to gel filtration chromatography using a Superdex 200 HR 10/30 column (GE Healthcare). The conditions for the chromatography were as follows: buffer, 20 mM Tris–HCl buffer, pH 7.5 containing 0.15 M NaCl; flow rate, 0.5 ml/min; fractionation, 0.25 ml/tube; detection of proteins, measurement of A_{280}. The purified proteins were dialysed against deionized water and stored at 4°C until used.

Expression of CBM of Alcaligenes sp. Strain XY-234—The expression of CBM of *Alcaligenes* sp. strain XY-234 (AlcCBM) was essentially conducted by the same method as described in (*8*). Briefly, *E. coli* strain BL21(λDE3) cells transformed with pCBM was grown at 25°C in 400 ml of Luria-Bertani medium supplemented with 100 μg/ml of

ampicillin with shaking. When the A_{600} reached approximately 0.6, IPTG was added to the culture at a final concentration of 1 mM. Cultivation was continued for another 3 h at 25°C, and then the harvested cells were suspended in deionized water containing 5 μg/ml each of leupeptine and pepstatin A. Purification of AlcCBM was carried out as described earlier.

Enzyme Assay—The activity of β-1,3-xylanase was measured as follows. The reaction mixture containing 0.5% β-1,3-xylan and an appropriate amount of enzyme in 300 μl of 50 mM sodium phosphate buffer, pH 7.5, was incubated at 37°C for 10 min. After incubation, the reaction mixture was centrifuged and 250 μl of the supernatant was recovered. The reducing sugars liberated by hydrolysis of the substrate in the supernatant were determined by the Somogyi–Nelson method (*15*). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of xylose per minute under the conditions described earlier. To determine the activity of deletion mutants, glycol-β-1,3-xylan was used as a substrate and the incubation period was changed to 30 min.

Protein Assay—Protein content was determined by the bicinchonic acid protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA) using bovine serum albumin as the standard (*16*).

SDS–PAGE and Western Blotting—Sodium dodecylsulfate-polyacrylamidegel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*17*). Proteins were separated by 12.5% SDS–PAGE and stained with Coomassie brilliant blue (CBB). A low-molecular-weight SDS–PAGE calibration kit (GE Healthcare) was used as the standard. To perform western blotting, proteins separated by 12.5% SDS–PAGE were transferred onto a nitrocellulose membrane using a semi-dry blotter (Bio-Rad Laboratories, Hercules, CA, USA). Then the membrane was blocked with 3% skim milk in Tris-buffered saline (TBS) for 1 h and washed with TBS containing 0.02% Tween 20 (T-TBS) three times. The membrane was incubated with anti-His (C-term) antibodies (Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature and washed with T-TBS three times. Then the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG antibodies for 1.5 h at room temperature. After washing with T-TBS three times, the membrane was stained with a peroxidase stain kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

Binding Assay for Insoluble Polysaccharides—The reaction mixture for the β-1,3-xylan-binding assay containing 5% β-1,3-xylan and an appropriate amount of mutant enzyme in 60 μl of 50 mM sodium phosphate buffer, pH 7.5, was incubated at 4°C for 30 min. Then the mixture was centrifuged and the amount of mutant enzyme in the supernatant was quantified by western blotting. For the analysis of binding specificity, the β-1,3-xylan in the reaction mixture was replaced with other insoluble polysaccharides, *i.e.* Avicel (microcrystalline β-1,4-glucan), β-1,4-xylan (from birchwood), β-1,4-mannan (from *Codium fragile*), curdlan (β-1,3-glucan), Chitin 500 and Chitin 1000 (β-1,4-*N*-acethylglucosaminan). The subsequent procedure was the same as described earlier.

Affinity Gel Electrophoresis Using Glycol-β-1,3-xylan—Native polyacrylamide gels (10% acrylamide), which contain 0, 0.1, 0.5 or 1.0% glycol-β-1,3-xylan, were prepared and loaded with 1.25 μg of purified protein in the native PAGE sample buffer. The electrophoresis was performed at 100 V and 4°C with the SDS-free running buffer. Subsequently, the gels were stained with CBB.

Binding Assay of XYL4 Mutant Enzymes and AlcCBM—The dissociation constants and binding capacity of XYL4 mutants [E138Q (*9*) and CBM-1-2] and AlcCBM were determined by the following procedures. A reaction mixture composed of 0.5% β-1,3-xylan and appropriate amounts of the purified protein (E138Q, 0.5–3.8 μM; CBM-1-2, 0.5–3.2 μM; AlcCBM, 2.0–14.9 μM) in 80 μl of 20 mM Tris–HCl buffer containing 0.15 M NaCl, pH 7.5, was incubated at 4°C for 1 h with gentle mixing. After the incubation, the mixture was centrifuged and the concentration of the unbound protein ([P], μM) in the supernatant was quantified by the Bradford method (*18*). The amount of protein forming the protein-β-1,3-xylan complex ([PC], in micromoles per gram of β-1,3-xylan) was determined by subtracting [P] from the total protein concentration. Adsorption parameters were obtained by using the equation of Sakoda and Hiromi (*19*), $[PC] = [P][PC]_{max}/(K_d+[P])$, where K_d (μM) and $[PC]_{max}$ (micromoles per gram of β-1,3-xylan) are the equilibrium dissociation constant and maximum amount of the bound protein, respectively.

Examination of Effects of NaCl on the Enzyme Activity and Binding Activity—Effects of NaCl on the activity of XYL4 and the mutant enzymes were examined as follows: a reaction mixture containing 0.5% substrate (β-1,3-xylan or glycol-β-1,3-xylan), 0.01 U of XYL4 or a mutant [catalytic module (CM) or CM-CBM-1)] and various concentrations (0–0.5 M) of NaCl in 300 μl of 5 mM sodium phosphate buffer, pH 7.5, was incubated at 37°C for 30 min. Then the mixture was centrifuged and the amount of reducing sugars in the supernatant was quantified by the Somogyi–Nelson method (*15*). Effects of NaCl on the binding ability of CBM-1-2 were examined as follows: a reaction mixture containing 0.5% β-1,3-xylan, an appropriate amount of CBM-1-2 and various concentrations (0–0.5 M) of NaCl in 100 μl of 5 mM sodium phosphate buffer, pH 7.5, was incubated at 4°C for 30 min. Then the mixture was centrifuged and the amount of CBM-1-2 in the supernatant was estimated by western blotting. Binding rate was calculated by the following equation: binding rate (%) = 100 × [1−(the amount of CBM-1-2 in the supernatant/the total amount of CBM-1-2)].

Purification of XYL4 Using β-1,3-xylan—Affinity chromatography using β-1,3-xylan as a solid phase was conducted as follows: the cell suspension from a 50-ml culture of pETXYL4-transformed *E. coli* was sonicated and cell debris was removed by centrifugation (8,000*g* for 10 min). The supernatant was added to 100 mg of β-1,3-xylan in 5 ml of binding buffer (20 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl) and gently mixed at 4°C for 30 min. Next the mixture was centrifuged and the supernatant (unbound fraction) was removed. The precipitated β-1,3-xylan-XYL4 complex was washed with the wash buffer (50 mM sodium

phosphate buffer, pH 7.5, containing 0.1% Lubrol PX) and then it was centrifuged and the supernatant (wash fraction) was discarded. After being washed three times, the β-1,3-xylan-XYL4 complex was dialyzed against deionized water to remove buffer salt, then centrifuged, and the XYL4 in the supernatant (elution fraction) was collected. All fractions were analysed by SDS–PAGE and western blotting as described earlier.

Detection of β-1,3-xylan-binding Proteins in the Extract of Turban Shell, T. cornutus—Approximately 20 g of viscera of *T. cornutus* was homogenized in 20 ml of deionized water containing 5 μg/ml of leupeptine and pepstatin A, and centrifuged at 8,000*g* for 15 min. The supernatant was further centrifuged at 12,000*g* for 15 min to remove precipitates and then subjected to affinity chromatography as described earlier with minor modifications. The supernatant was added to 20 ml of 20 mM sodium phosphate buffer, pH 7.5, containing 100 mg of β-1,3-xylan, 0.1 M NaCl and 0.1% Lubrol PX and gently mixed at 4°C for 30 min. Then the mixture was centrifuged and the precipitate was washed with the same buffer five times. Bound proteins were eluted from β-1,3-xylan by dialyzing against deionized water (water-eluted fraction) and then the proteins still bound to β-1,3-xylan were eluted by denaturing with SDS–PAGE sample buffer (SDS-eluted fraction). These proteins were analysed by SDS–PAGE. The β-1,3-xylanase activity of the eluted proteins was measured using β-1,3-xylan as the substrate by thin-layer chromatography (TLC). A reaction mixture containing 1% β-1,3-xylan and 10 μl of the water-eluted fraction in 100 μl of 20 mM sodium phosphate buffer, pH 7.5, was incubated at 37°C for 24 h. To evaluate the activity of the SDS-eluted fraction, the activity was measured using the complex of proteins and β-1,3-xylan before denaturation. After incubation, two volumes of ethanol were added to the mixture. Then the mixture was centrifuged and the supernatant obtained was evaporated with a Speed Vac concentrator. The dried material was dissolved in deionized water and spotted onto a Silica Gel 60 TLC plate, which was developed with 1-butanol/acetic acid/deionized water (10 : 5 : 1, by volume). After development, the TLC plate was sprayed with diphenylamine aniline phosphate reagent (*20*) and heated at 100°C for 10 min to visualize the digestion products.

RESULTS AND DISCUSSION

Alignment of CBMs and Construction of Various Deletion Mutants of XYL4—XYL4 has a modular architecture consisting of a catalytic module belonging to GH26 and two CBMs belonging to CBM31 (*9*). A sequence alignment of the CBMs of XYL4 and other CBMs in family 31 from different bacterial strains is shown in Fig. 1. The CBMs of XYL4 showed 40–55% identity with other CBMs belonging to CBM31. Recently, the CBM of *Alcaligenes* sp. strain XY-234 (*Alc*CBM31) was crystallized and its X-ray crystal structure was solved (*21*). *Alc*CBM31 has a β-sandwich structure composed of eight β-strands with a typical immunoglobulin fold. The folding topology of *Alc*CBM31 was found to differ from that of other CBMs, in which eight β-strands comprise a β-sandwich structure with a typical jelly-roll fold. The two CBMs of XYL4 show significant similarity with the members of CBM31, including *Alc*CBM31, and the amino acid residues which are speculated to play crucial roles in substrate binding are almost completely conserved (*21*) (Fig. 1). For example, four cysteine residues possibly forming two disulphide bonds are completely conserved in each CBM of XYL4, *i.e*. Cys-368, Cys-452, Cys-399 and Cys-404 in CBM-1; and Cys-490, Cys-574, Cys-521 and Cys-526 in CBM-2. In the case of several CBMs possessing polysaccharide-binding ability, the aromatic amino acid residues which are placed at the surface of the molecule have been shown to play an important role in their substrate binding (*4*, *6*, *22–24*). The aromatic amino acid residues, which are predicted to be located at the molecular surface of *Alc*CBM31, are also highly conserved in the two CBMs of XYL4, *i.e*. Tyr-376, Trp-395 and Tyr-397 in CBM-1; and Tyr-498, Trp-517, Tyr-519 and Tyr-525 in CBM-2, but one tyrosine is replaced by leucine (Leu-403) in CBM-1. These findings suggest that the two CBMs of XYL4 are very similar in structure and possibly functions to the CBMs of other members of family 31.

To investigate the function of the two CBMs in XYL4, a series of XYL4 deletion mutants were constructed as shown in Fig. 2A. The architectural features of the mutant enzymes are as follows: CM, which has a catalytic module but lacks both CBMs; CM-CBM-1, which has a catalytic module and CBM-1 but lacks CBM-2; CBM-1-2, which consists of the two CBMs but lacks a catalytic module; CBM-1 and CBM-2, which consist of only a single CBM.

The Binding Properties of Deletion Mutants of XYL4—Five deletion mutants and wild-type enzyme were expressed separately in *E. coli* strain BL21(λDE3) or BL21(λDE3)pLysS, and then their binding activities were assayed using water-insoluble β-1,3-xylan as the substrate (Fig. 2B). The wild-type and CBM-1-2, both containing two CBMs, showed similar activity to bind β-1,3-xylan, whereas CM-CBM-1, which lacks CBM-2, showed relatively weak binding activity. CM, which completely lacks the CBMs, showed no binding activity. Interestingly, CBM-1 and CBM-2 were found to not bind to β-1,3-xylan under the conditions used. These results suggest that recombinant CBM-1 or CBM-2 expressed in *E. coli* did not keep the correct folding/structure that should be required for binding to β-1,3-xylan.

The binding specificity of the CBMs of XYL4 was examined by an assay using various insoluble polysaccharides (Fig. 3). It was found that CBM-1-2 bound to β-1,3-xylan but not other insoluble polysaccharides, such as β-1,4-xylan, Avicel (microcrystalline β-1,4-glucan), β-1,4-mannan, curdlan (β-1,3-glucan) and two kinds of chitins (β-1,4-*N*-acethylglucosaminan; Chitin 500 and 1000). Next, to investigate whether CBMs of XYL4 bind to water-soluble substrates, affinity gel electrophoresis was performed using glycol-β-1,3-xylan as a substrate (Fig. 4). As a result, it was found that increasing concentrations of glycol-β-1,3-xylan did not affect the migration of CBM-1-2, while the migration of the wild-type and CM, both possess a catalytic module, was strongly inhibited by glycol-β-1,3-xylan in

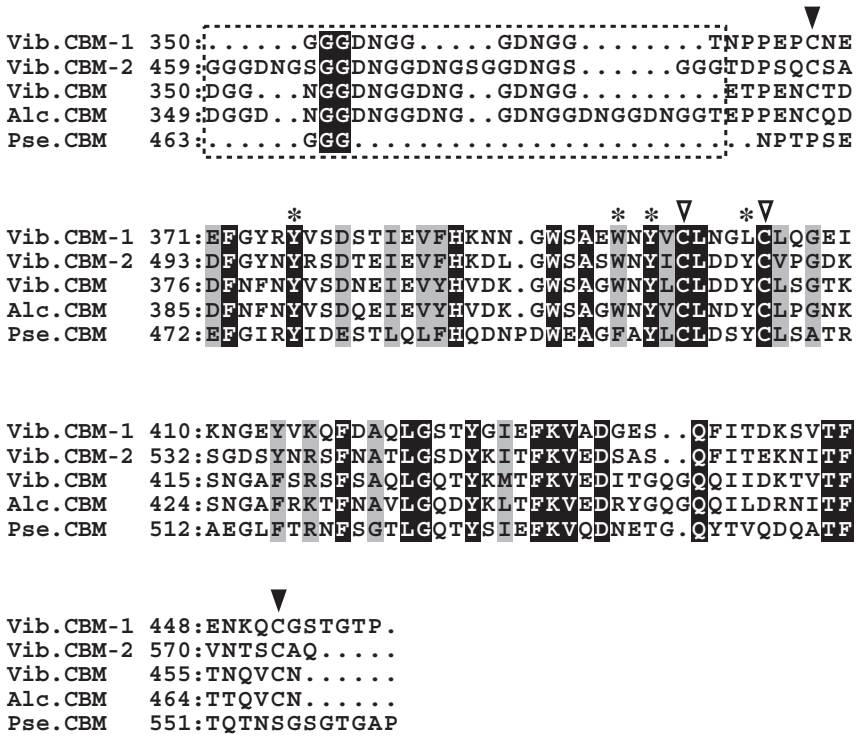

Fig. 1. **Sequence alignment of CBMs of β-1,3-xylanases.** Amino acid sequences of the CBMs belonging to family 31 were aligned using CLUSTAL W software (*13*). Identical and similar amino acid residues are shown by white letters on a black background and black letters on a grey background, respectively. The frame with a dotted line indicates the putative glycine-rich linker region and asterisks indicate well-conserved aromatic residues predicted to be located at the molecular surface of the CBMs (*21*). Black and white arrowheads indicate the cysteine residues predicted to form each disulphide bond, respectively. The numbers at the start of the respective lines indicate the amino acid numbers relative to Met-1. Gaps inserted into the sequences are shown by dots. Abbreviations used are as follows: Vib.CBM-1 and Vib.CBM-2, CBM-1 and CBM-2 of XYL4 from *Vibrio* sp. strain AX-4 (AB121027); Vib.CBM, CBM of TxyA from *Vibrio* sp. strain XY-214 (AB029043); Alc.CBM, CBM of TxyA from *Alcaligenes* sp. strain XY-234 (AB039953); Pse.CBM, CBM of AxnB from *Pseudomonas* sp. strain ND137 (AB063257).

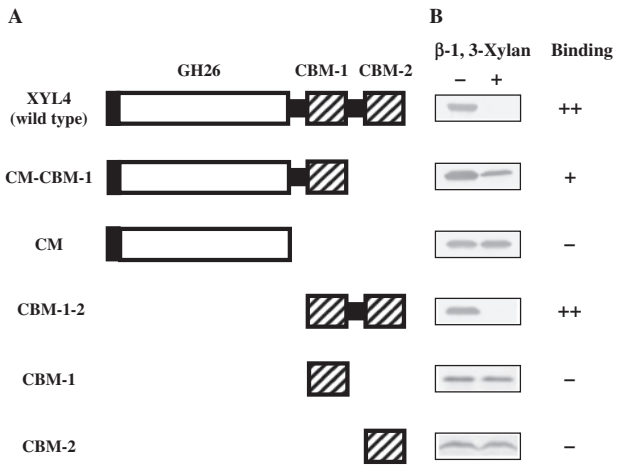

Fig. 2. **Construction of XYL4 and its deletion mutants and their activity to bind β-1,3-xylan.** (A) Modular architecture of XYL4 and the deletion mutants. Open and closed boxes indicate the catalytic modules and the signal peptides, respectively. CBMs are shown by slashed lines in open boxes. CBMs at the N-termini and C-termini are designated CBM-1 and CBM-2, respectively. Black bars indicate linkers that connect each module. (B) Activity of XYL4 and the deletion mutants to bind β-1,3-xylan. The binding assay was described in MATERIALS AND METHODS section. ++ and + indicate strong and weak binding, respectively. – indicates no binding.

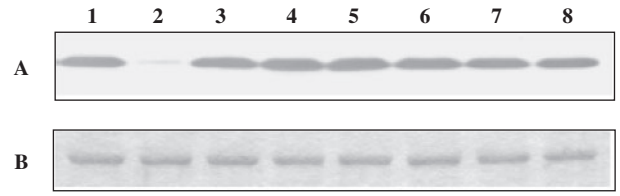

Fig. 3. **Analysis of the specificity of CBMs of XYL4 using insoluble polysaccharides.** CBM-1-2, which is the XYL4-deletion mutant consisting of two CBMs without a catalytic module, was used for the binding assay. (A) CBM-1-2 was mixed with various insoluble polysaccharides, the mixture was centrifuged, and the CBM-1-2 remaining in the supernatant was detected by western blotting. (B) BSA was mixed with various insoluble polysaccharides instead of CBM-1-2 as a control experiment. The BSA remaining in the supernatant was detected by CBB staining (negative control). Details are described in MATERIALS AND METHODS section. Proteins detected in the supernatant were 'not bound to polysaccharides'. Lane 1, no polysaccharide (control); lane 2, β-1,3-xylan; lane 3, β-1,4-xylan; lane 4, Avicel (microcrystalline β-1,4-glucan); lane 5, β-1,4-mannan; lane 6, curdlan (β-1,3-glucan); lane 7, Chitin 500; lane 8, Chitin 1000.

a concentration-dependent manner (Table 1). These results indicate that the specificity of the CBMs of XYL4 is very strict, *i.e.* they exclusively bind to insoluble β-1,3-xylan but not other insoluble polysaccharides or

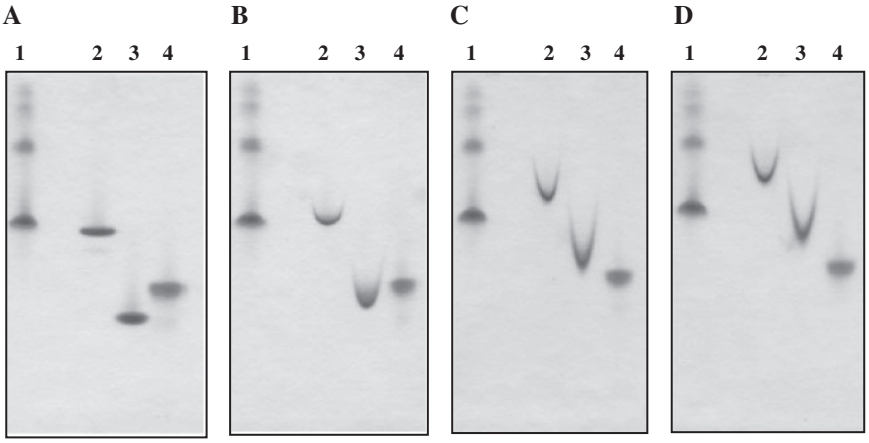

Fig. 4. **Binding of XYL4 and its deletion mutants to soluble glycol-β-1,3-xylan.** Polyacrylamide gels (SDS free) containing (A) 0%, (B) 0.1%, (C) 0.5% and (D) 1.0% glycol-β-1,3-xylan were used for PAGE to analyse the binding to soluble glycol-β-1,3-xylan. After electrophoresis at 4°C for 2 h, gels were stained with CBB. Lane 1, BSA (control); lane 2, XYL4 (wild type); lane 3, CM; lane 4, CBM-1-2. Details are described in MATERIALS AND METHODS section.

Table 1. **Binding of XYL4 and its mutants to glycol-β-1, 3-xylan on affinity gel electrophoresis.**

Glycol-β-1,3-xylan (%)	0.0	0.1	0.5	1.0
BSA (control)	1.00	1.00	1.00	1.00
XYL4 (wild-type)	1.07	0.97	0.78	0.71
CM	1.70	1.51	1.26	1.11
CBM-1-2	1.51	1.48	1.47	1.49

Values are the average of duplicate experiments and show relative migration on affinity gel, compared with BSA as a control. Details are described in MATERIALS AND METHODS section.

soluble glycol-β-1,3-xylan, though catalytic module of XYL4 binds to the soluble substrate.

To determine the dissociation constants of XYL4 mutants, their binding activity was measured using insoluble β-1,3-xylan as the substrate. Since a single CBM of XYL4 without a catalytic module (CBM-1 or CBM-2) was incapable of binding to β-1,3-xylan (Fig. 2B), we thus examined the binding activity of a CBM of *Alcaligenes* sp. (AlcCBM) (*8*) as a representative of a single CBM31 module in place of CBM-1 or CBM-2. The relationship between the [PC] and [P] of E138Q [point mutation of the catalytic residue of XYL4 abolishing the enzyme activity (*9*)], CBM-1-2 and AlcCBM was plotted in Fig. 5, and then their kinetic parameters were calculated from double-reciprocal plots (Table 2). The K_d of the mutants tested was as follows; E138Q, 0.87 μM; CBM-1-2, 3.57 μM; and AlcCBM, 8.15 μM, *i.e.* the affinity of the mutants possessing two CBMs (E138Q and CBM-1-2) for insoluble β-1,3-xylan was more than twice as that of the single CBM (AlcCBM). These results suggest that the two CBMs of XYL4 cooperatively function in the binding of insoluble β-1,3-xylan (*25–27*). On the other hand, the $[PC]_{max}$ of the mutants was as follows; E138Q, 0.26 μmol; CBM-1-2, 0.65 μmol; and AlcCBM, 1.93 μmol/g of β-1,3-xylan, indicating that the apparent capacity of the mutant adsorbing to β-1,3-xylan was larger for the single CBM of *Alcaligenes* sp. β-1, 3-xylanase than the two CBMs of XYL4. Assuming that

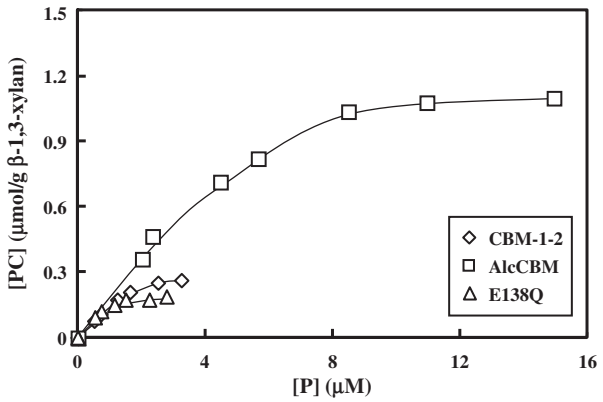

Fig. 5. **Stoichiometric analysis of binding of XYL4 mutants and AlcCBM to β-1,3-xylan.** Reaction mixtures containing 0.5% of β-1,3-xylan (w/v) and various concentrations of proteins were incubated at 4°C for 1 h with 80 μl of 20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl. After centrifugation, the amount of protein remaining in the supernatant was quantified by the Bradford method (*18*). CBM-1-2, lozenge; AlcCBM, square; E138Q; triangle. Details are shown in MATERIALS AND METHODS section.

Table 2. **Dissociation constants (K_d) and binding capacity ($[PC]_{max}$) of XYL4 mutants and AlcCBM.**

	K_d (μM)	$[PC]_{max}$ (μmol/g β-1,3-xylan)
E138Q	0.87 ± 0.19	0.26 ± 0.02
CBM-1-2	3.57 ± 1.35	0.65 ± 0.19
AlcCBM	8.15 ± 0.31	1.93 ± 0.03

Kinetic parameters of XYL4 mutants and AlcCBM were calculated according to the equation of Sakoda and Hiromi (*19*) from the double-reciprocal plot. Values are the average of triplicate experiments with SDs. Details are described in MATERIALS AND METHODS section.

a single CBM31 module occupies a binding site on β-1,3-xylan by one-to-one relationship, the amount of a repeating unit of CBM31 binding to a certain amount of β-1,3-xylan is less than half that of a single

CBM31 molecule. For this reason, the capacity of the two CBMs to bind insoluble β-1,3-xylan was lower as compared to that of AlcCBM. The X-ray crystal structure of the CBMs of XYL4 should be solved to understand the molecular mechanism underlying the binding of CBMs to β-1,3-xylan. It is worth noting that the catalytic module of β-1,3-xylanase has been crystallized and subjected to a preliminary X-ray analysis (*28*).

Effects of NaCl on Binding of CBMs to β-1,3-xylan—The role of the CBMs of XYL4 during the hydrolysis of insoluble β-1,3-xylan is significant. We previously reported that the CBMs *per se* did not hydrolyze β-1,3-xylan; however, their presence remarkably enhanced the enzyme activity of XYL4 when insoluble β-1,3-xylan was used as the substrate (*9*). The aromatic amino acid residues located at the molecular surface of CBMs binding to insoluble polysaccharide, such as CBM2a of xylanase 10A (*4*), ChBD2 of chitinase Pf-ChiA (*6*), CBM10 of xylanase Pf Xyn10A (*22*), CBM15 of xylanase Xyn10C (*23*) and ChBD of chitinase ChiC (*24*), have been reported to play an important role in the binding to substrates through hydrophobic interactions. In this study, we examined the effects of NaCl on the binding of the CBM with insoluble β-1,3-xylan in order to clarify whether the binding of the CBMs depends on hydrophobic interactions or not.

The binding of CBM-1-2 to insoluble β-1,3-xylan was found to increase in the presence of NaCl in a concentration-dependent manner (Fig. 6A). The enzyme activities of the wild-type and CM-CBM-1 were also increased by NaCl when the insoluble β-1,3-xylan was used for the enzyme assay. However, the enzyme activity of CM, which lacks the CBMs, was not affected by the concentration of NaCl (Fig. 6B). On the other hand, NaCl did not enhance the enzyme activities of the wild-type and mutant enzymes when soluble glycol-β-1,3-xylan was used as a substrate (Fig. 6C). These results suggest that the binding of the CBMs of XYL4 to insoluble substrates is controlled by hydrophobic interaction and increasing the concentration of NaCl enhances the binding, resulting in stronger enzyme activity towards the insoluble substrates.

Affinity Chromatography Utilizing the Interaction Between CBMs and β-1,3-xylan—Affinity chromatography utilizing the specific interaction between CBMs of XYL4 and β-1,3-xylan was used to purify the recombinant XYL4. The supernatant of the extract of *E. coli* expressing XYL4 was incubated at 4°C with β-1,3-xylan in the presence of 0.1 M NaCl. After being left at 4°C for 30 min, XYL4 adsorbed to β-1,3-xylan was collected by centrifugation and washed with NaCl-free buffer to remove the non-specifically bound proteins. Then, the XYL4-β-1,3-xylan complex was dialysed against deionized water to decrease the concentration of buffer salts. After centrifugation, it was examined whether XYL4 was recovered in the supernatant. We detected the 66-kDa single protein band in the supernatant when examined by SDS–PAGE (Fig. 7A, lane 6), and the protein was confirmed to be the XYL4 by western blotting (Fig. 7B, lane 6). These results indicated that the recombinant XYL4 was purified in a single step using β-1,3-xylan, and that the specific binding of the CBMs to β-1,3-xylan could be utilized for the purification of the recombinant XYL4.

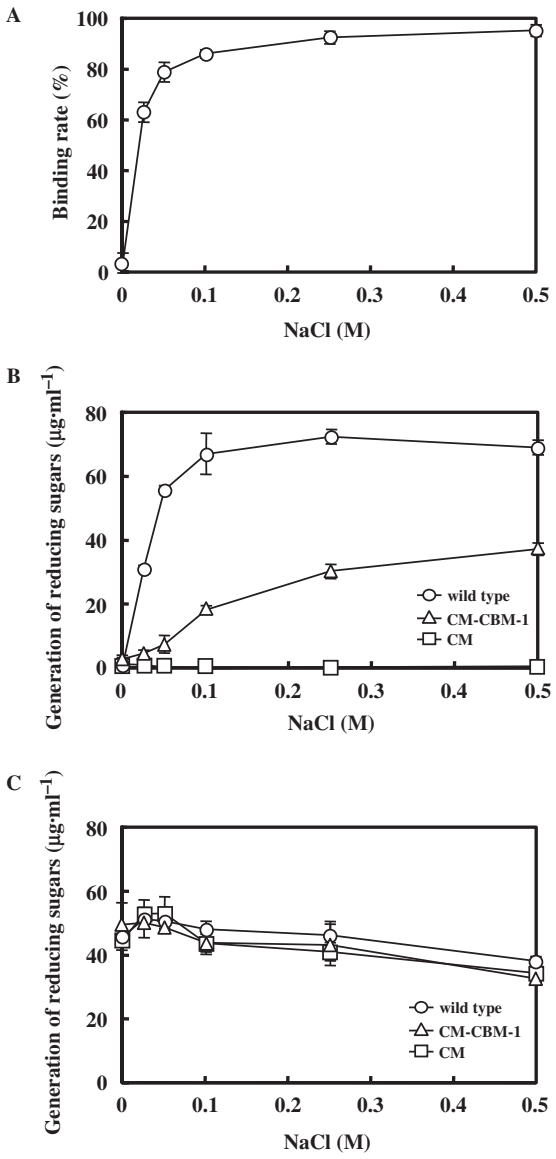

Fig. 6. **Effects of NaCl on the enzyme and binding activities of XYL4 and its deletion mutants.** (A) Effects of NaCl on the binding activity of CBM-1-2 were examined. Reaction mixtures containing 0.5% β-1,3-xylan, various concentrations of NaCl, and an appropriate amount of CBM-1-2 in 100 μl of 5 mM sodium phosphate buffer, pH 7.5, were incubated at 4°C for 30 min. After centrifugation, the CBM-1-2 remaining in the supernatant was detected by western blotting. Effects of NaCl on the enzyme activities of XYL4 and the mutant enzymes were examined using β-1,3-xylan (B) and glycol-β-1,3-xylan (C) as a substrate. Reaction mixtures containing 0.5% substrate, various concentrations of NaCl and 0.01 U of XYL4 or a mutant in 300 μl of 5 mM sodium phosphate buffer, pH 7.5, were incubated at 37°C for 30 min. Wild-type XYL4, circle; CM-CBM-1, triangle; CM, square. Values are the average of triplicate experiments with SDs. Details are described in MATERIALS AND METHODS section.

This method was further used to detect the β-1,3-xylan-binding proteins in the extract of a turban

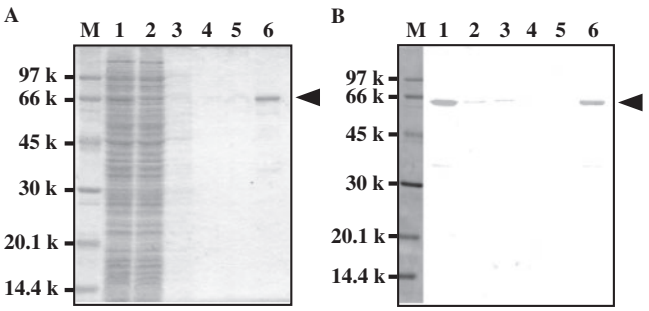

Fig. 7. **Purification of the recombinant XYL4 using β-1,3-xylan.** The supernatant of the extract from *E. coli* strain BL21(λDE3)pLysS transformed with pETXYL4 was incubated with β-1,3-xylan and then recombinant XYL4 was eluted from the β-1,3-xylan by decreasing the concentration of NaCl. After purification, the purity of each preparation was analysed by (A) SDS–PAGE with CBB staining and (B) western blotting with anti-His tag antibody staining. Lane M, low-molecular SDS–PAGE calibration kit; lane 1, crude extract of *E. coli* strain BL21(λDE3)pLysS transformed with pETXYL4; lane 2, unbound protein fraction; lane 3, wash fraction (1st); lane 4, wash fraction (2nd); lane 5, wash fraction (3rd); lane 6, eluted protein fraction. Details are shown in MATERIALS AND METHODS section.

shell, *T. cornutus*, which was likely to possess β-1, 3-xylanase in the viscera (*29*). The extract of *T. cornutus* viscera was incubated at 4°C with insoluble β-1,3-xylan in the presence of 0.1 M NaCl, and then the proteins bound to β-1,3-xylan were eluted by decreasing the salt concentration by dialysis as described in MATERIALS AND METHODS section. SDS–PAGE revealed that the eluate contained two proteins having a molecular mass of 97 and 120 kDa (water-eluted fraction) (Fig. 8A, lane 2). In addition, four additional proteins (66, 48, 35 and 17 kDa) were detected in the solution eluted from the β-1,3-xylan with SDS sample buffer (SDS-eluted fraction) (Fig. 8A, lane 3), suggesting that at least six β-1,3-xylan-binding proteins were present in the extract of *T. cornutus*. Next, the β-1,3-xylanase activity of the two fractions was analysed by TLC using β-1,3-xylan as the substrate (Fig. 8B). Oligosaccharides with different Rfs were generated from β-1,3-xylan when the substrate was incubated with the water-eluted fraction (Fig. 8B, lane 3) and SDS-eluted fraction (Fig. 8B, lane 5), suggesting that both fractions contain β-1,3-xylan-degrading enzymes. However, the enzymes involved in the water-eluted fraction seem to be different from those in the SDS-eluted fraction since the patterns of degradation of β-1,3-xylan by the two fractions were completely different (Fig. 8B, lane 3 versus lane 5). Collectively, insoluble β-1,3-xylan is useful to obtain β-1,3-xylan-binding proteins, which may have CBM(s), including β-1,3-xylanases from various samples. This method will facilitate further study of β-1,3-xylanase and β-1,3-xylan-binding proteins.

CONFLICT OF INTEREST

None declared.

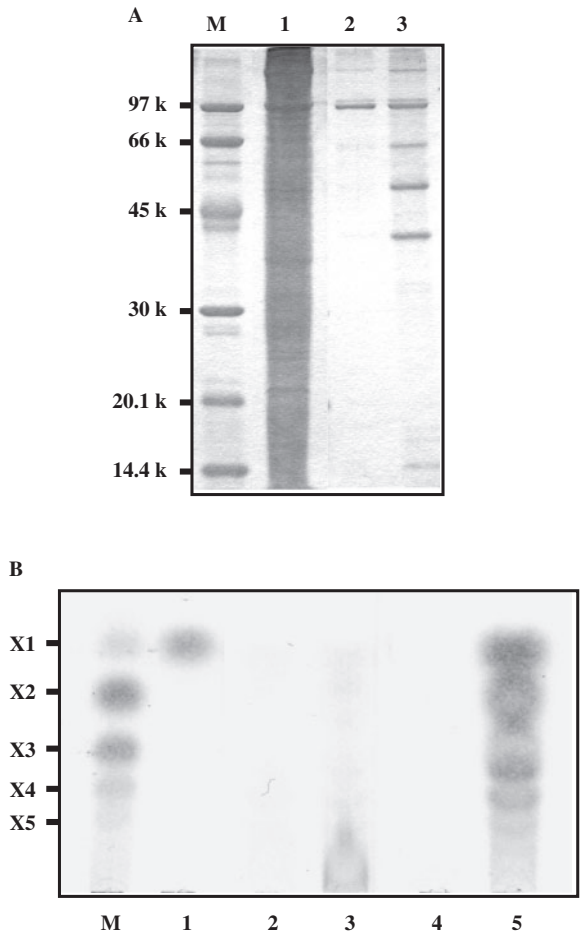

Fig. 8. **Detection of β-1,3-xylan-binding proteins (A), and β-1,3-xylan-degrading activity (B) in the extract of a turban shell, *T. cornutus*.** (A) SDS–PAGE showing the purification of β-1,3-xylan-binding proteins from the extract of *T. cornutus*. Lane M, low-molecular SDS–PAGE calibration kit; lane 1, crude extracts of *T. cornutus*; lane 2, fraction eluted from β-1,3-xylan with de-ionized water; lane 3, fraction eluted from β-1,3-xylan with SDS sample buffer. (B) TLC showing the β-1, 3-xylan-degrading activity of the fractions. Abbreviations used are as follows: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose. Lane M, xylooligosaccharide mixture (marker); lane 1, X1; lanes 2 and 4, elutes from β-1,3-xylan with de-ionized water (water-eluted fraction) and SDS sample buffer (SDS-eluted fraction), respectively; lane 3, β-1,3-xylan was incubated with the water-eluted fraction at 37°C for 24 h; lane 5, β-1,3-xylan was incubated with the SDS-eluted fraction at 37°C for 24 h. Lanes 3 and 5 show the hydrolysis of β-1, 3-xylan. Details are shown in MATERIALS AND METHODS section.

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