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Characterization of *Paenibacillus curdlanolyticus* B-6 Xyn10D, a Xylanase That Contains a Family 3 Carbohydrate-Binding Module▽†

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***Paenibacillus curdlanolyticus* B-6 Xyn10D is a xylanase containing a family 3 carbohydrate-binding module (CBM3). Biochemical analyses using recombinant proteins derived from Xyn10D suggested that the CBM3 polypeptide has an affinity for cellulose and xylan and that CBM3 in Xyn10D is important for hydrolysis of insoluble arabinoxylan and natural biomass.**

Most catalytic modules of xylanases (endo-1,4-β-xylanase; EC 3.2.1.8) are classified in family 10 or 11 of the glycoside hydrolases, based on the amino acid sequence (http://www.cazy.org/). Many xylanases consist of catalytic and ancillary modules, such as the carbohydrate-binding module (CBM) and the fibronectin type-3 homology (Fn3) module. CBMs are also classified into families based on amino acid sequence similarities. The family 3 CBMs (CBM3s) are often associated with family 9 and 48 catalytic modules or scaffolding proteins that are responsible for cellulosome (cellulolytic complex) assembly in anaerobic bacteria (3). CBM3s are sometimes found in complex modular xylanases, including *Caldibacillus cellulovorans* XynA (21), *Caldicellulosiruptor* sp. strain Rt69B.1 XynC (15), and *Caldicellulosiruptor* strain Tok7B.1 XynA (5) (Fig. 1A), which contain CBM22s in addition to CBM3s. The xylan-binding activity of CBM22 in *C. cellulovorans* XynA has been reported (21), but the function of the CBM3s was not investigated. The CBM3 of *Clostridium lentocellum* putative xylanase (GenBank accession no. ADZ82311) has a simple modular structure (Fig. 1A) but has not been biochemically characterized.

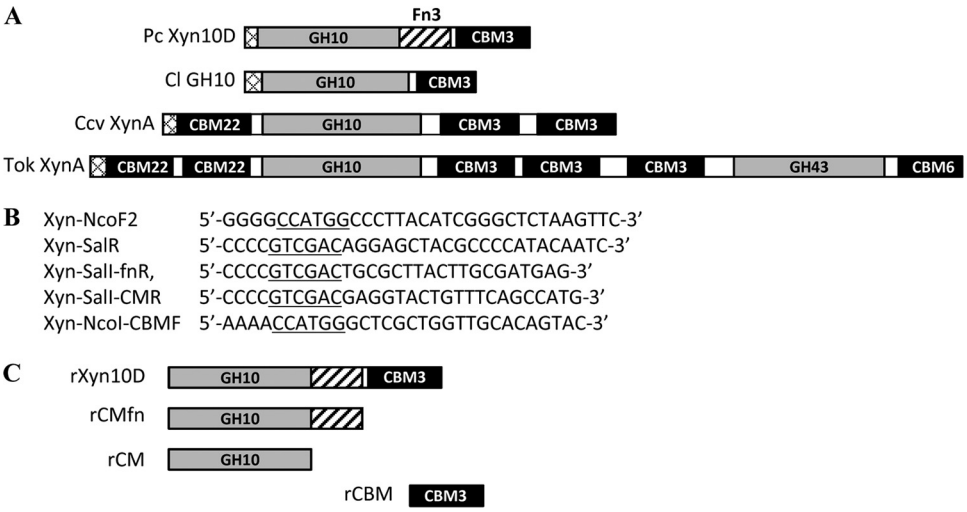

FIG. 1. Schematic of *P. curdlanolyticus* Xyn10D and some related xylanases (A), PCR primers for construction of rXyn10D (B), and the truncated derivatives (C). Pc, *P. curdlanolyticus*; Cl GH10, *C. lentocellum* putative family 10 glycoside hydrolase; Ccv, *C. cellulovorans*; Tok, *Caldicellulosiruptor* strain Tok7B.1; SP, signal peptide; doc, dockerin. NcoI and SalI sites in PCR primers are underlined. rXyn10D, rCMfn, rCM, and rCBM contain Xyn10D residues 32 to 577, 32 to 410, 32 to 317, and 429 to 577, respectively.

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TABLE 1. Activity of rCM, rCMfn, and rXyn10D toward xylans from different origins

Enzyme	Activity (μmol/min/μmol protein ± SD) toward:				
	Rye arabinoxylan[a]	Birchwood xylan[a]	4-*O*-Methyl-D-glucrono-D-xylan[a]	Oat spelt xylan[a]	Wheat arabinoxylan (insoluble)[b]
rXyn10D	26,500 ± 411	7,470 ± 98.0	9,110 ± 247	6,880 ± 0.00	2,470 ± 145
rCMfn	29,600 ± 408	8,330 ± 312	10,500 ± 478	6,040 ± 255	1,760 ± 100
rCM	31,200 ± 283	8,190 ± 170	10,400 ± 94.3	7,090 ± 233	1,770 ± 37.4

[a] Substrate concentration, 0.5%.
[b] Substrate concentration, 1.0%.

(All protein sequences cited in this paper are from the GenBank database.)

Paenibacillus curdlanolyticus strain B-6 is unique in that it produces extracellular xylanolytic-cellulolytic multienzyme complexes, mainly comprising xylanases, under aerobic conditions (17, 18, 22, 23), although the mechanism of multienzyme complex formation is unclear. Four xylanase genes, *xyn10A* (24), *xyn10B* (20), *xyn10C* (unpublished results), and *xyn11A* (17), were cloned from *P. curdlanolyticus* strain B-6, and the translated products were characterized. In this study, we cloned *xyn10D*, encoding a CBM3-containing xylanase (Xyn10D), from this bacterium and characterized the recombinant enzyme and its truncated derivatives.

A genomic library of *P. curdlanolyticus* strain B-6 constructed using a CopyControl fosmid library production kit (Epicenter, Madison, WI) has been described previously (20). We detected and excluded recombinant bacteria carrying the known xylanase genes by PCR using recombinant fosmid DNA as templates and specific primers. We identified *xyn10D* (DDBJ/EMBL/GenBank accession number AB600191), which consisted of 1,734 nucleotides (nt) and encoded 577 amino acid residues with a calculated molecular weight of 61,811, including a possible N-terminal signal peptide, as predicted by the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Searches using the Pfam database (http://pfam.sanger.ac.uk/) demonstrated that Xyn10D is a modular enzyme consisting of a signal peptide, a family 10 catalytic module of the glycoside hydrolases, an Fn3 module, and a CBM3, in that order, from the N terminus (Fig. 1A). CBM3 of Xyn10D showed high sequence identities to CBMs found in some characterized or hypothetical cellulolytic enzymes of *Paenibacillus* species, including the family 5 endoglucanase of *Paenibacillus* sp. KSM-N115 (64%; accession no. BAF62084) (16), *Paenibacillus lautus* CelA (60%; accession no. AAA22303) (8), and a putative cellobiohydrolase (69%; accession no. EFM08880) of *P. curdlanolyticus* YK9. It exhibited moderate sequence identities to some CBM3s present in xylanases and in scaffolding proteins that are involved in cellulosome formation, including *C. cellulovorans* XynA (45%; accession no. AAF61649) (21), *Caldicellulosiruptor* sp. strain Rt69B.1 XynC (44%; accession no. AAB95326) (15), *Caldicellulosiruptor* strain Tok7B.1 XynA (42%; accession no. AAD30363) (5), *Clostridium thermocellum* CipA (42%; accession no. CAA48312) and *Clostridium cellulolyticum* CipC (44%; accession no. AAC28899). There was a low identity with CBM3 of the putative *C. lentocellum* xylanase (27%; accession no. ADZ82311). CBM3s have been grouped into three subfamilies, 3a, 3b, and 3c (4, 9). Although the family 3a and 3b CBMs, both of which bind strongly to crystalline cellulose, are closely similar in their primary structures, family 3b CBMs lack a short region. The CBM of Xyn10D is a member of family 3b (see Fig. S1 in the supplemental material).

To investigate the function of CBM3 of Xyn10D, Xyn10D and its truncated derivatives were produced as recombinant proteins (rXyn10D, rCMfn, rCM, and rCBM) (Fig. 1C) by *Escherichia coli*. Plasmids used for the expression of these proteins were constructed by ligating a PCR fragment amplified using an appropriate combination of primers (Fig. 1B) into pET-28a(+) (Novagen, Madison, WI). *E. coli* BL21-CodonPlus (DE3)-RIPL (Novagen) cells carrying the recombinant plasmid were cultivated in Super broth (BD, Franklin Lakes, NJ) supplemented with chloramphenicol (50 μg/ml) and kanamycin (25 μg/ml) at 37°C. Cell extracts prepared by sonication were used for purification by a HisTrap HP column (GE Healthcare Japan, Tokyo, Japan). All purified proteins resulted in a single band following SDS-polyacrylamide gel electrophoresis (PAGE) (data not shown) (11).

Hydrolytic activities of the recombinant enzymes were measured with soluble xylans of different origins, such as rye arabinoxylan (Megazyme, Wicklow, Ireland), birchwood xylan (Sigma-Aldrich Japan, Tokyo, Japan), and 4-*O*-methyl-D-glucrono-D-xylan (Sigma-Aldrich Japan) by incubating for 10 min at 35°C in 50 mM sodium phosphate buffer (pH 7.0 for rCM, pH 6.0 for rXyn10B and rCMfn), followed by measuring reducing sugars released from the substrates by the 3,6-dinitrophthalic acid reagent (12). Enzyme assays were done in triplicate, and results were expressed as micromoles of reducing sugars/min/micromole of protein. rXyn10D was most active toward rye arabinoxylan and moderately active toward birchwood and oat spelt xylans (Sigma-Aldrich Japan, Tokyo, Japan) (Table 1). Derivatives of Xyn10D devoid of CBM, rCMfn, and rCM showed the same tendency in catalytic activities toward different xylans. They were most active toward rye arabinoxylan. These results suggest that Xyn10D and its deriva-

TABLE 2. Activity of rXyn10D, rCMfn, and rCM toward agricultural wastes

Enzyme	Activity (μmol/min/μmol protein ± SD) toward[c]:		
	Rice straw[a]	Rice husk[b]	Rice bran[b]
rXyn10D	32 ± 1.5	20 ± 0.5	91 ± 1.5
rCMfn	10 ± 7.5	2.0 ± 0.0	ND
rCM	ND	ND	26 ± 6.5

[a] Substrate concentration, 2.5%.
[b] Substrate concentration, 5.0%.
[c] ND, not detectable.

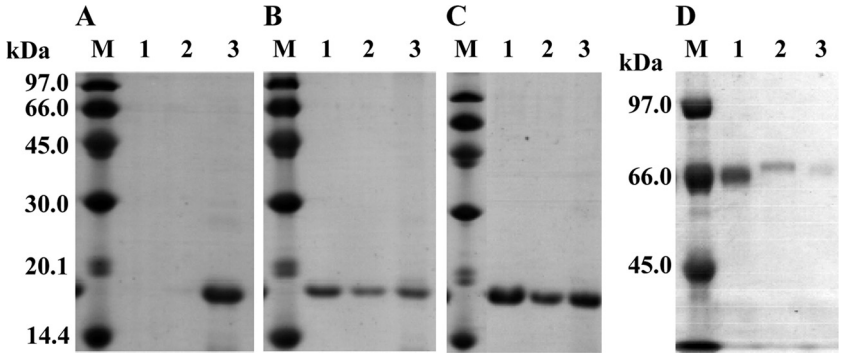

FIG. 2. Adsorption of rCBM to Avicel and insoluble xylan preparations. rCBM was incubated with Avicel (A), an insoluble fraction of oat spelt xylan (B), and insoluble wheat arabinoxylan (C). After centrifugation, proteins in the supernatant (lane 1), wash (lane 2), and precipitate (lane 3) fractions were analyzed by SDS-PAGE. In panel D, BSA as a control protein was incubated with an insoluble fraction of oat spelt xylan, and each fraction was subjected to SDS-PAGE. In panels A to C, 15% polyacrylamide gels were used. In panel D, a 7.5% polyacrylamide gel was used.

tives prefer highly arabinosylated xylan to less substituted xylans since rye arabinoxylan is more arabinosylated than oat spelt xylan (19). However, since rXyn10D hydrolyzed xylooligosaccharides (data not shown), it does not necessarily require arabinose residues for substrate recognition. Specific activities of these enzymes were similar (Table 1). These results suggest that the presence of CBM3 in rXyn10D does not affect the catalytic activity toward soluble substrates. In contrast, when insoluble wheat arabinoxylan was used as a substrate, rXyn10D showed higher activity toward the insoluble substrate than the truncated enzymes (Table 1). It should be noted, furthermore, that rXyn10D hydrolyzed agricultural waste, such as rice straw, rice husk, and rice bran, more efficiently than the truncated enzymes (Table 2). These results strongly suggest that CBM3 in rXyn10D plays an important role in hydrolysis of insoluble xylan and native biomass materials. Although Kataeva et al. (10) found that the Fn3 repeat from the *C. thermocellum* cellobiohydrolase CbhA promoted hydrolysis of cellulose by modifying its surface, the function of the Fn3 module in Xyn10D is unclear, because activities of rCMfn and rCM were similar toward xylans from different origins (Table 1).

The binding of rCBM to Avicel and insoluble fractions of oat spelt xylan and wheat arabinoxylan was determined by mixing rCBM (88 mg) and insoluble polysaccharides (100 mg) in 50 mM sodium phosphate buffer (pH 7.0) and then incubating on ice for 1 h with occasional stirring. After centrifugation, the supernatant fraction was recovered, and the precipitate was resuspended in sodium phosphate buffer. The suspension was centrifuged to separate wash and precipitate fractions, which were all analyzed by SDS-PAGE. Almost all protein coprecipitated with insoluble cellulose (Avicel), and very little protein was detected in the supernatant and wash fractions (Fig. 2A), indicating that rCBM was tightly bound. The rCBM moderately bound to the insoluble fraction of oat spelt xylan (Fig. 2B) and wheat arabinoxylan (Fig. 2C). The control protein (bovine serum albumin [BSA]) only slightly bound to xylan (Fig. 2D).

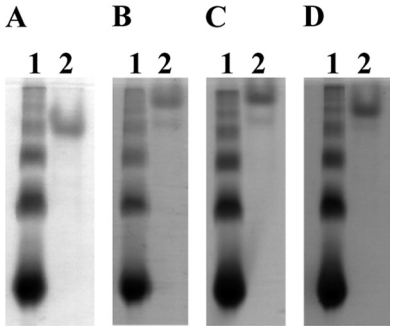

FIG. 3. Adsorption of rCBM to soluble xylans of different origins. Affinities of rCBM (lane 2) for rye arabinoxylan (B), birchwood xylan (C), and oat spelt xylan (D) were analyzed by native affinity gel electrophoresis. Lane 1 contains BSA as a control protein. (A) A gel without a polysaccharide served as a reference.

The affinity of rCBM for rye arabinoxylan, birchwood xylan, and oat spelt xylan was examined by native affinity PAGE as described previously (2). Protein samples (2.4 μg) were loaded onto gels, and BSA was used as a control. Migration of the proteins was significantly retarded by inclusion of rye arabinoxylan and birchwood xylan in gels and less affected by oat spelt xylan (Fig. 3). Affinity of rCBM for xylans was in contrast to the observations for CBMs from *C. thermocellum* CipB (13) and *Clostridium cellulovorans* CbpA (7); that is, the latter CBMs showed no affinity for xylan.

Importance of CBMs in hydrolysis of xylan and complex substrates has been sometimes found in modular enzymes and enzyme complex (1, 6, 14). It is interesting that CBM3 has an affinity for xylan and plays an important role in hydrolysis in soluble substrates. Structural analysis of the CBM of Xyn10D should be carried out to determine substrate recognition by CBM3.

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