Essential role of the family-22 carbohydrate-binding modules for β-1,3-1,4-glucanase activity of *Clostridium stercorarium* Xyn10B

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Abstract ***Clostridium stercorarium*** **Xyn10B is a modular enzyme comprising two family-22 carbohydrate-binding modules (CBMs), a family-10 catalytic module of glycoside hydrolases, a family-9 CBM, and two S-layer homologous modules consecutively from the N-terminus. To investigate the role of the family-22 CBMs, truncated proteins were constructed: a recombinant catalytic module polypeptide (rCD), a CBM polypeptide composed of two family-22 CBMs (rCBM) and a polypeptide composed of the family-22 CBMs and the catalytic module (rCBM-CD). We found that rCBM-CD was highly active toward β-1,3-1,4-glucan; however, rCD was negligibly active toward the same substrate. The V_{max}/K_m value of rCBM-CD for β-1,3-1,4-glucan was 7.8 times larger than that for oat-spelt xylan, indicating that rCBM-CD should be specified as a β-1,3-1,4-glucanase rather than a xylanase despite the fact that family-10 catalytic modules are well-known xylanase modules. These results indicate that the family-22 CBMs in rCBM-CD are essential for hydrolysis of β-1,3-1,4-glucan.**

Key words: Xylanase; Carbohydrate-binding module; Substrate specificity; Thermostabilizing module; *Clostridium stercorarium*

1. Introduction

Many glycoside hydrolases consist of two or more functional modules, such as a catalytic module and carbohydrate-binding module (CBM) [1]. Based on amino acid sequence similarities, catalytic modules of glycoside hydrolases can be classified into 92 families, and CBMs into 34 families ([2]; http://afmb.cnrs-mrs.fr/CAZY/index.html). It is generally believed that each functional module in a polypeptide can function normally even after separation from an adjacent module. Therefore, many catalytic modules and CBMs, which can be expressed independently of other functional modules by gene manipulation techniques, have been analyzed for structural and biochemical characterization.

Family-22 CBMs were originally identified and specified as thermostabilizing modules since their removal from thermophilic enzymes resulted in a decrease in the thermostability and/or optimal temperature of the enzymes [3–5]. Later, however, these modules were found to have an affinity for soluble [6–9] or insoluble [10] polysaccharides, and were consequently compiled and classified in family-22 CBMs.

Clostridium stercorarium Xyn10B (formerly XynC) is a typical modular enzyme that comprises two family-22 CBMs, a family-10 catalytic module of glycoside hydrolases, a family-9 CBM, and two S-layer homologous modules consecutively from the N-terminus [11]. Modular enzymes whose module organization is similar to Xyn10B have been reported as xylanases in several bacteria such as *Caldicellulosiruptor* sp. [12], *Clostridium thermocellum* [9], *Thermoanaerobacterium saccharolyticum* [3], *Thermoanaerobacterium thermosulfurigenes* [13] and *Thermotoga maritima* [8]. The binding affinities of the family-22 CBMs from *C. thermocellum* XynY [4] and *T. maritima* XynA [8] to soluble saccharides have been investigated and the tertiary structure of the CBM from *C. thermocellum* XynY has also been disclosed [8].

In the present study, we have found that a derivative of Xyn10B comprising two family-22 CBMs and a catalytic module is highly active toward β-1,3-1,4-glucan, however, the catalytic module polypeptide lacking CBMs was not. This indicates that the presence of the family-22 CBMs adjacent to the catalytic module plays an essential role in hydrolysis of β-1,3-1,4-glucan.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli M15 (Qiagen) was cultivated in Luria broth supplemented with ampicillin (50 μg/ml) for use as the host of derivatives of plasmid pQE-60T, which contains a short stretch of nucleotides encoding a thrombin recognition sequence (LVPRGS) between the multiple cloning sites and His tag-coding region of pQE-60 (Qiagen). *E. coli* BL21(DE3) was used as the host for a derivative of pET-28a (Novagen). Plasmid pMA-5.1 containing the full-length *xyn10B* gene was described previously [11].

2.2. Construction of p2CBM-CD10, pCD10, and p2CBM

To construct p2CBM-CD10, which encodes an enzyme composed of the family-22 CBMs and the family-10 catalytic module (rCBM-CD) (Fig. 1A), the coding region was amplified from a pMA-5.1 template with a combination of two synthetic oligonucleotide primers; primer 1 containing an *Afl*III recognition sequence, 5′-TTACATGTCAGAAACAACGGTTTATCATGAGAC-3′, and primer 2 containing an *Bgl*II recognition sequence, 5′-CGCAGATCTTTTCTCAATGAATTCGTCCGG-3′. The amplified DNA fragment was digested with *Afl*III and *Bgl*II, and cloned between the *Nco*I and *Bgl*II sites of pQE-60T, yielding p2CBM-CD10. A combination of primer 3 containing a *Nde*I site, 5′-GGGGCATATGATTGACGACATCAACTTTGAACC-3′, and primer 4 containing a *Sal*I recognition sequence, 5′-GGGCAGCTGCTAATATTTCTCAATGAATTCGTCCGGATC-3′, was used to construct pCD10. The amplified DNA fragment was digested with *Nde*I and *Sal*I, and cloned between the *Nde*I and *Sal*I sites of pET-28a, yielding pCD10. This plasmid encodes the

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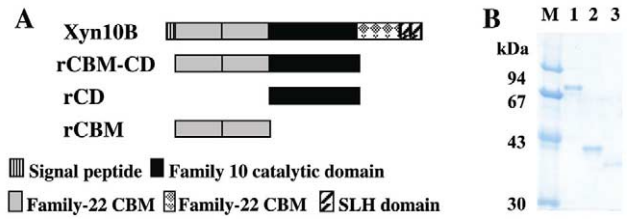

Fig. 1. Schematic diagram of *C. stercorarium* Xyn10B and its derivatives (A) and SDS–PAGE of the purified proteins (B). B: Lane 1, rCBM-CD; lane 2, rCD; lane 3, rCBM; lane M, protein mass standards.

family-10 catalytic module devoid of the CBM (rCD) (Fig. 1A). To construct p2CBM encoding a polypeptide composed of two family-22 CBMs (rCBM) (Fig. 1A), the coding region was amplified from a pMA-5.1 template with a combination of two synthetic oligonucleotide primers; primer 5 containing a *Nco*I recognition sequence, 5′-GGCCATGGCAGAAACAACGGTTTATCATGA-3′, and primer 6 containing a *Bgl*II recognition sequence, 5′-GGGAGATCTTTTTA-AACCTTTTTCAATCTCTTC-3′. The amplified DNA fragment was digested with *Nco*I and *Bgl*II, and cloned between the *Nco*I and *Bgl*II sites of pQE-60T, yielding p2CBM.

2.3. Site-specific polymerase chain reaction (PCR) mutagenesis

rCBM-CD was inactivated by converting each of two catalytic Glu residues conserved in all family-10 catalytic modules to Ala as follows. Site-directed mutagenesis of plasmid p2CBM-CD10 was accomplished using an overlap extension PCR mutagenesis procedure [14] with a pMA-5.1 template. The primers used to create the mutation to convert Glu-490 to Ala (E490A) were 5′-GGAAGGATTGTCGCT-CATTGCTGCGTTTACAACA-3′, its complementary sequence, and primers 1 and 2; the primers used to create the mutation to convert Glu-614 to Ala (E614A) were 5′-GCCCGCACGGATATCCAGTGC-GCTTATGCTGACCTC-3′, its complementary sequence, and primers 1 and 2. The amplified DNA fragments were digested with *Afl*III and *Bgl*II and cloned between the *Nco*I and *Bgl*II sites of pQE-60T, yielding pMU-E490A and pMU-E614A, respectively, and producing recombinant proteins rE490A and rE614A. The entire genes were sequenced to confirm the desired mutation and to check PCR fidelity.

2.4. Purification of recombinant proteins

Recombinant proteins rCBM-CD, rCD, rCBM, rE490A, and rE614A were purified from the cell-free extract prepared from each *E. coli* M15 or BL21(DE3) harboring one of the recombinant plasmids. Chromatography on a HiTrap chelating column (Amersham Pharmacia Biotech) was conducted following the supplier's protocol. Active fractions were combined and desalted by dialysis against 20 mM Tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.5). Samples were treated with thrombin protease (10 units per mg of each recombinant protein) and placed on the HiTrap chelating column. Proteins that did not adsorb to the column due to removal of their affinity tag were further purified on RESOUCE PHE and MonoQ HR5/5 column chromatographies. Protein concentration was determined using the Micro BCA protein assay reagent kit (Pierce) with bovine serum albumin as the standard.

2.5. Enzyme assays

Xylanase activity was measured by incubation in 50 mM sodium phosphate–12 mM citric acid buffer (pH 6.3) in the presence of oat-spelt xylan (15 mg/ml; Fluka Ag, Buchs, Switzerland) at various temperatures for 10 min unless otherwise stated. Released reducing sugars were measured with 3,5-dinitrosalicylic acid reagent [15] with xylose or glucose as the standard. β-1,3-1,4-Glucanase activity was determined at 60°C with lichenan (Sigma) and barley β-glucan (Sigma) as described above. One international unit (IU) corresponds to 1 μmol of xylose or glucose equivalent released per min.

2.6. Native affinity gel electrophoresis

The affinity of rCBM for soluble polysaccharides, namely oat-spelt xylan, birchwood xylan (Sigma), barley β-glucan, CMC (low viscosity; Sigma), laminarin (Nacalai Tesque, Kyoto, Japan), and chitin (Nacalai Tesque) were examined by native affinity gel electrophoresis as described previously [16].

3. Results

3.1. Thermostabilizing effect of the family-22 CBMs

rCBM-CD, rCD, and rCBM were purified from recombinant *E. coli* clones to homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS–PAGE (Fig. 1B). Since family-22 CBMs were originally identified as thermostabilizing modules [3–5], the effect of the family-22 CBMs on the xylanase activity of the catalytic module was determined. As a result, the optimum temperature of rCBM-CD was determined to be approximately 75°C but that of rCD was between 65 and 70°C, suggesting that family-22 CBMs of Xyn10B also contribute to thermal stabilization of the catalytic module adjacent to the CBMs.

3.2. Binding of rCBM to polysaccharides

The affinity of rCBM to a series of different soluble polysaccharides was qualitatively evaluated using native affinity gel electrophoresis (Fig. 2). Protein migration was significantly retarded by the inclusion of oat-spelt xylan and barley β-glucan in the gels, and was slightly affected by birchwood xylan. rCBM showed no affinity to CMC, laminarin or chitin (data not shown). Similarly, mutant proteins E490A and E614A also showed a high affinity for oat-spelt xylan and barley β-glucan. These results clearly indicate that the family-22 CBMs present in the N-terminus of Xyn10B have a binding affinity for polysaccharides such as oat-spelt xylan and barley β-glucan.

3.3. Substrate specificity of rCBM-CD and rCD

Since the family-22 CBMs showed an affinity for xylan, it was presumed that the CBMs play an important role in xylan hydrolysis and therefore the hydrolytic activities of rCBM-CD and rCD toward several substrates were compared. Both rCD and rCBM-CD showed similar specific activities toward oat-

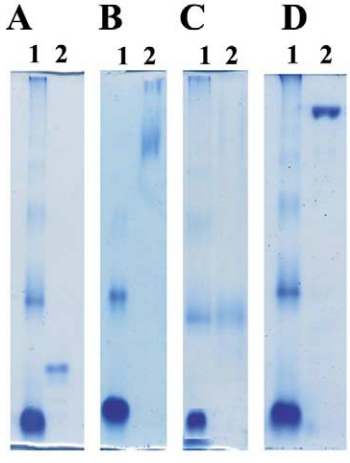

Fig. 2. Adsorption of rCBM to soluble polysaccharides. Affinities of rCBM for various soluble polysaccharides including oat-spelt xylan (B), birchwood xylan (C), and barley β-glucan (D) were analyzed by native affinity gel electrophoresis. A gel without a polysaccharide served as a reference (A). Lane 1, bovine serum albumin as a control protein; lane 2, rCBM.

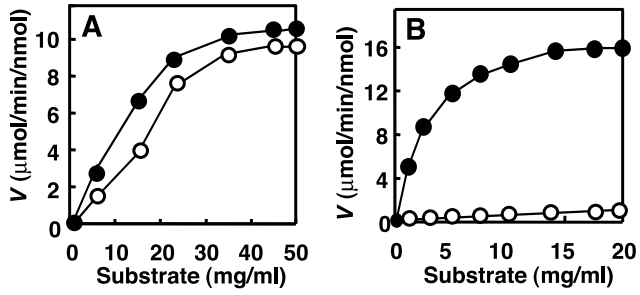

Fig. 3. Hydrolysis of oat-spelt xylan (A) and barley β-glucan (B) by rCBM-CD and rCD. The initial rates of the reaction of rCD (open circle) and rCBM-CD (closed circle) were measured at 60°C on various concentrations of oat-spelt xylan and barley β-glucan.

spelt xylan and birchwood xylan, i.e. specific activities of rCD were 136 and 157 IU/mg for oat-spelt xylan and birchwood xylan, respectively, and those of rCBM-CD were 113 and 114 IU/mg for the same substrates. When β-1,3-1,4 mixed glucan allomorphs such as barley β-glucan and lichenan were used as substrates, rCBM-CD hydrolyzed these two substrates with specific activities (120 and 93 IU/mg) similar to those toward oat-spelt and birchwood xylans. On the other hand, rCD without the family-22 CBMs showed relatively low activities toward these substrates (8 and 2 IU/mg).

The initial rates of the reaction of rCD and rCBM-CD were measured at 60°C in various concentrations of oat-spelt xylan and barley β-glucan. From Fig. 3, the V_{max} and K_m values of rCD were estimated to be 9.6 μmol/min/nmol protein and 17 mg/ml for xylan, respectively (Table 1). Similar values were obtained for rCBM-CD on xylan; a V_{max} of 10.6 μmol/min/nmol protein and a K_m of 12 mg/ml. The V_{max} and K_m values of rCBM-CD for β-glucan were determined as 15.9 μmol/min/nmol protein and 2.3 mg/ml, respectively. In contrast, the kinetic parameters of rCD with β-glucan could not be determined since reducing sugars released from the substrate gradually increased with increasing concentrations of the substrate, up to 20 mg per ml, and did not reach a plateau. As seen in Fig. 3, the amount of reducing sugars released from β-glucan by rCD was quite low compared to those released by rCBM-CD. It should be noted that the V_{max}/K_m value of rCBM-CD for barley β-glucan (6.9) was relatively larger than that for xylan (0.88).

Mutation of the possible catalytic Glu residues at either 490 or 614 into Ala in rCBM-CD abolished its catalytic activity toward both xylan and barley β-glucan (data not shown). rCBM itself showed no catalytic activity toward any substrates tested including xylan and barley β-glucan. These results strongly suggested that two Glu residues in the family-10 catalytic module were responsible for hydrolysis of xylan and barley β-glucan.

4. Discussion

Many cellulases and xylanases are modular enzymes consisting of a catalytic module and a CBM, however, additional modules often occur in an enzyme molecule. It is now believed that each functional module such as the catalytic module, which originally occurs in a modular enzyme, can function normally even after they have been artificially separated from an adjacent module such as a CBM. This assumption is the fundamental principle that allows the detailed construction and characterization of a single functional module isolated from other adjacent modules.

The removal of CBM(s) from modular glycoside hydrolases such as cellulase and xylanase [9,17,18] or the artificial connection of a CBM and a catalytic module [19,20] often affects enzyme activity towards insoluble and even soluble substrates in a case. The role of a CBM is expected to place the enzyme on a substrate, resulting in an increase in substrate concentration around the enzyme, and/or to disrupt the crystalline structure of the substrates thus supplying the catalytic module with more easily digestible substrates.

On the other hand, the truncation of some ancillary modules often results in a shift in optimal temperature to a lower range, and a decrease in thermostability, e.g. the removal of family-22 CBMs, which were originally named thermostabilizing modules [6], decreased optimal temperature and thermostability of family-10 catalytic modules originally adjacent to the CBMs. Recently, it was shown that module interactions contribute to the thermostability of modular cellulase CbhA modules from *C. thermocellum* [21,22].

In this study, we found that the removal of family-22 CBMs from the catalytic module drastically reduced catalytic activity towards β-1,3-1,4 mixed glucan, and decreased the thermostability of the catalytic module. Although the catalytic module by itself showed relatively high activity towards xylan, the K_m/V_{max} values of rCD and rCBM-CD for oat-spelt xylan were quite smaller than that of rCBM-CD towards barley β-glucan (Table 1). It can be denied that the family-22 CBMs themselves mediate the hydrolysis of β-1,3-1,4 mixed glucan by the observations that rCBM itself showed no hydrolytic activity and two catalytic residues, Glu-490 and Glu-614 in Xyn10B, which are conserved in all family-10 enzymes, were involved in hydrolysis of β-1,3-1,4 mixed glucan. This phenomenon may be explained as follows: the family-22 CBMs bind more strongly to glucan than to xylan (Fig. 2), therefore it is likely that the CBMs act to direct its polysaccharide ligand into the active site of the enzyme, thus creating a very large effective concentration of substrate at the active site.

In conclusion, this is the first report indicating that CBMs strongly affect the substrate specificity of a catalytic module adjacent to it. Furthermore, it should be noted that we must be more circumspect with regards to the truncation of a mod-

Table 1
K_m and V_{max} values of rCBM-CD and rCD for xylan and barley β-glucan

Enzyme	Oat-spelt xylan			Barley β-glucan		
	K_m (mg/ml)	V_{max} (μmol/min/nmol)	V_{max}/K_m	K_m (mg/ml)	V_{max} (μmol/min/nmol)	V_{max}/K_m
rCBM-CD	12	10.6	0.88	2.3	15.9	6.9
rCD	17	9.6	0.56	–	–	–

–: Kinetic parameters could not be determined.

ular enzyme when characterizing a module of interest since there is a possibility that separation of functional modules affects the biochemical and structural characteristics of one or both of the separated modules not only in Xyn10B but also in many other modular enzymes.

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