Characterization of Family 3 Carbohydrate-binding Module from *Clostridium josui*

S. Ichikawa[1] and S. Karita[1]

[1] Graduate School of Regional Innovation Studies, Mie University, Tsu city, Japan
karita@innov.mie-u.ac.jp

Abstract—**Binding properties of the family 3 carbohydrate-binding module which is one of the modules of a scaffold protein of the cellulosomes from *Clostridium josui* (*Cj*CBM3) was evaluated. Although it is classified to a type of binding to surface of crystalline cellulose, it bound to celluloses which have broad amorphous regions and to soluble xyloglucan. Furthermore it is showed *Cj*CBM3 bound to them though its flat binding site same as it interacts with crystalline surface. This CBM possesses a wider binding surface which includes more hydrophilic residues than other CBMs which are also categorized as crystalline surface binding type. It is revealed that polar hydrophilic residues in the binding site are important to bind to xyloglucan. Additionally it implied they interact with xyloglucan side chains.**

Keywords— **cellulase, carbohydrate-binding module.**

I. Introduction

Lignocellulose, a major component of the plant cell wall, is produced through photosynthesis. Photosynthesis fixes atmospheric CO_2 to produce living carbon compounds (mainly, lignocellulose); biodegradation of lignocellulose sends gaseous CO_2 back to the atmosphere. Cellulose, the primary component of lignocellulose, is the most abundant polymeric carbohydrate that human beings do not fully utilize. Sustainable production of biofuels from cellulosic materials provides benefits to the environment and economy. Efficient biological conversion of lignocellulosic biomass to biofuels and biobased chemicals involves three sequential steps: lignocellulose pretreatment, enzymatic cellulose hydrolysis, and fermentation, where enzymatic cellulose hydrolysis plays a central role in producing soluble fermentable sugars from the pretreated solid cellulosic feedstock.

The enzymatic degradation of lignocellulose is one of the most important reactions on earth. Despite this, glycoside hydrolases attack lignocellulose relatively inefficiently because their target glycosidic bonds are often inaccessible to the active site of the appropriate enzymes. In order to overcome these problems, many of the glycoside hydrolases that degrade insoluble substrates are modular, comprising catalytic modules appended to one or more non-catalytic carbohydrate-binding modules (CBMs). CBMs promote the association of the enzyme with the substrate. In view of the central role that CBMs play in the enzymatic hydrolysis of plant-structural and -storage polysaccharides, the ligand specificity displayed by these protein modules and the mechanism by which they recognize their target carbohydrates have received considerable attention since their discovery almost 25 years ago.

The family 3 CBM from *Clostridium josui*, anaerobic cellulolytic bacterium isolated from Thailand compost[1], is one of the modules of a scaffold protein of the cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. The cellulosome shows high enzymatic activity because it is composed of various enzymes, degrading plant cell wall synagistically. In this report, binding properties of *Cj*CBM3 was characterized.

II. Materials and methods

A. Plasmid constructions

The DNA fragments encording for *Cj*CBM3 and CBM1 from *Trichoderma reesei* Cel7A (*Tr*CBM1)[2],[3] were amplified from genomic DNAs of *C. josui* and *T. reesei* by using the primer A and B, and the primer C and D, respectively (Table 1). The amplified DNA fragment was ligated into pCR2.1 (Invitrogene). Resulting plasmids were sequenced forconfirming the absence of artificial mutations. The confirmed plasmid samples were digested with restriction enzymes. *Cj*CBM3 coding fragment was ligated into pQE30 (Qiagen) and *Tr*CBM1 coding region was ligated into pRSET/CFP (Invitrogen). Additionally, in this research, two *Cj*CBM3 mutants, W124A and Y71A/W124A, were prepared by the site direct mutagenesis method. The primer C and D, and the primer E and F were used to convert 71th tyrosine to alanine, and 124th tryptophan to alanine, in *Cj*CBM3 respectively. Yielding plasmids that each *Cj*CBM3 region presents in pQE30 and *Tr*CBM1 coding region presents in pRSET/CFP were used for transforming *Escherihia coli* JM109 (Toyobo) and BL21(DE3) (Invitrogen), respectively. DNA manipulation was performed according to standard techniques.

Table 1 Primers used for the DNA constructions

A:	5'-AAGGATCCGCAGCTGATACTGGCG-3'
B:	5'-AAGCTTTCAACCATTAGGTGTTGAACCA-3'
C:	5'-GGATCCCCTACCCAGTCTCACTACGG
D:	5'-GAATTCGGGGGAGGTCAGGCACTGAGAGTAGTAAGG
E:	5'-AACGCTATGGATGTTACTTCAAAGGT-3'
F:	5'-TGAAGTAACATCCATAGCGTTGTT-3'
G:	5'-GACGCTTCTAATTTTGATCAATCAAA-3'
H:	5'-TTGATCAAAATTAGAAGCGTCGTT-3'

Incorporated *Bam*HI, *Hin*dⅢ and *Eco*RI restriction sites are underlined.

B. Protein purifications

Transformed *E. coli* cells were grown for overnight at 37°C in Luria-Bertani (LB) broth supplemented with 50μg/ml ampicillin. The cultivated cells were collected with centrifugation (6000×g) and disrupted with sonication. The expressed proteins in cell free extracts were purified with Ni-NTA agarose resin (Qiagen) according to the manufacturer's instruction. The purified proteins were dialyzed with 20mM potassium phosphate buffer (KPB) pH.7 for overnight at 4°C. The dialyzed proteins were centrifuged at 18,000×g for 2 min. The supernatants were dispensed in conveniently sized packets and stored at -80°C. The final preparations were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and measured its amounts from the absorbance at 280 nm. The molar extinction coefficients for *Cj*CBM3, W124A, Y71A/W124A and *Tr*CBM1-CFP (34410, 28860, 27520 and 30760, respectively) were predicted from the tryptophan, tyrosine and cysteine contents of the proteins.[4]

C. Substrate preparations

Microcrystalline cellulose (Avicel; Funakoshi), carboxymethyl-cellulose (CMC; Sigma chemical), xyloglucan (Megazyme), birch wood xylan (Fluka), oat-spelt xylan (Fluka), chitin (Wako) were purchased. Acid swollen cellulose (ASC) was prepared by phosphoric acid treatment of Avicel. Ball-milled cellulose (BMC) was prepared by ball-mill processing of the KC Flock (Nippon Paper Chemicals) with distilled water for 72h. Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* as described previously.[5] Each prepared substrate was stored at 4°C.

D. Macroarray assays

Tested polysaccharides were applied as 1μl aliquots to untreated nitrocellulose sheets (Millipore) in a 5-fold dilution series. Sheets were left to dry at room temperature for 30 min prior to blocking for 1 h with 5% (w/v) skim milk protein in 5×phosphate-buffered saline (PBS; prepared from a 10×stock solution containing 80g NaCl, 2g KCl, 28.6g Na_2HPO_4 $12H_2O$ and 2g KH_2PO_4 in 1L H_2O, pH 7.2). The nitrocellulose sheets were incubated with 1.25μg/ml of the appropriate CBM in 5×PBS for 1 h. The nitrocellulose sheets were then washed with distilled water extensively prior to incubation in a 5000-fold dilution of anti-His horseradish peroxidase (HRP) conjugate (Qiagen) in 5×PBS. After washing with distilled water, sheets were incubated in freshly prepared HRP substrate to detect CBM binding (0.5% 3-3'-Diaminobenzidine, tetrahydrochloride (DAB) and 0.005% H_2O_2). The reaction was stopped by washing the nitrocellulose sheets with pure water.

E. Affinity gel electrophoreses

The affinities of *Cj*CBM3 for soluble polysaccharides were also determined by affinity gel electrophoresis. The method was essentially as described by Araki *et al.*[6] using xyloglucan or CMC at a concentration of 0.1% (w/v). Electrophoresis was carried out for 30 min at room temperature in native polyacrylamide gels containing 12% (w/v) acrylamide. Bovine serum albumin (BSA) was used as non-binding control protein.

F. Adsorption isotherm measurements

All adsorption isotherm measurements were carried out at room temperature in 1.5-ml Proteo Save Tubes (Sumitomo Bakelite) containing various concentration of CBM mixed with 0.0017% ASC in 20mM KPB, to a final aqueous volume of 0.3 ml. Each solution was gently mixed for ~3 sec and incubated for 30 minutes, during which time, it was again mixed in the same way after 15 minutes from the start of incubation, to allow the adsorption system to equilibrate. The samples were then centrifuged at 25°C and 18,000×g for 2 min to remove the protein-covered substrates. The samples were centrifuged at 18,000×g for 1 min again and the clear supernatant was collected to determine unbound CBM concentrations with the absorbance at 280 nm. Each measurement was done in triplicate. Bound CBM concentrations were calculated with unbound CBM concentrations and total CBM concentrations.

III. Results

A. Production of *Cj*CBM3 and its mutants

*Cj*CBM3, its mutants and *Tr*CBM1-CFP were prepared in accordance with the procedure in "MATERIALS AND METHODS". Because *Tr*CBM1 was not expressed in *E. coli* JM109, it was fused with cyan fluorescence protein (CFP) and successfully was expressed. Purity of each prepared CBMs was confirmed by SDS-PAGE (Fig. 1). The amount of the prepared CBMs was enough to perform the following experiments. The CD spectrum showed that the structures of each *Cj*CBM3s were never changed by amino acid substitution (data not shown).

B. Affinity of *Cj*CBM3 to various polysaccharides

Bindings to various polysaccharides were detected by macroarray assays. *Cj*CBM3 bound to BMCC, BMC, ASC, xyloglucan, and chitin, but it failed to bind to CMC, birch wood xylan and oat-spelt xylan (Fig. 2). Binding properties of *Cj*CBM3 to xyloglucan and CMC were also reconfirmed in aqueous condition (Fig. 3). On the other hand, *Tr*CBM1-CFP bound to BMCC and BMC, but could not bind to other polysaccharides. CFP couldn't bind to all polysaccharides (data not shown).

The amino acids substitutions in *Cj*CBM3 lost affinities to xyloglucan as well as to BMC.

C. Adsorption isotherm analysis

Adsorption isotherm analyses between *Cj*CBM3 and ASC were performed in the following four conditions to evaluate effects of xyloglucan and ion intensity: 1. *Cj*CBM3 and ASC. 2. *Cj*CBM3, ASC and xyloglucan. 3. *Cj*CBM3 and ASC in 1M NaCl addition. 4. *Cj*CBM3, ASC and xyloglucan in 1M NaCl addition.

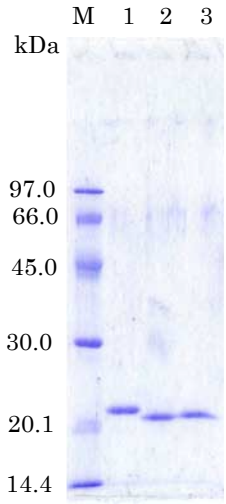

Fig. 1 SDS-PAGE of *Cj*CBM3 and its mutants
The gel was stained with Rapid Stain CBB Kit (Nakalai tesque). M, molecular weight marker (LMW Electrophoresis Calibration Kit; Amersham); lane 1, *Cj*CBM3; lane 2, W124A; lane 3, Y71A/W124A was presented, respectively.

When 0.1% xyloglucan was added to the system (condition 2.), the binding amount of *Cj*CBM3 to ASC decreased compared with them in condition 1 (Fig. 4). It indicates that *Cj*CBM3 was sequestered by soluble xyloglucan from insoluble ASC. However, in high salt concentration condition (condition 4), *Cj*CBM3 bound to ASC as similar to it in condition 3. Therefore, the high salt concentration condition interrupts the binding between *Cj*CBM3 and xyloglucan.

IV. Discussions

CBMs were classified to the three types by its binding mode[7]: type A has a flat surface to bind to crystalline cellu-

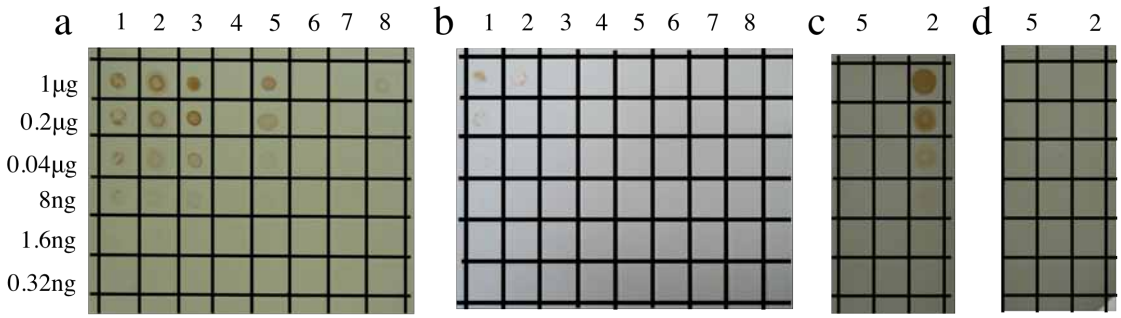

Fig. 2 CBM macroarray assay
CBM macroarray assay of the binding of (a)*Cj*CBM3, (b)*Tr*CBM1-CFP, (c)W124A or (d)Y71A/W124A to a series of BMCC (lane 1), BMC (lane 2), ASC (lane 3), CMC (lane 4), xyloglucan (lane 5), birchwood xylan (lane 6), oatspelt xylan (lane 7), and chitin (lane 8) was represented. Samples were applied to nitrocellulose in the dilution series indicated and probed with 1.25 μg/ml of the CBM.

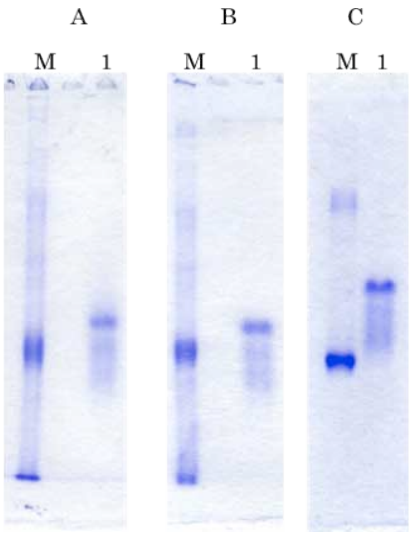

Fig. 3 Affinity gel electrophoresises of *Cj*CBM3 and its mutants
The affinity of *Cj*CBM3 for soluble cellulose was analyzed by affinity gel electrophoresis with gel containing CMC (B), xyloglucan (C) or not (A). M, BSA; lane 1, *Cj*CBM3 was applied, respectively.

lose, type B has a cleft or clefts and shows affinity for amorphous polysaccharides with the cleft(s), and type C has a groove to interact with reducing ends of cellulose chains. *Cj*CBM3 is classified as type A,[7] however, it could bind to ASC and xyloglucan that are not crystalline polysaccharides (Fig. 2). The 3D structure of family 3 CBM, from *Clostridium thermocellum*, whose sequence of amino acids is similar to that of *Cj*CBM3, demonstrates presence of a groove which may bind to amorphous single cellulose chain on the other side from the planar binding surface.[8] However, *Cj*CBM3 couldn't bind to CMC (Fig. 2 and 3) and cellooligosaccharides (data not shown). Thus, it is indicated that the groove may not function. Furthermore, decrease of binding amount of *Cj*CBM3 mutants which have the amino acids substitutions in flat surface ensure that *Cj*CBM3 binds to non-crystalline polysaccharides though its flat surface. While *Tr*CBM1-CFP, which is also classified as type A, could not bind to non-crystalline polysaccharides. These results indicate that, even if CBMs have a flat binding surface, some of them can bind to non-crystalline polysaccharides.[9] Model for the interaction of some type A CBMs with cellulose was interpreted that *Clostridium thermocellum* CBM3 possesses a broader binding surface, which includes more hydrophilic residues than *Tr*CBM1 does.[8] To demonstrate the mechanism of xyloglucan binding ability of *Cj*CBM3, adsorption isotherm analyses were carried out. These analyses verified *Cj*CBM3 binding ability to xyloglucan and implied importance of polar hydrophilic

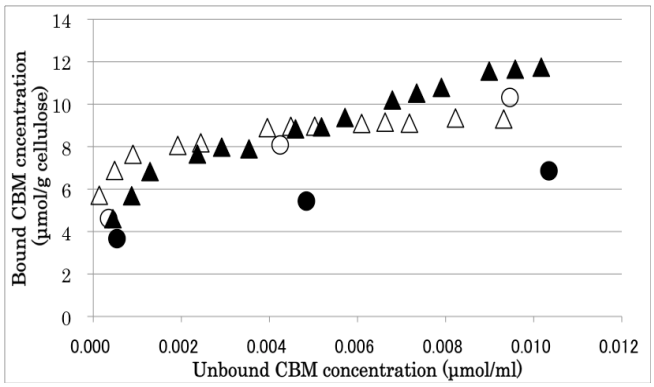

Fig. 4 The adsorption isotherms with *Cj*CBM3 to ASC
The adsorption isotherms with *Cj*CBM3 to ASC (▲), ASC in 1M NaCl addition (△), ASC and xyloglucan (●) and ASC and xyloglucan in 1M NaCl addition (○) were illustrated.

residues with this ability. Xyloglucans are composed of (1→6)-linked α-xylosyl residues along a 1,4-β-glucan backbone and minor additional side chains containing 1,2-β-galactosyl residues and/or α -fucosyl-(1→2)-β-galactosyl residues attached to the 2-position of the xylosyl residues.[10] Thus, polar residues in *Cj*CBM3 binding site may interact with these side chains because it could not bind to CMC with no oligosaccharides side chains.

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