Functions of Family-22 Carbohydrate-Binding Module in Clostridium thermocellum Xyn10C

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Clostridium thermocellum xylanase Xyn10C (formerly XynC) is a modular enzyme, comprising a family-22 carbohydrate-binding module (CBM), a family-10 catalytic module of the glycoside hydrolases, and a dockerin module responsible for cellulosome assembly consecutively from the N-terminus. To study the functions of the CBM, truncated derivatives of Xyn10C were constructed: a recombinant catalytic module polypeptide (rCM), a family-22 CBM polypeptide (rCBM), and a polypeptide composed of the family-22 CBM and CM (rCBM–CM). The recombinant proteins were characterized by enzyme and binding assays. Although the catalytic activity of rCBM–CM toward insoluble xylan was four times higher than that of rCM toward the same substrate, removal of the CBM did not severely affect catalytic activity toward soluble xylan or β-1,3-1,4-glucan. rCBM showed an affinity for amorphous celluloses and insoluble and soluble xylan in qualitative binding assays. The optimum temperature of rCBM–CM was 80°C and that of rCM was 60°C. These results indicate that the family-22 CBM of C. thermocellum Xyn10C not only was responsible for the binding of the enzyme to the substrates, but also contributes to the stability of the CM in the presence of the substrate at high temperatures.

Key words: xylanase; carbohydrate-binding module; thermostabilizing module; Clostridium thermocellum

Many glycoside hydrolases including xylanase consist of two or more functional modules, such as a catalytic module (CM) and a carbohydrate-binding module (CBM). Based on amino acid sequence similarities, CMs of glycoside hydrolases can be classified into 97 families and CBMs into 39 families (http://afmb.cnrs-mrs.fr/CAZY/index.html).1,2) CMs of xylanases can be divided into two substantial groups: families 10 and 11 of the glycoside hydrolases.

Family-22 CBMs are often found with a family-10 CM in a modular enzyme, e.g., Caldibacillus cellulosivorans XynA,3) Clostridium josui Xyn10A,4) Clostridium stercorarium Xyn10B,5) Clostridium thermocellum Xyn10B,5) Polyplastron multivesiculatum Xyn10B,7) or Thermotoga maritima XynA.8) 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.6,8,9) Later, however, these modules were compiled and classified in family 22 of CBMs since they were found to have affinities for different types of substrates, e.g., soluble xylan,10–13) barley β-glucan11–13) or insoluble cellulose.7) Recently, we have found that a truncated derivative of C. stercorarium Xyn10B, composed of two family-22 CBMs and a CM, preferred β-1,3,1,4-glucan such as barley β-glucan and lichenan to xylan as a substrate and removal of the CBMs from this enzymes drastically reduced catalytic activity toward β-1,3,1,4-glucan but not toward xylan, suggesting that the family-22 CBMs in C. stercorarium Xyn10B are essential for hydrolysis of β-1,3,1,4-glucan.13) These observations suggest that functions of the family-22 CBMs are not necessarily common in different enzymes.

C. thermocellum xylanase Xyn10C (formerly XynC)4) has been found in the cellulosome (a highly organized enzyme complex containing cellulases and hemicellulases) of C. thermocellum as one of major catalytic components, although this bacterium cannot grow on xylan. Although Xyn10C is known to be a modular enzyme, comprising a family-22 CM, a family-10 CM of the glycoside hydrolases, and a dockerin module responsible for cellulosome assembly consecutively from the N-terminus (Fig. 1), the function of the family-22 CM has not yet been characterized. In this study, we constructed three truncated derivatives of

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Abbreviations: ASC, acid-swollen cellulose; BMC, ball-milled cellulose; CBM, carbohydrate-binding module; CMC, carboxymethyl-cellulose; CD, catalytic module; FPLC, fast protein liquid chromatography; HMC, hydroxymethyl-cellulose; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Tris, Tris(hydroxymethyl)aminomethane
Xyn10C, a recombinant CM polypeptide (rCM), a CBM polypeptide composed of the family-22 CBM (rCBM), and a polypeptide composed of the family-22 CBM and the catalytic module (rCBM–CM) and evaluated the function of the family-22 CBM by measuring the thermal stability, binding affinity for various polysaccharides, and substrate specificity of these polypeptides.

Materials and Methods

Strains and growth conditions. The Escherichia coli strains used in this study were XL1-Blue (Stratagene) and M15 (Qiagen). Recombinant E. coli strains were cultured at 37 °C in Luria broth supplemented with ampicillin (50 μg/ml) or both ampicillin (50 μg/ml) and kanamycin (50 μg/ml).

Plasmids and plasmid constructions. The plasmid pKS103 carrying the C. thermocellum xyn10C gene was described previously. The plasmid vectors used in this study were pT7-Blue (Novagen) and pQE-30T. Plasmids used to produce the truncated derivatives of Xyn10C were digested with BamHI and SalI sites of pQE-30T and BamHI sites of pKS103 constructs as follows: DNA fragments encoding the family-22 CBM region and/or the family-10 CM (CM), and a dockerin module. The artificial BamHI and SalI sites in PCR primers are underlined.

Purification of rCBM, rCM and rCBM–CM. To produce the recombinant proteins, 1.5-liter cultures of E. coli recombinants were grown to mid-log phase (absorbance at 600 nm, 0.6) and then isopropyl-β-D-thiogalactopyranoside was added to the cultures to give a final concentration of 1 mM. After an additional incubation of 3 h, the cells were harvested, washed, and disrupted by sonication. Cell debris was removed by centrifugation, and the cell extracts thus obtained were used for purification of the recombinant proteins. These proteins were purified by HiTrap Chelating HP (Amersham) column chromatography. The 6xHis tag was removed from the recombinant proteins by thrombin digestion and the remaining digestion and proteins that did not adsorb to the column due to the removal of the affinity tag were collected and further purified with a Resource Q column (Amersham) by using FPLC. The purity of each fraction was analyzyed by SDS–polyacrylamide gel electrophoresis (PAGE). Protein concentration was determined with bovine serum albumin as the standard, using the Micro BCA protein assay reagent kit (Pierce).

Enzyme assays. Xylanase activity was measured by a 10-min incubation at 60 °C in Britton and Robinson’s universal buffer (50 mM phosphoric acid, 50 mM boric acid, and 50 mM acetic acid (the pH was adjusted to 2 to 12 with 1 M NaOH)). Reducing sugars released from birchwood xylan (Sigma Chemical) or oat-spelt xylan (Fluka Ag, Buchs, Switzerland) were determined with the 3,6-dinitrophenol acid reagent, as described by Miller. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of xylose equivalent per min from xylan. Enzyme activities towards barley β-glucan (Sigma), lichenan (Sigma), hydroxyethyl-cellulose (HMC; Fluka), carboxymethyl-cellulose (CMC; Sigma), were assayed as described above, except that xylan and xylose were replaced by each substrate and product.

The optimum temperatures of rCM and rCBM–CM were determined by assaying their enzyme activities with oat-spelt xylan as the substrate at various temperatures at pH 7.0 for 10 min. Optimum pH was measured by assaying their enzyme activities at 60 °C in Britton and Robinson’s universal buffer solutions (pH 6–8.5). Thermostability and pH stability of the purified enzyme were measured by incubating the enzyme solution at agarose gel using GFX PCR DNA and Gel band purification kit (Amersham). These sites were ligated between the BamHI and SalI sites of pQE-30T and introduced into E. coli M15. The combinations of the primers were as follows: primers Xyn10C-N and Xyn10C-CBM to construct pCBM, yielding rCBM; primers Xyn10C-CM and Xyn10C-C to construct pCM, yielding rCM; primers Xyn10C-N and Xyn10C-C to construct pCBM–CM, yielding rCBM–CM. Schematic diagrams of these proteins and nucleotide sequences of the PCR primers are shown in Fig. 1.

Fig. 1. Schematic Diagram of C. thermocellum Xyn10C and Its Derivatives (A), and PCR Primers Used for the Construction of the Truncated Derivatives (B).

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<td>Xyn10C</td>
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<td>G6CCGCGATCCCGAAGCTTTGATG6-3’</td>
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<td>Xyn10C-CBM</td>
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Results and Discussion

Qualitative carbohydrate-binding assays. Binding of rCBM to insoluble polysaccharides was determined as follows: rCBM (40 μg) was mixed with insoluble polysaccharides (10 mg) in a 10 mM sodium phosphate buffer (pH 6.3) in a final volume of 0.2 ml and incubated on ice for 1 h with occasional stirring. After centrifugation, the supernatant and the precipitated polysaccharides were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). The polysaccharides tested were Avicel (FMC), acid-swollen cellulose (ASC), ball-milled cellulose (BMC), the insoluble fraction of oatspelt xylan (Fluka), lichenan, and agar (Nacalai Tesque). ASC and BMC were prepared in this laboratory from KC Floc cellulose powder (Nippon Paper Chemicals), as described previously.17)

The affinities of these proteins for soluble polysaccharides, viz. CMC, HMC, barley β-glucan (Sigma), and birchwood xylan (Sigma), were examined by native affinity PAGE as described by Meissner et al.,12) with some simplifications. We used the Laemmli system for electrophoresis, excluding SDS from all solutions. The separating gel contained 10% acrylamide. A control gel without any polysaccharides was prepared and run simultaneously. Protein samples were loaded onto gels in a standard loading buffer without SDS. Electrophoresis was run at 4°C and 100 V for 2 h. Proteins were visualized by Coomassie blue staining.

Results and Discussion

Construction and production of the Xyn10C derivatives

Truncated forms of Xyn10C were constructed as described in “Materials and Methods”. rCBM is the family-22 CBM polypeptide, rCM is the family-10 CM polypeptide, and rCBM–CM is the polypeptide composed of the CBM and the CM collectively (Fig. 1). These proteins were purified from the whole-cell lysates of E. coli recombinants using HiTrap Chelating HP column and Resource Q column at FPLC. Each of the purified preparations gave a single band on SDS–PAGE, and the molecular sizes of the purified rCBM–CM, rCM, and rCBM were in good agreement with those deduced from the nucleotide sequences (MW: 58,111 for rCBM–CM; 40,140 for rCM; and 18,134 for rCBM) (Fig. 2). These purified proteins were used for enzyme and polysaccharide-binding assays.

Effects of temperature and pH on the activity and stability of rCM and rCBM–CM

Family-22 CBMs were originally identified as thermostabilizing modules, i.e., shifts (10–20°C) in optimum temperature upon artificial removal of family-22 CBMs from thermophilic modular xylanases were observed in C. cellulovorans XynA (from 90 to 70°C),3) C. stercorarium Xyn10B (75 to 60°C),13) C. thermocellum XynX (70 to 60°C) and XynY (75 to 60°C),6) and T. saccharolyticum XynA (75 to 65°C) in the presence of the substrate. Furthermore, an increase in thermostability in the absence of the substrate was observed in C. cellulovorans XynA, C. thermocellum XynX, and C. thermocellum XynX and XynY. Therefore, the effects of temperature and pH were determined on the activity and stability of rCM and rCBM–CM. When the xylanase activity of the truncated enzymes was determined by a 10-min incubation at various temperatures, the optimum temperature of rCBM–CM was determined to be approximately 80°C but that of rCM was approximately 60°C (Fig. 3A). This observation confirms the thermostabilizing effect of family-22 CBMs in the presence of the substrate. By contrast, no significant difference was observed between the thermal stability of rCBM–CM and that of rCM (Fig. 3B) when they were heated in the absence of the substrate for 10 min. It is possible that the interaction between the CM and the CBM increases in the presence of xylan, resulting in stabilization of the CM only in the presence of the substrate. As shown in Fig. 3C and D, although the presence of the family-22 CBM adjacent to the CM negligibly affected the optimum pH of the enzyme, rCBM–CM was slightly more stable in acidic pH range and less stable in the alkaline pH range than rCM. In mesophilic xylanase Xyn10B from P. multivesiculatum, removal of the CBM did not affect its optimum temperature (40°C) but shifted its optimum pH from 7.0 to 8.0.7)

Binding of rCBM to insoluble and soluble polysaccharides

To investigate the function of the family-22 CBM of Xyn10C in hydrolytic action, we qualitatively examined the ability of rCBM to bind insoluble polysaccharides by incubating the protein with the polysaccharides and various temperatures (50 to 90°C) for 10 min at pH 7.0 or incubating the enzyme solution in Britton and Robinson’s buffer (pH 4–11) for 1 h at 60°C, followed by measurement of residual enzyme activities under the standard conditions.
comparing the protein concentrations in the supernatant fraction (unbound protein) and in the precipitate fraction (bound protein) by SDS–PAGE. As shown in Fig. 4, rCBM showed relatively high affinity for BMC as compared with Avicel, suggesting that this CBM polypeptide prefers amorphous cellulose to crystalline cellulose. rCBM was found to associate with the insoluble fraction of oat-spelt xylan. On the other hand, rCBM showed only low affinity for lichenan and negligible affinity for agar.

To confirm the affinity of rCBM for soluble polysaccharides, this protein was subjected to native affinity PAGE analysis. As shown in Fig. 5, migration of the protein was strongly retarded in the gel containing birchwood xylan as compared to the control gel, indicating that it has a strong affinity for birchwood xylan. By contrast, this CBM showed only a slight affinity for barley β-glucan and CMC and no affinity for HMC.

Although family-22 CBMs were specified at first as thermostabilizing modules, some of these modules were found to have an affinity for soluble xylan and β-1,3-1,4-glucan by qualitative and quantitative analyses and were reassigned to family 22 of CBMs, e.g., the CBM of C. thermocellum XynY showed a high affinity for rye arabinoxylan \( (K_a, 1.1 \times 10^7) \) and a low affinity for barley β-glucan \( (K_a, 7.8 \times 10^2) \). One of two repeated CBMs of T. maritima XynA was shown by native affinity PAGE to have an affinity for various kinds of
xylans such as oat-spelt xylan and birchwood xylan, barley \(\beta\)-glucan, lichenan, hydroxyethyl-cellulose, and methyl-cellulose but not for CMC,\(^{12}\) the CBM of \textit{C. cellulovorans} selectively bound to soluble xylan and weakly to hydroxyethyl-cellulose;\(^{9}\) and the CBMs of \textit{C. stercorarium} were qualitatively shown to have a high affinity for barley \(\beta\)-glucan in addition to soluble xylan.\(^{13}\) Although many family-22 CBMs showed affinities only for soluble polysaccharides, it was found that the CBM of \textit{P. multivesiculatum} bound strongly to celluloses of various crystallinities.\(^{7}\) The binding specificity of \textit{C. thermocellum} Xyn10C is similar to that of \textit{P. multivesiculatum} Xyn10B in that they have affinities for xylan and insoluble cellulose (Fig. 4) although the former appears to prefer amorphous cellulose to crystalline cellulose.

In the CBM of \textit{C. thermocellum} XynY, Arg-583, Trp-611, Tyr-660, Tyr-136, and Glu-138 were identified as ligand-binding sites and/or critical residues for maintaining the structural integrity of the binding cleft,\(^{20}\) and these five residues were conserved in \textit{C. thermocellum} Xyn10C and the other related CBMs.

**Substrate specificity of rCM and rCBM–CM**

The substrate specificities of rCM and rCBM–CM were compared toward a series of different polysaccharides such as oat-spelt xylan and barley \(\beta\)-glucan. As shown in Fig. 6, the hydrolytic activity of rCBM–CM toward the insoluble fraction of oat-spelt xylan was about 4 times greater than that of rCM although that of the former toward birchwood xylan was only 1.4 times higher than that of the latter, indicating that the presence of the family-22 CBMs enhanced the catalytic activity of the CM toward the insoluble substrate but not the soluble substrate. Similar phenomena were reported for \textit{C. stercorarium} Xyn10B,\(^{21}\) i.e., removal of a family-9 CBM from the truncated enzyme composed of a family-10 CM and the CBMnegated its cellulose- and xylan-binding abilities and severely reduced its enzyme activity towards insoluble xylan but not soluble xylan.

Artificial connection of a family-6 CBM derived from \textit{C. stercorarium} XynA to \textit{Ruminococcus albus} endoglucanase EGVI improved the hydrolytic activity of the \textit{R. albus} enzyme toward insoluble cellulose but not soluble cellulose.\(^{23,24}\) It was also found that the CBMs of the \textit{Pseudomonas fluorescens} subsp. \textit{cellulosa} xylanase XynA and arabinofuranosidasexyIC potentiated catalytic activity toward complex substrates.\(^{23,24}\) In these cases, it is likely that these CBMs contribute to the enrichment of the enzymes on the surface of the insoluble substrates, resulting in increased contact of the enzymes with the substrates.

The CBM of Xyn10C showed an affinity not only for xylan but also for cellulose. The presence of the family-22 CBD in Xyn10C places the enzyme on cellulose in plant cell walls, resulting in an increase in substrate concentration around the enzyme, since plant cell walls consist of cellulose, hemicellulose including xylan, and lignin.

Recently we found that the family-22 CBMs in \textit{C. stercorarium} Xyn10B have an essential role in hydrolysis of \(\beta\)-1,3-1,4-glucan such as barley \(\beta\)-glucan and lichenan. A truncated derivative of Xyn10B comprising two family-22 CBMs and a family-10 CM showed stronger activity on \(\beta\)-1,3-1,4-glucan than on xylan although the family-10 of glycoside hydrolases is well known as a group of xylanases, and removal of the CBMs from this enzyme drastically reduced hydrolytic activity toward \(\beta\)-1,3-1,4-glucan but not toward xylan.\(^{15}\) This phenomenon was explained in the previous paper as follows: the family-22 CBMs bind more strongly to glucan than to xylan, and hence, 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. By contrast, as shown in Fig. 6, the hydrolytic activity of rCBM–CM toward barley \(\beta\)-glucan and lichenan was substantially lower than that toward oat-spelt xylan or birchwood xylan, and removal of the CM from rCBM–CM did not strongly affect activity toward \(\beta\)-1,3-1,4-glucan. Since rCBM showed a weaker binding affinity for \(\beta\)-1,3-1,4-glucan than for xylan (Figs. 4 and 5), it is possible that the CM cannot provide effectively the CM adjacent to the CM with \(\beta\)-1,3-1,4-glucan as the substrate. Alternatively, it is likely that \textit{C. thermocellum} Xyn10C is an ordinary xylanolytic enzyme with substrate specificity restricted to xylan but that \textit{C. stercorarium} Xyn10B is adapted for hydrolysis of \(\beta\)-1,3-1,4-glucan rather than xylan. This supposition is perhaps supported by the finding that the xylanase activity of rCBM–CM was about 10 times stronger than that of \textit{C. stercorarium} Xyn10B.\(^{15}\) The results obtained for \textit{C. thermocellum} Xyn10C and \textit{C. stercorarium} Xyn10B suggest that the function and role of family-22 CBMs must be different in different enzymes.

In this study, we found that family-22 CBM of \textit{C. thermocellum} Xyn10C bound to soluble and insoluble xylan as well as to insoluble cellulose and that the
presence of the CBM enhanced the hydrolytic activity of rCBM–CM toward xylan but not toward β-1,3-1,4-glucan.

References