Functional characterization and mutation analysis of family 11, Carbohydrate-Binding Module (*Ct*CBM11) of cellulosomal bifunctional cellulase from *Clostridium thermocellum*

S. Bharali · R. K. Purama · A. Majumder · C. M. G. A. Fontes · A. Goyal

Received: 14 November 2006 / Final revision: 25 March 2007 / Accepted: 18 April 2007

Abstract The non-catalytic, family 11 carbohydrate binding module (*Ct*CBM11) belonging to a bifunctional cellulosomal cellulase from *Clostridium thermocellum* was hyper-expressed in *E. coli* and functionally characterized. Affinity electrophoresis of *Ct*CBM11 on nondenaturing PAGE containing cellulosic polysaccharides showed binding with β -glucan, lichenan, hydroxyethyl cellulose and carboxymethyl cellulose. In order to elucidate the involvement of conserved aromatic residues Tyr 22, Trp 65 and Tyr 129 in the polysaccharide binding, site-directed mutagenesis was carried out and the residues were changed to alanine. The results of affinity electrophoresis and binding adsorption isotherms showed that of the three mutants Y22A, W65A and Y129A of *Ct*CBM11, two mutants Y22A and Y129A showed no or reduced binding affinity with polysaccharides. These results showed that tyrosine residue 22 and 129 are involved in the polysaccharide binding. These residues are present in the putative binding cleft and play a critical role in the recognition of all the ligands recognized by the protein.

Keywords Carbohydrate binding module · Cellulase · *Clostridium thermocellum* · Cellulose · Affinity-electrophoresis · Adsorption isotherm · Site-directed mutagenesis

Introduction

The biosphere is provided with an extensive resource of organic carbon by the active role of microbial glycoside hydrolases, which play an important role in the plant cell wall degradation process. However, the entire network of polysaccharides in the plant cell wall is inaccessible to enzyme attack. Most of the enzymes hydrolysing plant cell wall polysaccharides have modular structures comprising catalytic module(s) and non-catalytic carbohydrate binding module(s) (CBMs). Based on primary structure similarity, CBMs have been grouped into 43 different families¹. It is believed that CBMs have a tendency to be associated with catalytic domains of a particular glycoside hydrolase (GH) family and also remain located in a particular place in the polypeptide. In general, glycoside hydrolases (GH) that catalyse plant cell wall degradation contain non-catalytic carbohydrate binding modules (CBMs) which interact with polysaccharides². The CBMs enhance the activity of the catalytic module against insoluble polysaccharides by mediating an intimate and prolonged association of the enzyme with its target substrate^{3–4}. Therefore, CBMs play a major role in potentiating the capacity of cellulases and hemicellulases to degrade plant cell wall polysaccharides. Based on structural studies, it has been observed that the topology of the ligand binding site varies. On the basis of ligand binding capabilities, CBMs have been grouped into two categories. The CBMs interacting with the flat surfaces of crystalline polysaccharides usually contain a planar hydrophobic carbohydrate binding site and are referred to as Type A CBMs^{5–8}. While the CBMs that bind to single polysaccharide chains accommodate these ligands in extended clefts and are defined as Type B CBMs^{9–12}. Type B CBMs have been described that bind to a diversity

S. Bharali · R. K. Purama · A. Majumder · A. Goyal (✉)
Department of Biotechnology, Indian Institute of Technology
Guwahati,
North Guwahati - 781 039,
Assam, India

C. M. G. A. Fontes
CIISA-Faculdade de Medicina Veterinária,
Rua Prof. Cid dos Santos - 1300 477,
Lisbon, Portugal

e-mail: arungoyl@iitg.ernet.in
Tel: +91 / 361 / 258 2208

of ligands, with some modules displaying plasticity in their capacity to accommodate heterogeneity in the sugar backbone both in terms of the identity of the polysaccharides and the nature of the linkage. In general the ligand specificity of Type B CBMs reflects the substrate hydrolysed by the associated catalytic modules¹. It is however unclear in case of enzymes that contain multiple catalytic domains with distinct substrate specificities, as to how these different activities influence the evolution of the ligand recognition profile of the appended CBM. In order to investigate whether CBMs can display greater affinity for mixed linked glucans and to understand the evolutionary trend exerted on the ligand specificity of CBMs in enzymes that contain distinct catalytic modules, the study on family 11 CBM was carried out. *CtCBM11* is a carbohydrate binding module associated with a bifunctional cellulosomal cellulase (Lic26A-Cel5E-CBM11) enzyme from *Clostridium thermocellum*. It displays a modular architecture containing an N-terminal family 26 GH module (Lic26A), internal family 5 GH (Cel5E) and family 11 CBM (*CtCBM11*) modules and a C-terminal dockerin. Only three other members of CBM family 11 are known. The cellulosomal cellulase complex comprising GH5¹³ and Lic26A¹⁴ catalytic domains display β -1,4 and β -1,3-1,4-mixed-linked endoglucanase activity, respectively. *CtCBM11* does indeed display a preference for specific β -1,3-1,4-mixed linked glucans although the protein is able to bind to β -1,4 glucose polymers. Mutagenesis studies on the ligand binding cleft of *CtCBM11* revealed the residues involved in binding soluble and insoluble polysaccharides.

Materials and Methods

Bacterial strains, culture conditions and plasmids

The *Escherichia coli* strains used in this study were BL-21 (Novagen) for protein expression. The plasmid used in this work was recombinant of the expression vector

pET21a (Novagen) encoding for *CtCBM11* protein containing the His₆ tag at the C-terminal. The *E. coli* cells containing this plasmid were cultured in Luria Bertani (LB) liquid media at 37°C and 180 rpm unless otherwise stated.

Hyper-expression and purification of *CtCBM11*

The *E. coli* cells harbouring the *CtCBM11* gene containing plasmid pET21a¹⁵ were cultured in LB medium supplemented with 100 µg/mL ampicillin at 37°C and grown to mid-exponential phase (A_{550} 0.6) at which point isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM and the cultures were incubated for further 8h for protein induction¹⁵. The cells were collected by centrifugation and the cell pellet was resuspended in 50 mM sodium phosphate buffer pH 7.0. The cells were then sonicated and centrifuged at 4°C. The supernatant containing soluble protein was collected and passed through a 0.45 µm filter membrane¹⁵. The *CtCBM11* protein was purified to homogeneity by metal ion affinity-chromatography¹⁶. The purified proteins were dialysed against 20 mM sodium phosphate buffer pH 7.0 with at least three changes using the same buffer. The concentration of the purified protein was determined by the method of Bradford¹⁷ and the purity was analysed by SDS-PAGE.

Construction of mutants of *CtCBM11* by site-directed mutagenesis

The three mutants Y22A, Y129A and W65A of *CtCBM11* were generated using the PCR based Quick-change site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. The primers listed in Table 1 were designed in such a manner so as to incorporate only nucleotides coding for alanine in place of tyrosine and tryptophan in *CtCBM11*. The DNA sequences incorporated with the mutations were sequenced to ensure that only the appropriate mutations had been incorporated into the nucleotide.

Table 1 Oligonucleotides used for site-directed mutagenesis of *CtCBM11*.

Construct	Codon change	Oligonucleotide sequences (5'→3')	Location	Direction
W65A	TGG→GCG	CCGGACGGCGAT <u>GCG</u> TCAAAATGGCTT*	2304-2330	Forward
		AAGCCATTTTGAC <u>GCG</u> ATCGCCGTCCGG*		Reverse
Y22A	TAC→GCC	TGGGGTTCA <u>GCC</u> TCCGGTGAAGGTGCA*	2178-2204	Forward
		TGCACCTTCAACCGAG <u>GCT</u> GAACCCCA*		Reverse
Y129A	TAT→GCT	AGACTTGAT <u>GCT</u> CAGCCGCCTGGACAG*	2499-2525	Forward
		CTGTCCAGGCGGCTG <u>AGC</u> ATCAAGTCT*		Reverse

*Mutations are shown bold and underlined.

Affinity-electrophoresis of *CtCBM11* and its mutants with soluble polysaccharides

The ligand specificity of *CtCBM11* for a range of soluble polysaccharides was determined by affinity gel-electrophoresis (AGE). The method was essentially as described by Tomme *et al.* 2000¹⁸ using the polysaccharide ligands at varying concentrations in % (w/v). The native polyacrylamide gel electrophoresis was carried out using 10% (w/v) acrylamide gels containing varying concentrations of ligand. Bovine serum albumin was used as the non-specific binding negative control. Quantitative assessment of ligand binding characteristics of *CtCBM11* and its mutants using affinity electrophoresis was carried out as described previously¹⁹.

Binding adsorption isotherms of *CtCBM11* and mutants with insoluble polysaccharides

The quantitative and qualitative assessments of *CtCBM11* binding to BMCC, Avicel and acid-swollen were carried out. The protein (30 µg) in 50 mM Tris-HCl buffer, pH 7.5, containing 0.05 % (v/v) Tween 20 and 5 mM CaCl₂ (Buffer A) was mixed with 1 mg of ligand in a final reaction volume of 200 µl. The reaction mixture was incubated for 2 h at 4°C with gentle shaking, after that the insoluble ligand was precipitated by centrifugation at 13000xg for 5 min. The supernatant, containing the unbound protein, was removed and the pellet was washed three times with 200 µl of Buffer A. The bound protein from the washed pellet was eluted by boiling the polysaccharides in 200 µl of 10% (w/v) SDS containing 10 % (v/v) β-mercaptoethanol for 10 min. The pellet for bound protein and the supernatant for unbound protein were analysed by SDS-PAGE using a 13% (w/v) acrylamide gel. The control was the gel containing protein and no polysaccharide and the electrophoresis was performed in parallel to ensure also that no precipitation occurred. For quantitative analysis the free or unbound protein concentration in unbound fraction obtained after centrifugation was determined by Bradford method¹⁷ and the bound protein was estimated by subtracting the free protein from the initial protein concentration. Adsorption isotherm was generated and K_r and K_a were calculated using an equation as described elsewhere^{20–21}.

Results and Discussion

Qualitative binding analysis of *CtCBM11* with soluble ligands

The ligand binding characteristics of *CtCBM11* were determined with various soluble ligands using affinity

gel electrophoresis. For identification of soluble polysaccharides binding to *CtCBM11*, qualitative affinity electrophoresis was carried out using nondenaturing-PAGE on 10% (w/v) acrylamide gel containing 0.1% ligand. The *CtCBM11* showed retardation or no movement into the gel (implying binding the polysaccharide in the gel) on non-denaturing native polyacrylamide gels containing lichenan, β-glucan, hydroxyethyl cellulose (HEC), glucomannan, xylan or carboxymethyl cellulose, CMC (Table 2). Incidentally, in bifunctional cellulase, the associated catalytic module Cel5E (GH5¹³) showed high activity with lichenan, β-glucan, HEC and CMC and the other associated catalytic module Lic26A showed high activity with lichenan and β-glucan²². This corroborates the contention that the CBMs reflect the substrates specificity of the associated glycoside hydrolases.

Quantitative binding analysis of *CtCBM11* and its mutants with soluble ligands

It is established that very subtle amino acid substitutions can dramatically alter the ligand specificity of CBMs, and therefore the accumulation of those mutations could have contributed for the evolution of the ligand specificities required by the various enzymes²². It is understood that in the binding cleft some of the conserved aromatic amino acid residues of CBMs take part in substrate binding. In order to determine the importance of these conserved aromatic amino acids in binding to the substrate, site directed mutagenesis was carried out to alter three of the conserved aromatic residues of *CtCBM11* to Alanine to produce mutants W65A, Y22A and Y129A. The mutants were expressed

Table 2 Qualitative binding analysis of *CtCBM11* to soluble polysaccharides (0.1%, w/v) by affinity gel electrophoresis

Ligand	<i>CtCBM11</i>
Arabinan	–
Carboxymethyl cellulose	+
Galactomannan	–
β-Glucan	+
Glucomannan	+
Glucoronoxylan	–
Hydroxyethyl cellulose	+
Laminarin	–
Lichenan	+
Rhamnogalacturan	–
Rye arabinoxylan	–
Xylan, oat splot	+

+ binding, – No binding.

Fig. 1 Quantitative binding analysis of *CbCBM11* and its mutants with lichenan by affinity-gel electrophoresis. (A) Non-denaturing-PAGE displaying lanes: (1) Bovine serum albumin, (2) wild-type *CbCBM11*, (3) W65A, (4) Y22A and (5) Y129A contained varying concentration of lichenan in gels: (A) 0%; (B) 0.001%; (C) 0.002%; (D) 0.005%; (E) 0.02%; (F) 0.05%. (B) a plot of normalized relative mobility (R) of *CbCBM11* and mutants versus lichenan concentration. (C) shows a plot of $1/r$ versus lichenan concentration, where r is the relative migration distance of proteins in presence of affinity ligand in the gel.

in *E. coli* BL-21 cells. The mutants were purified following the same protocol as that of wild-type *CtCBM11*. The three mutants were analysed for affinity towards soluble polysaccharides. The three mutants along with the wild-type *CtCBM11* as control were run on native-PAGE containing varying ligand concentration in the gel. The quantitative analysis of binding affinity of *CtCBM11* and mutants for β -glucan, lichenan (Fig. 1A), HEC (Fig. 2), glucomannan (Fig. 3), xylan (Fig. 4) and CMC (Fig. 5) was carried out using affinity gel electrophoresis. *CtCBM11* gets retarded in the gels containing increasing concentrations of lichenan (Fig. 1a), HEC (Fig. 2), glucomannan (Fig. 3), xylan (Fig. 4) and CMC (Fig. 5). Figure 1B shows normalized relative

mobility (R) of *CtCBM11* and mutants versus lichenan concentration. Figure 1A and 1B clearly show that the mutants Y22A is least retarded in the gels containing polysaccharides, followed by Y129A and W65A. Similar order of retardation of the proteins was observed with lichenan (Fig. 1A), HEC (Fig. 2), glucomannan (Fig. 3) or xylan (Fig. 4). The affinities of *CBM11* and its mutants for ligands were quantified and the association constants (K_a) were determined by using an equation described elsewhere¹⁹. Fig. 1C shows a sample plot of $1/r$ versus lichenan concentration, where r is the relative migration distance of proteins in presence of affinity ligand in the gel. The values of K_a were calculated by extrapolating the lines

Fig. 2 Quantitative binding analysis of *CtCBM11* with hydroxyethyl cellulose by affinity-gel electrophoresis. Lanes: (1) Bovine serum albumin, (2) *CtCBM11*, (3) W65A, (4) Y22A, (5) Y129A in gels containing varying concentrations of HEC: (A) 0.0 %; (B) 0.02 %, (C) 0.04%; (D) 0.1%; (E) 0.2% and (F) 0.4%.

Fig. 3 Quantitative binding analysis of *CtCBM11* and its mutants with glucomannan by affinity gel electrophoresis. Lanes: (1) Bovine serum albumin, (2) wild type *CtCBM11*, (3) Y22A, (4) W65A and (5) Y129A in gels containing different concentrations of glucomannan: (A) 0%; (B) 0.01%; (C) 0.02% and (D) 0.04%.

Fig. 4 Quantitative binding analysis of *CtCBM11* with xylan by affinity-gel electrophoresis. Lanes: (1) Bovine serum albumin, (2) *CtCBM11*, (3) W65A, (4) Y22A and (5) Y129A in gels containing different concentrations of xylan: (A) 0.0 %; (B) 0.1 %; (C) 0.2%; (D) and 0.4%.

and taking inverse of value of intercept on x-axis of the binds lichenan and other soluble polysaccharides with sig-

Fig. 5 Quantitative binding analysis of *CtCBM11* with carboxymethyl cellulose by affinity-gel electrophoresis. Lanes: (1) Bovine serum albumin, (2) *CtCBM11* in gels containing varying concentrations of CMC: (A) 0.0 %; (B) 0.1 %; (C) 0.2%; (D) 0.3% and (E) 0.4%.

plot as described by Takeo¹⁹. Table 3 lists the K_a values of *CtCBM11* and mutants with soluble substrates used. The K_a of β -glucan and lichenan are much higher than those of other ligands (Table 3). Moderate binding of *CtCBM11* was observed in gels containing HEC, glucomannan and xylan whereas CMC showed very weak binding (Table 3). The results showed that the mutant W65A still binds the soluble polysaccharides almost as the wild-type *CtCBM11*, but with around 25–50% reduced binding affinity as compared to the wild-type *CtCBM11* (Table 3). The mutant Y129A

nificantly reduced affinity as the values of K_a were lowered by 80–90% as compared to that of wild-type *CtCBM11* for corresponding soluble polysaccharides (Table 3). In the case of mutant Y22A the affinities for β -1,3 and β -1,4 polysaccharides were completely abolished and K_a values were less than 2% of K_a values of wild-type *CtCBM11* for the corresponding polysaccharides (Table 3). Moreover, Y22A did not bind at all to glucomannan or HEC and binding to xylan or CMC was insignificant (Table 3). From the results, it can be summarized that the ligand binding affinities (K_a)

Table 3 Binding of wild type *CtCBM11* and its mutants to soluble polysaccharides quantified by affinity gel electrophoresis.

Polysaccharide	K_a (% w/v)			
	Wild-type	W65A	Y129A	Y22A
β -Glucan	1195	587	56.2	15.5
Lichenan	701.6	400	68.1	9.7
Glucomannan	25.9	19.5	1.8	NB
Hydroxyethyl cellulose	24.4	14.6	0.47	NB
Xylan, oat splot	17.5	13.9	1.6	0.47
Carboxymethyl cellulose	7.6	5.0	1.2	0.7

NB; not bound.

of Y22A and Y129A mutants are much lowered as compared to those of wild-type *CtCBM11* or the mutant W65A. Therefore, these results show that the tyrosine residues 22 and 129 could effectively be involved in polysaccharides binding. These two tyrosine residues 22 and 129 present in the putative binding cleft have a definite role in the recognition of all the ligands recognized by the protein. However, unlike the tyrosine residue, the tryptophan residue W65 may not necessarily be involved in ligand binding, but may be involved in facilitating the association of the carbohydrate binding module with the ligand.

Binding of *CtCBM11* and its mutants to insoluble polysaccharides

Affinities of proteins for insoluble polysaccharides were determined by binding adsorption isotherm analysis. *CtCBM11* displayed affinity for insoluble cellulose derivatives such as BMCC, Avicel and acid-swollen cellulose. Fig. 6A and 6B shows quantitative binding of *CtCBM11* to bacterial microcrystalline cellulose (BMCC) and avicel. The data were analysed by GraphPad (Prism 2.01) software using non-linear regression analysis and one binding-site equation. The measured equilibrium association constants (K_a) and binding capacities (N_o) of *CtCBM11* to BMCC, Avicel and acid-swollen cellulose (ASC) are listed in Table 4. The binding capacity (N_o) of *CtCBM11* ranged from 0.31 for ASC to 3.1 $\mu\text{mol/g}$ for Avicel. The binding capacity for Avicel and BMCC was around 10 times higher than that of

ASC. This shows that *CtCBM11* displays stronger binding capacity with crystalline cellulose as compared to the amorphous cellulose substrates. The binding affinity, i.e. the values of equilibrium association constant (K_a) of *CtCBM11* with BMCC, avicel and ASC were of the similar order with a mean value of 1.23×10^5 for avicel.

The qualitative binding analysis of *CtCBM11* and mutants with BMCC was also performed using SDS-PAGE as described in the Methods section. The SDS-PAGE analysis showed that wild-type *CtCBM11* and W65A retain significant content of bound protein (Fig. 7, lanes 3 & 5), whereas in the case of mutant Y22A (Fig. 7, lane 7) it is not able to retain or bind BMCC and the affinity for binding BMCC is completely lost and that of mutant Y129A (Fig. 7, Lane 9) the binding affinity is significantly abolished. The mutants Y22A and Y129A showed almost no binding with BMCC and this confirmed that tyrosine residues 22 and 129 have a critical role to play in binding of the *CtCBM11* to insoluble polysaccharides.

Conclusion

CtCBM11 is a carbohydrate binding module that binds strongly the soluble β -1,3- β -1,4 polysaccharides such as β -glucan and lichenan and moderately with glucomannan, xylan and CMC. It has already been reported earlier that interactions with aromatic rings in both type A and type B, CBMs play a critical role in polysaccharide binding. It is evident now that both the tyrosine residues 22 and 129 of *CtCBM11* are involved in polysaccharide binding. The catalytic modules Lic26A and Cel5E in tandem do not co-operate during soluble substrate hydrolysis²². However, the catalytic module Cel5E and *CtCBM11* both co-operate during insoluble polysaccharide hydrolysis. *CtCBM11* shows high affinity towards β -glucan and lichenan and both being the substrates of the associated catalytic modules *CtLic26A*^{13,22} and *Cel5E*¹⁴. This shows that the CBMs reflect the substrate specificity of associated

Table 4 Affinity of *CtCBM11* for insoluble polysaccharides by adsorption isotherm analysis.

Polysaccharide	K_r (l/g)	N_o ($\mu\text{mol/g}$)	K_a (M^{-1})
Bacterial micro-crystalline cellulose (BMCC)	0.227	2.7	0.63×10^5
Avicel	3.7	3.07	1.23×10^5
Acid-swollen cellulose (ASC)	0.055	0.31	1.8×10^5

Fig. 6 Quantitative binding analysis of CtCBM11 to insoluble polysaccharides. Adsorption isotherm for CtCBM11 binding to (A) Bacterial micro-crystalline cellulose (BMCC) and (B) Avicel was performed by incubating the protein with known concentration (% w/v) of polysaccharides and incubated for 2h at 4°C. The free or unbound protein concentration was determined after centrifugation and the bound protein was estimated by subtracting the free protein from the initial protein concentration.

catalytic modules. Quantitative and qualitative binding adsorption isotherm analysis of CtCBM11 displayed binding affinity and binding capacity with insoluble polysaccharides such as BMCC, avicel and ASC. The qualitative binding adsorption analysis of CtCBM11 and mutants with BMCC showed that the wild type protein and W65A can still retain the insoluble polysaccharide whereas the mutants Y22A and Y129A can not, again corroborating a potential role or involvement of tyrosine residues Y22 and Y129 in polysaccharide binding.

Acknowledgement The research project in part and SB were supported by a grant from Council of Scientific and Industrial Research (CSIR), India to AG.

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Fig. 7 Qualitative analysis of binding of *C/*CBM11 and its mutants to Bacterial micro-crystalline cellulose (BMCC) assessed by SDS-PAGE. Lanes: (1) Mol wt. Marker, (2) Free *C/*CBM11, (3) Bound *C/*CBM11, (4) Free W65A, (5) Bound W65A, (6) Free Y22A, (7) Bound Y22A, (8) Free Y129A and (9) Bound Y129A. The protein was mixed with BMCC and incubated for 2h at 4°C. The free or unbound protein was removed after centrifugation (Lanes 2,4,6,8), and the pellet containing the bound protein was washed three times in 50 mM Tris-HCl buffer, pH 7.0, containing 0.05% Tween 20 and 5 mM CaCl₂. The bound protein was eluted by boiling the pellet in 10% (w/v) SDS (Lanes 3,5,7,9).

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