Carbohydrate-binding properties of a separately folding protein module from β -1,3-glucanase Lic16A of *Clostridium thermocellum*

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The multi-modular non-cellulosomal endo-1,3(4)- β -glucanase Lic16A from *Clostridium thermocellum* contains a so-called X module (denoted as CBMX) near the N terminus of the catalytic module (191–426 aa). Melting of X-module-containing recombinant proteins revealed an independent folding of the module. CBMX was isolated and studied as a separate fragment. It was shown to bind to various insoluble polysaccharides, including xylan, pustulan, chitin, chitosan, yeast cell wall glucan, Avicel and bacterial crystalline cellulose. CBMX thus contains a hitherto unknown carbohydrate-binding module (CBM54). It did not bind soluble polysaccharides on which Lic16A is highly active. Ca^{2+} ions had effects on the binding, e.g. stimulated complex formation with chitosan, which was observed only in the presence of Ca^{2+} . The highest affinity to CBMX was shown for xylan (binding constant $K=3.1 \times 10^4 \text{ M}^{-1}$), yeast cell wall glucan ($K=1.4 \times 10^5 \text{ M}^{-1}$) and chitin ($K=3.3 \times 10^5 \text{ M}^{-1}$ in the presence of Ca^{2+}). Lic16A deletion derivatives lacking CBMX had lower affinity to lichenan and laminarin and a slight decrease in optimum temperature and thermostability. However, the specific activity was not significantly affected.

Received 22 December 2008

Revised 24 March 2009

Accepted 3 April 2009

INTRODUCTION

Multimodular enzymes are frequently found in micro-organisms that hydrolyse complex mixed carbohydrates (Coutinho & Henrissat, 1999; Lynd *et al.*, 2002). Microbial glycosyl hydrolases are often members of this class of proteins (Bayer *et al.*, 2000; Schwarz *et al.*, 2004). The modules of such enzymes are linked by flexible spacers and represent independently folded structures (Boraston *et al.*, 1999; Carrard *et al.*, 2000). It is possible to separate such a module from a protein without affecting its properties. Carbohydrate-binding modules (CBMs) are the most numerous among non-catalytic modules. According to Boraston *et al.* (1999), the main functions of CBMs in a multimodular enzyme are as follows. (i) Substrate proximity – the CBM binds to a polysaccharide, thus putting the catalytic module in close proximity to its substrate. (ii) Carbohydrate targeting – a CBM having an affinity to the same polysaccharide as the catalytic module can increase its efficiency; this function is more important

for micro-organisms that use complex carbohydrates. (iii) Disruptive function – some CBMs dissolve the crystal structure of a microfibril to make it more accessible to the catalytic module. (iv) Enzyme stabilization – the presence of auxiliary modules stabilizes the enzyme structure and enhances its temperature resistance. (v) Activity modulation – the CBM affects the type and parameters of the catalytic module activity. In addition some CBMs are responsible for anchoring carbohydrate hydrolases to the surface of the bacterial cell (Ezer *et al.*, 2008). According to the classification presented in the CAZy database (http://www.cazy.org/fam/acc_CBM.html), the presently known CBMs are classified into 53 families (Cantarel *et al.*, 2009).

Clostridium thermocellum is a moderately thermophilic anaerobic bacterium commonly found in soil, self-heated rotting biomass and hot springs (Johnson *et al.*, 1982; Chuvil'skaya *et al.*, 1986; Belaich *et al.*, 1997). This bacterium produces multienzyme complexes (cellulosomes) specialized in cellulose degradation (Bayer & Lamed, 1986). Most of the glycosyl hydrolases of *C. thermocellum* are parts of the cellulosome (Zverlov *et al.*, 2005). However, there are exoenzymes that do not belong to this complex (Schwarz *et al.*, 2004; Berger *et al.*, 2007). One of these non-cellulosomal enzymes is laminarinase

Abbreviation: CBM, carbohydrate-binding module.

A supplementary table listing homologues of the CBMX module of Lic16A of *C. thermocellum* is available with the online version of this paper.

Lic16A (GenBank accession no. CAC27412), with a molecular mass of 147 kDa. The structure and some properties of Lic16A have been investigated (Fuchs *et al.*, 2003). Biochemically, Lic16A is characterized as a 1,3- β -D-glucan glucanohydrolase (EC 3.2.1.73) or endo-1,3(4)-glucanase (EC 3.2.1.6). It has a complex structure (see Fig. 1a) and shows high activity on polysaccharides with mixed 1,3–1,4- β -linkages. It is composed of a leader peptide, a threefold repeat of a module homologous to proteins of the cell surface layer (SLH), the CBMX module, a GH16 catalytic module and a fourfold repeat of a CBM belonging to the CBM4A subfamily (Zverlov *et al.*, 2001).

Here the properties of the separated CBMX and of Lic16A deletion derivatives were investigated, as well as the influence of CBMX on the enzymic features of the catalytic module. We have demonstrated that CBMX is a separately folding protein unit which contains a hitherto unknown carbohydrate-binding module.

METHODS

Recombinant DNA techniques and protein purification. DNA preparation, cloning, restriction and PCR were carried out by standard procedures (Sambrook *et al.*, 1989) and in accordance with recommendations of the manufacturers. Chromosomal DNA of the *C. thermocellum* F7 strain (Chuvil'skaya *et al.*, 1986) was used as PCR template. The oligonucleotide primers used for PCR amplifications are specified in Table 1. The resulting DNA fragments were inserted into the pQE32 expression vector (Qiagen), and their sequences were verified by sequencing double-stranded plasmid DNA on both strands by using ABI BigDye Terminator v. 3.1 reagents and an ABI Prism 3100-Avant genetic analyser (Applied Biosystems). For cloning and expression, *Escherichia coli* XL-1 Blue was used. *E. coli* cells were grown at 37 °C in Luria–Bertani broth (LB) medium supplemented with necessary antibiotics. *E. coli* XL1 Blue cells carrying recombinant

Table 1. Oligonucleotide primers used in this study

Construct	Sequence (5'–3')
LicA-full	for: ctaatcctggatccaccatgaatccgaatgaag rev: caaaaccgaagctttagatagatctgccgtcc
LicA-xcat	for: ccaccaggatccgccgaaacgctctgattaacacc rev: ccaccaggatccgccgaaacgctctgattaacacc
LicA-catchbd	for: gggtctagagtggcgtctggttggagcg rev: gggtctagagttgccgaaagaagcggagg
LicA-cat	for: gggtctagagtggcgtctggttggagcg rev: ggggacgcctcctaagaggtctctt
CBMX	for: caagtcggatccacaataaaattctgaat rev: aatgaagagtggcgtaaaagcttgataa

plasmids (pQE32::LicA-full, pQE32::LicA-xcat, pQE32::LicA-cat, pQE32::LicA-catchbd) (Fig. 1b) and a helper plasmid pACYC-RIL, containing IleX and ArgU tRNA genes, which code for rare tRNAs in *E. coli* (Stratagene), were grown in LB medium supplemented with ampicillin (100 mg l⁻¹) and chloramphenicol (50 mg l⁻¹), collected by centrifugation, washed twice in 20 mM Tris/HCl, pH 8.0, and sonicated on ice to obtain crude cell extract. Proteins were purified on Ni-NTA-agarose as recommended by the manufacturer. The purity and molecular mass of protein fractions were verified by SDS-PAGE (Laemmli, 1970).

Enzyme assays. Enzyme solution aliquots in standard assays were incubated in a phosphate/citrate buffer (50 mM) at various pH values and temperatures with polysaccharides in concentrations of 0.05–0.5 % (w/v). The reducing sugars released from polymeric substrates were detected by the 3,5-dinitrosalicylic acid method (Wood & Bhat, 1988). One unit of enzyme (U) liberates 1 μ mol glucose equivalent per minute under standard conditions. All tests were performed in triplicate, and the coefficients of variation (ratio of the standard deviation to the mean) were less than 10 %. Specific activities and Michaelis constants were calculated using the Microcal Origin 7.5

Fig. 1. (a) Multimeric structure of Lic16A from *C. thermocellum*. (b) Deletion derivatives of Lic16A. (c) Multimeric structure of LamA from *Paenibacillus* sp.

software, from the reaction curves at the optimum temperature and pH values.

Binding to soluble carbohydrates. This was analysed using the affinity electrophoresis technique (Tomme *et al.*, 1996). Native polyacrylamide gels (10%), with or without soluble substrates ($1 \text{ mg} \cdot \text{ml}^{-1}$) added prior to polymerization, were prepared separately and run in parallel at 4°C . Bovine serum albumin was used as a negative control. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Binding of proteins to insoluble carbohydrates. This was investigated as described by Carvalho *et al.* (2004). Proteins in 50 mM Tris/HCl, pH 7.0, 0.05% Tween 20 buffer solutions were incubated with insoluble polysaccharides for 3 h at 4°C with stirring. The samples were then centrifuged, and the supernatant was removed for measurement of the protein concentration by the Bradford assay (Bradford, 1976). The pellets were washed three times with the same buffer, dissolved in the sample buffer, boiled for 10 min and then loaded onto a gel for electrophoresis. The amount of bound protein was calculated as the difference between the added amount and the remaining amount in the supernatant. Binding constants and target binding capacity were computed using the single binding site model from the equation

$$[B] = \frac{[N_0][F]}{\frac{1}{K_a} + [F]}$$

where $[N_0]$ is the concentration of the carbohydrate-binding sites, $[F]$ the concentration of free (unbound) protein, $[B]$ the concentration of bound protein, and K_a the binding constant (M^{-1}).

Protein melting. Protein melting curves in 50 mM MES buffer, pH 6.0, were determined spectrophotometrically by measuring UV absorption of protein solutions at different temperatures, by using the Agilent 8453 UV-visible Spectroscopy system, with the thermostatic cell Agilent 89090A Peltier temperature controller (Agilent). Measurements were done at 280 nm (tryptophan absorption maximum) and 1°C steps within a temperature range of $25\text{--}90^\circ\text{C}$. At each step the solutions were allowed to reach equilibrium. Measurements were repeated three to five times to achieve a 5% margin of error. Melting points were determined using the Agilent ChemStation software.

Polysaccharides. Activity and binding of Lic16A and its deletion derivatives were evaluated on lichenan, laminarin, Avicel CF1 and carboxymethylcellulose (Sigma-Aldrich), as well as on chitin, chitosan, xylan, bacterial crystalline cellulose from Sigma-Aldrich, pachyman from Megazym and pustulan from Roth. Phosphoric acid swollen cellulose was prepared from Avicel CF1 by the method of Wood (1988). Insoluble β -glucan from the yeast cell wall of *Saccharomyces cerevisiae* was prepared according to Bacon *et al.* (1969).

RESULTS

Lic16A primary structure and construction of truncated proteins

Fig. 1(a) illustrates the modular organization of the enzyme Lic16A (Fuchs *et al.*, 2003). A fragment of about 25 kDa, corresponding to amino acid residues 191–426 of this sequence, is denoted as the X module or CBMX. Amino acid sequences containing areas homologous to CBMX have been identified by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (see Supplementary Table S1, available with the online version of this paper). The most homologous genes code for bacterial proteins which contain N-terminal SLH modules, some of them combined with a putative catalytic module of family GH16. The only other related enzyme which has been experimentally characterized is the endo-1,3- β -glucanase LamA from *Paenibacillus* sp. (GenBank accession no. ABJ15796). It shows 65% sequence similarity in the CBMX-encoding portion of the gene. This enzyme degrades β -1,3-linkages in glucans, e.g. in laminarin and curdlan (Hong & Meng, 2003). It has a multimodular structure remarkably similar to Lic16A (see Fig. 1a, c). The flanking sequences of the CBMX module of Lic16A are enriched for proline and threonine residues. Similar sequences often serve as linkers separating modules in multimodular proteins (Gilkes *et al.*, 1991; Howard *et al.*, 2004). To characterize the function of the X module, five deletion mutants of Lic16A were constructed using PCR (Fig. 1b). The recombinant proteins were produced in *E. coli* cells with a His₆-tag sequence upon induction, allowing for one-step purification from the cell extracts. All the recombinant proteins were devoid of a leader peptide. LicA-full represents the intact protein (144 kDa), CBMX the isolated X module (25 kDa), LicA-xcat a tandem of the X and catalytic module (53 kDa), LicA-cat the separate catalytic module (34 kDa), and LicA-catchbd a tandem of CBM4A-1 and the catalytic module (48 kDa). The recombinant proteins were purified for further characterization.

The ability of Lic16A to degrade various β -glucans has been demonstrated previously (Fuchs *et al.*, 2003). Lic16A hydrolyses substrates containing β -1,3- and mixed β -1,3–1,4-linkages and has the largest activity on lichenan. Here, we investigated the activity of the deletion derivatives on lichenan, laminarin and a number of insoluble substrates (Table 2). A comparison of Michaelis constants for soluble substrates shows that the modular composition of the deletion derivatives predominantly affects their affinity for carbohydrates, whereas their specific activity per molecule A_a ($\text{U } \mu\text{mol}^{-1}$) remains practically unchanged (Table 2).

Biochemical characterization of deletion mutants

The temperature and pH optima for LicA-full and LicA-xcat coincided (70°C and 6.0 respectively) but differed from those for LicA-cat and LicA-catchbd, not containing CBMX (60°C and 6.5 respectively). The half-inactivation times of LicA-full, LicA-xcat, LicA-cat and LicA-catchbd at 70°C were found to be 40, 30, 20 and 20 min respectively (data not shown).

The effect of various additives on the activity of proteins on lichenan was investigated. EDTA (5 mM), Mg^{2+} (2 mM) and 10% ethanol did not have appreciable influence on the constructs. A 1.5–2-fold activity increase occurred for 2% glycerol and 2 mM β -mercaptoethanol. However, 2 mM Ca^{2+} and 10 mM SDS stimulated only those polypeptides that had the X module in their structure, increasing their activity 1.5-fold (data not shown).

Table 2. Enzyme activities of constructs on various substrates

A_a , enzyme activity ($\text{U } \mu\text{mol}^{-1}$); K_m , Michaelis constant (mg ml^{-1}); ND, not detectable.

Substrate		LicA-full	LicA-xcat	LicA-cat	LicA-catcbd
Lichenan	A_a	964	888	983	1020
	K_m	0.45	0.98	1.41	1.54
Laminarin	A_a	61	91	111	103
	K_m	2.11	1.69	3.83	3.56
Yeast cell wall β -glucan	A_a	ND	0.43	0.52	0.48
Pachyman	A_a	0.43	0.23	0.32	12.5
Pustulan	A_a	ND	0.12	0.17	5.5

Protein melting

Enzyme assays with soluble substrates showed that an increase in enzyme affinity could be attributed to the presence of the X module (Table 2). To investigate whether the X module acts as an independent subunit or forms a complex with the catalytic module, the protein stability and the stabilizing effect of various additives was studied by UV spectroscopy (see Fig. 2). Melting of a protein module is a cooperative process (Branden & Tooze, 1999; Dobson *et al.*, 1994; Finkelstein & Ptysin, 2005). Cooperation results in a narrow melting peak, as in the isolated catalytic module LicA-cat, with a melting point of about 83 °C (Fig. 2a). LicA-catcbd includes two modules connected by a flexible linker and, in contrast to LicA-cat, gives two overlapping melting peaks with apparent melting points of 69 and 83 °C and a low-temperature small peak at 38 °C. Considering the result for the LicA-cat module, it is reasonable to associate the peak at 69 °C with the melting of CBM4A-1. The peak at 38 °C may reflect the interaction of the catalytic module and CBM4A-1 at low temperatures far from the temperature optimum. The melting curve for LicA-xcat also contains three apparent melting peaks, at 40,

65 and 80 °C (Fig. 2a). Therefore, LicA-xcat seems to include two modules essentially melting independently.

Among all the additives tested, calcium had the greatest effect on the recombinant proteins (Fig. 2b). During the melting of LicA-xcat in the presence of 10 mM CaCl_2 , only two peaks were observed; the low-temperature peak disappeared, and the melting points of the CBMX and catalytic modules shifted (to 68 and 78 °C respectively). Calcium had no effect on the melting rate of LicA-cat and LicA-catcbd, although in the latter case the three characteristic melting points were shifted upward slightly (40, 72 and 81 °C from 38, 69 and 83 °C).

Polysaccharide binding of the isolated X module

The isolated X module was prepared by PCR (Fig. 1b), cloning and expression in *E. coli* and affinity purification to homogeneity (designated CBMX). Its molecular mass of about 25 kDa corresponded to the theoretically predicted value. Its binding property to various target ligands was studied qualitatively (Fig. 3) and quantitatively (Table 3). CBMX demonstrated the highest affinity to yeast cell wall

Fig. 2. Melting curves for Lic16A deletion fragments: (a) in the absence of calcium; (b) in the presence of 10 mM CaCl_2 . Absorbance was measured at 280 nm. Derivative absorption coefficients were normalized relative to their maximum values.

Fig. 3. Qualitative carbohydrate binding of CBMX. After the binding of CBMX to the insoluble polysaccharides and washing of unbound protein in 50 mM Tris/HCl, pH 7.0, 0.05 % Tween 20 buffer solution, the bound protein was eluted by 10 min boiling in sample buffer. I, initial protein before binding; F, free protein in supernatant after binding; B, protein bound to polysaccharide after washing. The presence of two protein bands with molecular masses ~25.2 kDa and ~17.2 kDa was due to the degradation of CBMX during binding procedures and was discussed earlier (Fuchs *et al.*, 2003). β -Glucan, glucan from yeast cell wall; BCC; bacterial crystalline cellulose.

β -glucan, xylan and chitin, with the affinity to other polysaccharides being markedly lower. Calcium strongly stimulated chitin and chitosan binding of CBMX but decreased the affinity to yeast cell wall β -glucan. Binding of other polysaccharides was not significantly affected by the presence of calcium. CBMX did not bind to soluble target molecules like lichenan, laminarin and CMC (data not shown), even though lichenan and laminarin are the polysaccharides for which Lic16A has substantially higher activity.

DISCUSSION

Lic16A is an enzyme with unusually high activity on soluble substrates. The enzyme has a complex structure consisting of one catalytic and eight non-catalytic modules. It is not clear how the non-catalytic modules support the catalytic activity. The CBMX module adjoins the catalytic module. The isolated CBMX module was shown to bind a wide range of different insoluble polysaccharides, including

xylan, yeast cell wall β -glucan and crystalline cellulose. It has the highest affinity to xylan and yeast cell wall β -glucan. High affinity to chitin and chitosan is dependent on the presence of calcium ions. A similar ability to bind such a wide range of heterogeneous polysaccharides is characteristic of carbohydrate-binding modules of family CBM37 (Ezer *et al.*, 2008).

We have identified a number of proteins with high homology to CBMX (Supplementary Table S1). As none of the already known CBMs has significant homology to CBMX, the latter seems to contain a new family of CBMs (CBM54). The corresponding areas of the proteins listed in Supplementary Table S1 can also be postulated to have carbohydrate function. According to results from the retardation experiments, LicA-catcbd failed to bind the soluble target substances laminarin and lichenan, on which the catalytic module showed the highest activity. LicA-catcbd was slightly more active on insoluble substrates than the other constructs studied and deletion of CBMX led to a decrease in the affinity of Lic16A for soluble carbohydrates.

Table 3. Target binding capacity (N_0) and binding constants (K) for module CBMX

Polysaccharide target	With calcium		Without calcium	
	N_0 ($\mu\text{mol g}^{-1}$)	K (M^{-1})	N_0 ($\mu\text{mol g}^{-1}$)	K (M^{-1})
Xylan	34 ± 1	$(5 \pm 1) \times 10^4$	14 ± 1	$(3.1 \pm 0.5) \times 10^4$
Chitin	48 ± 1	$(3.3 \pm 0.7) \times 10^5$	10 ± 0.3	$(1 \pm 0.1) \times 10^3$
Pustulan	16 ± 0.6	$(3.4 \pm 1) \times 10^3$	12 ± 0.5	$(2.1 \pm 0.5) \times 10^3$
Avicel	40 ± 2	$(8.5 \pm 1.6) \times 10^3$	16 ± 0.6	$(3.4 \pm 1) \times 10^3$
Yeast cell wall β -glucan	18 ± 0.5	$(5 \pm 0.5) \times 10^4$	18 ± 0.3	$(1.4 \pm 0.2) \times 10^5$
Chitosan	20 ± 0.6	$(6.6 \pm 0.5) \times 10^4$	24 ± 1	21 ± 5
Bacterial crystalline cellulose	16 ± 0.6	$(2.8 \pm 0.6) \times 10^3$	14 ± 0.6	$(3 \pm 0.6) \times 10^3$

cth	192	IKILNKIIPSLYNEKGDYKNEE----	VAGNALINTEG-VILK	DTVINGDLYLAQGIQNGDVTLDG																																																			
ep2	210	ASILDRIAVEVVANNGVHKLGR---	VAGNVVISAMGNTGLQD	TVIIGNLYIPAGVNGQIFLDN																																																			
geo	202	SLEYKVPTDITYDKAGTYGPTAGVETIKGN	VIVAAPG-VTLQNVVIEGNLTLTE	EAVGEGDAFFKK																																																			
pb3	228	ESGGIVPAALNLS	EAGTYGPEAGTFTVAGDLVVDVPG-VTL	RNLAVKGNLTIGEGVGEAEFLDH																																																			
dre	203	QQATAAEKTVFYAKPGTYGPATGRETINGN	VTISVAD-VTLQNTTISGDLLLA	EGVNGDVKLKN																																																			
pb4	203	AVLLSGMIGDLVAKPGKYDAAQ----	VEGNLIIRSTD-VTVDR	TAVYGNLLLAEGIKEGDATLKG																																																			
pb1	229	VVLLDRLAGEIIRQPGSYDGVK----	SDSGLLIASAD-TILKQAEVKG	NVLITAGVGEGETLDG																																																			
csa	199	VKLLSSIGAVIVTKPGVYQNIK----	KEGFVLINSQD-VELKE	AVIKGNVYINQSVGKGTVTLTN																																																			
pb2	207	VVLLSRLAGEVYVKAQTYKDQK----	LAGNGLVNLDG-VTIENS	SAIGNLYLTAGIGEGDFTLKN																																																			
bca	185	VTILDNIFNANYNTSGEYTG-----	DVDGSAVISADN-VTLK	DMHITGDLIVAEGVGEHVELDN																																																			
cc2	196	VAIINNIVKGYTTKAGTYDD-----	NVTGTVIIKVPD-VTLK	GVNITENLIIAEGVGEDEVTLDS																																																			
cph	193	ITMIDSIVIKYENEDGTYSK-----	SVTGTAVINTGN-ITL	KKMQINGNLIIEGVGSGDVTLDN																																																			
cc1	190	LIMLDKCVGQIALKSGTIKT-----	NAAGNMFISVGA-VTLR	GISISGDLIIEGVGDGVVTLES																																																			
ep1	188	ITMVSKVSEKIIINRPGEYVEG----	YVDSSVVINCTN-VTLK	NTTIRGNLYINEGIGAGDVTLEG																																																			
cth		VNVK-GTV	EVN	GGGS	DS	IE	FIN	T	KINR	VV	NKTG	----	V	R	I	V	T	S	G	N	T	S	V	E	S	V	V	K	S	G	A	K	L	E	307																				
ep2		VEVR-GTV	FE	EGGX	---	I	ENT	K	I	E	T	L	I	V	N	NED	----	T	T	I	K	G	N	F	K	S	E	V	E	V	A	D	I	R	T	A	N	I	D	323															
geo		VNVK-GTTT	I	QGGG	ANSV	HE	F	E	D	S	V	L	V	R	V	-S	V	D	K	Q	T	-G	T	V	R	V	V	V	G	E	S	A	I	Q	H	V	V	V	H	S	P	V	K	L	E	323									
pb3		VKVS-GST	E	IRGGG	VNSV	HL	D	D	S	E	L	G	T	V	-V	I	E	K	D	-G	K	V	R	V	V	S	G	T	T	V	E	Q	M	D	V	K	S	N	A	S	V	E	349												
dre		VKVS-GKT	I	I	KGGG	ANSV	T	L	E	D	C	S	L	P	N	I	-T	V	S	K	E	--	G	-V	R	V	V	A	S	G	N	T	T	V	K	V	R	L	E	S	G	A	T	L	V	322									
pb4		VTVT-GKT	V	I	KGGG	SHSV	HL	R	NAK	V	G	K	V	-V	N	K	S	G	-G	P	V	R	V	V	L	E	G	S	S	K	V	G	E	M	S	L	E	T	G	A	V	V	E	346											
pb1		LSAD-GTL	V	N	GGGS	HSV	HL	R	NAK	V	G	K	V	-V	N	K	S	G	-G	P	V	R	V	V	L	E	G	S	S	K	V	G	E	M	S	L	E	T	G	A	V	V	E	346											
csa		VSIENGKLFV	F	GGGS	N	SV	K	L	NN	T	K	V	E	K	E	V	Y	V	A	N	M	V	S	-E	K	V	R	L	S	I	E	G	K	S	E	V	E	H	L	I	V	I	S	P	A	S	V	E	318						
pb2		TEVN-GT	I	F	I	S	G	G	V	H	S	I	HL	NN	V	H	V	P	A	I	-I	V	D	K	E	-G	P	V	R	V	I	V	D	G	-S	E	V	G	T	M	D	L	N	S	D	T	V	L	E	323					
bca		VTVD-GRL	V	R	G	G	G	A	N	S	I	V	I	K	G	D	S	K	I	T	Q	V	I	I	A	R	Q	E	-G	K	V	R	V	A	V	E	D	G	A	Q	V	S	V	V	V	E	E	G	S	D	D	V	302		
cc2		VTVK-GNT	V	V	R	G	G	E	N	S	I	H	I	T	G	S	S	N	I	S	N	I	K	I	E	K	NN	-N	K	L	R	I	V	I	L	D	G	N	T	V	K	E	I	E	I	A	K	G	E	E	I	313			
cph		VEIS-GQ	L	I	V	R	G	G	G	K	N	S	I	H	I	G	S	T	K	V	A	N	I	I	V	D	K	K	E	A	P	V	S	L	K	F	S	S	S	Y	E	S	S	S	V	T	V	I	N	G	E	L	Y	L	311
cc1		VKLA-GRL	I	I	R	G	G	G	P	N	G	V	I	I	K	N	S	Q	I	G	N	L	I	V	E	K	S	A	-G	N	V	Y	I	R	V	G	S	T	T	I	K	Q	A	Y	L	K	S	G	C	T	I	E	307		
ep1		VTVL-GTV	E	I	N	G	G	G	D	K	P	I	V	L	K	X	-C	K	M	A	E	M	V	D	K	Y	-G	N	V	N	I	T	G	D	A	N	T	K	I	T	N	X	X	F	K	S	A	G	T	V	A	305			

Fig. 4. Multiple alignment of the conserved region from CBMX (new family CBM54). Abbreviations: cth, *licA* *C. thermocellum*, CAC27412; ep2, *Epulopiscium* sp., ZP_02692123; geo, *Geobacillus* sp., Y412MC10, ZP_03036254; pb3, *Paenibacillus* sp. JDR-2, ZP_02846396; dre, *Desulfotomaculum reducens* MI-1, YP001113692; pb4, *Paenibacillus* sp. JDR-2, ZP_02849476; pb1, *Paenibacillus* sp. CCRC17245, ABJ15796; csa, *Caldicellulosiruptor saccharolyticus* DSM 8903, YP_001181313; pb2, *Paenibacillus* sp. JDR-2, ZP_02847034; bca, *Bacteroides capillosus* ATCC 29799, ZP_02036105; cc2, *Clostridium cellulolyticum* H10, ZP_01575502; cph, *Clostridium phytofermentans* ISDg, YP_00158884; cc1, *Clostridium cellulolyticum* H10, ZP_01576976; ep1, *Epulopiscium* sp., ZP_02693375. Black boxes designate conserved residues potentially involved in sugar binding, grey boxes polar aromatic residues potentially involved in calcium binding.

According to the Lic16A primary structure analysis and to the melting experiments, CBMX is a separately folding module flanked by linker sequences. The temperature and pH optima, as well as the thermostability of the catalytic activity, of the LicA-cat module and the Cat-CBM4A-1 tandem of Lic16A coincided, suggesting that CBM4A-1 does not have any functions beyond binding polysaccharides. This conclusion was supported by the melting experiments. The binding module in Lic16A, CBM4A-1, belongs to the CBM4A family, members of which are known to bind soluble and partially also insoluble polysaccharides (Zverlov *et al.*, 2001). Preliminary data show that CBM4A-1 might be specialized in binding insoluble polysaccharides. This observation and its influence on the catalytic activity of Lic16A will be the subject of further studies.

The activity of many cellulolytic enzymes of *C. thermocellum* increases in the presence of calcium ions (Lamed & Bayer, 1988; Grepinet *et al.*, 1988). The highest increase in the activity of recombinant truncated Lic16A proteins due to calcium stimulation was observed for constructs that contained CBMX. The analysis of LicA-xcat melting was in favour of a stabilizing effect of Ca^{2+} on CBMX. Addition of Ca^{2+} ions resulted in a more than 100-fold increase of the CBMX target binding capacity and binding constant on chitin and chitosan. Binding to the other polysaccharides studied here was not affected by calcium. CBMX probably contains more than one carbohydrate-binding site. This may also explain the wide target recognition spectrum of CBMX. In turn, chitin- and chitosan-binding centres of CBMX may include a Ca^{2+} -binding site that affects the affinity to the target and provides

stabilization of the module. The presence of CBMX in the constructs resulted in an increase in their temperature optimum, and the X-Cat tandem was determined to have a thermostability between that of Lic16A and the isolated catalytic module. Thus, it can be assumed that CBMX carries out a stabilizing function in Lic16A and moderates its activity.

A sequence alignment among the sequences from Supplementary Table S1 revealed a conserved core sequence in the N-terminal part of the 14 sequences most homologous to the CBMX module (Fig. 4). This sequence covers 115 amino acid residues in CBMX and thus has the size of a typical CBM. However, the sequence is not homologous to hitherto known CBMs. Although none of the homologous sequences has been shown so far to have carbohydrate-binding capacity, it should be noted that a pattern of conserved aromatic amino acid residues can be identified (Fig. 4); these residues have been shown in other CBM families to be responsible for binding to target sugar residues (Xie *et al.*, 2001; Boraston *et al.*, 2004). Moreover, in the new family CBM54 some polar amino acid residues are more or less conserved which have a potential to be involved in target binding by hydrogen bonds (Notenboom *et al.*, 2001; Xie *et al.*, 2001; Pell *et al.*, 2003) or in calcium ion binding (Bolam *et al.*, 2004; Chauvaux *et al.*, 1995; Jamal-Talabani *et al.*, 2004).

It is interesting to note that in all 14 homologous proteins CBMX is located at the N terminus of the respective proteins, but preceded by another protein module, a duplicated SLH module which is involved in cell wall anchoring (Schwarz *et al.*, 2004). The invariable juxtaposition of these two modules in the homologous proteins suggests a function of CBMX comparable to that of SLH in cell wall binding, similar to that of the CBM37 modules in ruminococci (Ezer *et al.*, 2008). This function could be enhanced by the SLH module. However, the function of the conserved submodule CBM54 defined in Fig. 4 has to be investigated in other bacteria.

NOTE ADDED IN PROOF

After acceptance of this paper a subsequence of CBMX was added to the list of CBMs in the CAZy database as a new family, CBM54. The kind help of B. Henrissat in this matter is gratefully acknowledged.

ACKNOWLEDGEMENTS

This work was supported by the Russian Foundation for Basic Research (Project 06-04-48351a).

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Edited by: H. J. Flint