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Stable linker peptides for a cellulose-binding domain–lipase fusion protein expressed in *Pichia pastoris*

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Fusion proteins composed of a cellulose-binding domain from *Neocallimastix patriciarum* cellulase A and *Candida antarctica* lipase B were constructed using different linker peptides. The aim was to create proteolytically stable linkers that were able to join the functional modules without disrupting their function. Six fusion variants containing linkers of 4–44 residues were expressed in *Pichia pastoris* and analysed. Three variants were found to be stable throughout 7-day cultivations. The cellulose-binding capacities of fusion proteins containing short linkers were slightly lower compared with those containing long linkers. The lipase-specific activities of all variants, in solution or immobilized on to cellulose, were equal to that of the wild-type lipase.

Keywords: *Candida antarctica*/cellulose-binding domain/lipase/proteolysis

Introduction

Many naturally occurring enzymes are composed of two or more distinct modules that are joined into a single macromolecule by stretches of amino acids referred to as linkers. Recent studies have provided examples of linkers that are important in establishing the structural and functional assembly of multi-domain proteins, as reviewed by Gokhale and Khosla (Gokhale and Khosla, 2000). Enzymes active on insoluble substrates typically carry various substrate-binding modules. For example, most cellulose-degrading enzymes consist of a catalytic module and a cellulose-binding domain (CBD) joined by a linker, which may be susceptible to proteolysis (Linder and Teeri, 1997). These interdomain linkers are relatively long and probably flexible in order to allow the two modules to perform independent functions. Although structural studies of different linkers are still lacking, they are thought to adopt extended conformations. Many microbial cellulases and xylanases contain linkers rich in prolines and hydroxyamino acids that are often *O*-glycosylated, which probably helps to maintain an extended conformation and may also protect the linker from proteolysis (Gilkes *et al.*, 1991). The small, polar residues frequently observed in the linkers also contribute to their flexibility and stability through hydrogen bonding to water (Argos, 1990). Linker engineering studies have shown that the length of the linker is important for the activity of some cellulases (Shen *et al.*, 1991; Srisodsuk *et al.*, 1993; Wilson *et al.*, 1995; Black *et al.*, 1996).

Recombinant production of chimeric enzymes requires stable linkers for joining the fusion partners without interfering with their function. The use of long linkers may result in low yield of active fusion protein since unprotected and flexible regions are often susceptible to proteolytic cleavage during recombinant protein production. Use of a shorter linker might overcome problems associated with protease degradation. On the other hand, there is a risk that a short linker brings the modules too close to each other, resulting in a loss of function.

Lipases have great potential in the pulp and paper industry for pitch removal and deinking processes. Unfortunately, the use of enzymes adds a high cost to the papermaking process. This could be reduced by allowing the enzyme to react more efficiently; one way could be to anchor the enzyme to its substrate using a cellulose-binding domain (CBD).

We have previously shown that a fusion between the CBD from *Neocallimastix patriciarum* cellulase A (Cel6A) and *Candida antarctica* lipase B (CALB) using a linker based on the linker of cellobiohydrolase Cel6A from *Trichoderma reesei* exhibits normal lipase activity and good cellulose-binding ability (Rotticci-Mulder *et al.*, 2001). Heterologous expression of the CBD–CALB fusion protein was carried out in the methylotrophic yeast *Pichia pastoris*. For our research purposes we chose to work with *P.pastoris* as a host for recombinant protein production since it is easy to use and can give very high product yields as well as high levels of secretion into an almost protein-free medium. The CBD–CALB fusion protein was found to be unstable owing to proteolytic cleavage at the linker, which resulted in a mixture of CBD–CALB and a degradation product equal in size to the wild-type CALB produced in *P.pastoris*.

Here we describe the production and performance of a series of engineered linkers designed to enhance the proteolytic stability of the CBD–CALB fusion protein during long-term cultivations. The parameters considered were the length, the amino acid content and the presence of potential glycosylation sites in the linkers. Six fusion proteins with different linker peptides were cloned and produced in *P.pastoris*. The stabilities of the fusion proteins against proteolysis were analysed in a 7-day time course study of expression using Western blot analysis. The lipase activity and the cellulose-binding property displayed by the fusion partners were investigated in order to determine whether there was any loss in module function. The effect on the cellulose-binding capacity of the FLAG peptide previously attached to the N-terminus of CBD–CALB (Rotticci-Mulder *et al.*, 2001) was also studied.

Materials and methods

Strains, plasmid and media

Escherichia coli strain TOP10F′ (Invitrogen, Carlsbad, CA) was used for all plasmid constructions and *P.pastoris* protease reduced strain SMD1168H (Invitrogen) for protein expression. Vector pPICZαA and Zeocin were purchased from Invitrogen. *E.coli* was grown in LB medium (10 g Tryptone, 5 g yeast extract, 5 g NaCl and 1 ml 1 M NaOH per litre) containing

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Zeocin (25 μg/ml). *P.pastoris* was grown in YPD medium (10 g yeast extract, 20 g peptone and 20 g dextrose per litre) or BMGY medium (10 g yeast extract, 20 g peptone, 13.4 g yeast nitrogen base, 0.4 mg biotin, 10 ml glycerol and 100 ml 1 M K_2HPO_4/KH_2PO_4, pH 6.0 per litre). BMMY medium (10 g yeast extract, 20 g peptone, 13.4 g yeast nitrogen base, 0.4 mg biotin, 5 ml methanol and 100 ml 1 M K_2HPO_4/KH_2PO_4, pH 6.0 per litre) was used for induction of protein production. YPDS plates (10 g yeast extract, 20 g peptone, 20 g dextrose, 182.2 g sorbitol and 20 g agar per litre) containing Zeocin (100 μg/ml) were used for selection of transformants.

Vector constructions and protein production

The gene coding for CALB was amplified from plasmid YpCALB (Rotticci-Mulder *et al.*, 2001) using forward primer A, 5′CTTCGAATTC**CTACCTTCCGGTTCGGACC**, and reverse primer B, 5′GCGGATACAGCGGCCGCTCA-TCA**GGGGGTGACGATGCCGGA**, introducing cleavage sites for the restriction enzymes *Eco*RI and *Not*I, respectively. The cleavage sites are underlined in all sequences and the regions of homology are in bold. DNA encoding the CBD was prepared by PCR amplification of plasmid pBSFCA containing the *N.patriciarum* Cel6A gene (Denman *et al.*, 1996). Forward primer C, 5′CTTCCTCGAG*AAAAGAGAGGCTGAAGCT***TG-TGGTGGTGCCTGGG**, and reverse primer D, 5′GGGG-TACC**TTGACATTGTGAATACCATTG**, introduced sites for cleavage with *Xho*I and *Kpn*I to the 5′- and 3′-end of the CBD, respectively (the sequence encoding the reconstruction of the KEX2 site is in italics). For the CBD–CALB 2 construct containing the complete linker from *N.patriciarum* Cel6A, reverse primer E, 5′CTTCGAATTC**ACCACCATTGTTATTGTT-ATTG**, was used together with the forward primer C mentioned above for amplification of the CBD together with its natural linker. The fragment coding for the CBD together with a linker based on the *T.reesei* Cel6A linker was amplified from vector pTCL (a kind gift from Dr Gang-Ping Xue, CSIRO, Australia) using reverse primer F, 5′CTTCGAAT TC**ACCATTGTTAA-CACG**, together with the forward primer C mentioned above. Forward primer G, 5′CTTCCTCGAG*AAAAGAGAGGCT-GAAGCT***GATTACAAAGACGAT**, was used together with the reverse primer D mentioned above in order to amplify the CBD together with an N-terminal FLAG peptide for the construct FLAG–CBD–CALB 4. The sense and antisense strands of three linkers were purchased as oligonucleotides (Interactiva, Ulm, Germany) and annealed together by placing a mixture of complementary strands in boiling water which was left to cool to room temperature. Suitable 3′- and 5′-overhangs allowed annealing to *Kpn*I- and *Eco*RI-digested DNA using standard ligation procedures. Cleaved CALB and CBD PCR products were mixed with linker fragments and allowed to ligate for 30 min at room temperature before addition of digested plasmid pPICZαA. The final ligation mixture was incubated at 15°C overnight. The ligated constructs were transformed by heat-shock into TOP10F′ cells. After selective growth on LB Zeocin plates at 37°C overnight, colonies were picked and screened for plasmids containing inserts using PCR. Sequencing on both strands was performed with a MegaBACE sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) to confirm correct sequences in frame with the α-factor secretion signal. A 5 μg amount of DNA linearized with *Pme*I was transformed into *P.pastoris* strain SMD1168H by electroporation (Gene Pulser II, Bio-Rad, Hercules, CA) and spread on selective YPDS Zeocin plates. Screening of transformants for CBD–CALB secretion was performed according to the procedure described by Holmquist *et al.* (Holmquist *et al.*, 1997) using anti-CALB antibodies. Protein expression and purification was performed as described by Rotticci-Mulder *et al.* (Rotticci-Mulder *et al.*, 2001).

Analysis of protein stability

Cultures of CBD–CALB variants were grown in baffled shake flasks and protein expression was allowed to continue for 7 days. Methanol was added to the culture medium every 24 h to a final concentration of 0.5% (v/v) and 1 ml samples were taken for protein analysis. The stabilities of the fusion proteins were examined on Western blots using anti-CALB antibodies.

Analysis of lipase activity

Active site titration (Rotticci *et al.*, 2000) was performed using a methyl *p*-nitrophenyl *n*-hexylphosphonate inhibitor in order to determine the concentration of active sites in preparations of pure CBD–CALB variants. The lipase hydrolytic activity of a fusion enzyme was measured at 25°C and pH 7.5 using a pH-stat (TIM900 Titration Manager, Radiometer, Copenhagen, Denmark) and 100 mM NaOH.

Tributyrin substrate solution (2% gum arabic, 0.2 M $CaCl_2$ and 0.2 M tributyrin) was emulsified by sonication (Branson 250, 30 W) for 1 min. To start the reaction, enzyme was added to 1.5 ml of substrate solution.

For the activity measurements on CBD–CALB variants anchored to cellulose, a soluble ethyl butyrate substrate solution was used to eliminate any effect involved in the use of emulsions for measurements on immobilized enzymes. Ethyl butyrate was mixed with 5 mM MOPS buffer, pH 7.5, to reach a final concentration of 100 mM. CBD–CALB was stirred at 800 r.p.m. with bacterial microcrystalline cellulose (BMCC) in an Eppendorf tube for 1 h at room temperature. A reference sample containing enzyme mixed with buffer instead of BMCC was prepared for each fusion protein. To make the activity measurements reliable, the enzyme concentration and BMCC amount were adjusted so that >50% of CBD–CALB bound to the cellulose. Half of an enzyme–BMCC mixture was centrifuged and the activity present in the supernatant was determined and compared with that of the reference sample in order to confirm this.

An aliquot of enzyme–BMCC mixture was added directly to the ethyl butyrate substrate solution in a pH-stat and the activity was compared with that present in the reference sample. To ensure that the fusion enzyme remained attached to cellulose, washed BMCC pellets containing bound CBD–CALB were stirred for 3 min at 25°C to mimic the conditions during an activity measurement. After centrifugation, the amount of released fusion enzyme was determined by measuring the lipase activity present in the supernatant.

Active site titration was performed in order to determine if the concentration of active CALB sites was different in a sample containing CBD–CALB anchored to cellulose compared with its reference sample. Inhibitor was added to an enzyme–BMCC sample and the solution was mixed by inverting the tube five times. The cellulose was removed by centrifugation and the supernatant was collected and analysed. Reference samples containing enzyme mixed with buffer were treated in the same way.

Cellulose-binding studies

The purification of BMCC from coconut gel (Snowhouse, Thailand) was performed according to the method described by Gilkes *et al.* (Gilkes *et al.*, 1992). Dilutions of 50 μM

Table I. Linker candidates for the design of a stable CBD–CALB fusion enzyme

Linker	Amino acid sequence
1	PGGAPS**NRS**TTSRVSPTTSRSSSVTPPPGSTTTRVN*NGEF
2	PGGAPSN**NAS**NNNNNNNNNNNNNNNNNNNNNHNNNNNNNNNNNNNGGEF
3	GTNNNHNNNNNNNNNNNNNGGEF
4	GTPTPTPTPTGEF
5	GTPTPT**NRS**PTPTGEF
6	GTPF

enzyme stock solutions were made in 20 mM MOPS buffer, pH 7.5 containing 50 mM NaCl. Enzyme and BMCC (1 g/l) were mixed in Eppendorf tubes at 800 r.p.m. and 4°C for 90 min. Reference samples were made containing buffer instead of BMCC. All samples were filtered through Millex PVDF Durapore filters, 0.45 μm (Millipore, Bedford, MA) and the concentration of unbound protein was determined by comparing the absorbance (280 nm) of the filtrate with that of its reference sample.

Results

Cloning and expression of CBD–CALB variants

In order to increase the proteolytic stability, six different CBD–CALB fusion proteins were constructed using linkers, which varied in the presence or absence of potential *N*-glycosylation sites, in length and in amino acid composition (Table I). *N*-glycosylation sites are marked in bold in Table I. The genes were placed under the control of the methanol inducible alcohol oxidase gene (AOX1) promotor and the plasmids were transformed into *P.pastoris* strain SMD1168H. Western blot analysis of CBD–CALB 1 culture media (Figure 1A) revealed a protein of 45 kDa representing CBD–CALB 1 glycosylated at the potential *N*-glycosylation site present in CALB and a 75 kDa protein corresponding to CBD–CALB 1 with *N*-glycosylations at both the CALB site and the site present in the linker, as have been shown previously (Rotticci-Mulder *et al.*, 2001). Constructs 2 and 5 were also *N*-glycosylated at the site present in the linker (data not shown). Protein purification was performed using hydrophobic interaction chromatography followed by gel filtration as described previously (Rotticci-Mulder *et al.*, 2001), which allowed the isolation of pure intact CBD–CALB. The expression level was calculated to be ~10 mg/l.

Analysis of fusion protein stability

Proteolysis in the linker region was significant for CBD–CALB 1 during cultivation (Figure 1A). The major degradation product equal in size to wild-type CALB (36 kDa) produced in *P.pastoris* (Rotticci-Mulder *et al.*, 2001) could be detected from day three onwards. The amount of produced fusion protein reached a steady-state level at day three, while the amount of degradation product increased throughout the cultivation, suggesting that the rate of degradation was as high as the production rate after day three. After 7 days of expression, >50% of the produced fusion protein was proteolytically cleaved at the linker. Expression of CBD–CALB 2 resulted in a similar degradation behaviour (data not shown). As can be seen in Figure 1B, CBD–CALB 3 was also degraded but to a lesser extent. In the case of CBD–CALB 4, minimal proteolysis was only detected towards the end of the time course study and the amount of expressed fusion protein increased steadily throughout the cultivation (Figure 1C).

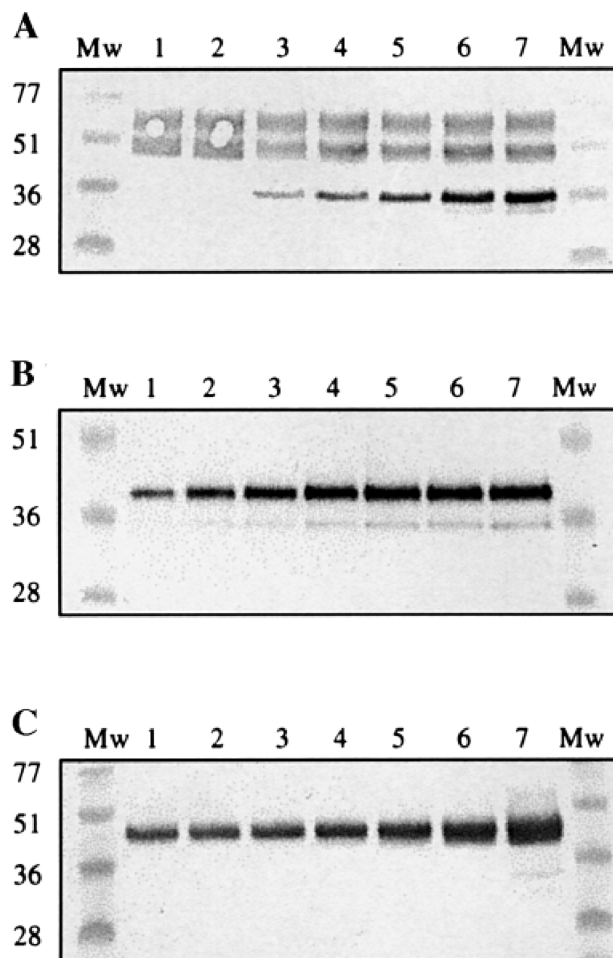

Fig. 1. Western blot analysis using anti-CALB antibodies showing the degree of proteolysis for CBD–CALB variants. Culture media containing secreted protein were analysed at 24 h intervals for 7 days of production starting at lane 1. Lane Mw represents the molecular weight marker in kDa. (**A**) CBD–CALB 1; (**B**) CBD–CALB 3; (**C**) CBD–CALB 4.

Expression of CBD–CALB 5 and CBD–CALB 6 resulted in a similar stable production (data not shown).

The isolated degradation product from expression of CBD–CALB 1 was collected after gel filtration and the N-terminus of the protein was sequenced (Protein Analysis Centre, Stockholm, Sweden). The analysis showed that only four amino acids of linker 1 remained attached to the lipase (this site is marked with an asterisk in Table I).

Analysis of lipase activity

The specific lipase hydrolytic activity towards emulsified tributyrin was essentially the same for the wild-type CALB and the six different fusion proteins (Table II). The activities

Table II. Specific hydrolytic activity of wild-type CALB and the CBD–CALB variants in solution and when immobilized on cellulose via the CBD

Enzyme	Activity, tributyrin (s^{-1})	Activity, ethyl butyrate (s^{-1})	
		Reference sample	Enzyme–BMCC mix
CALB	370 ± 32	n.d.	n.d.
CBD–CALB 1	347 ± 23	266 ± 6	273 ± 16
CBD–CALB 2	345 ± 21	263 ± 9	269 ± 7
CBD–CALB 3	374 ± 15	277 ± 7	275 ± 7
CBD–CALB 4	330 ± 33	260 ± 14	267 ± 8
CBD–CALB 5	359 ± 27	277 ± 11	272 ± 15
CBD–CALB 6	370 ± 32	277 ± 8	275 ± 5

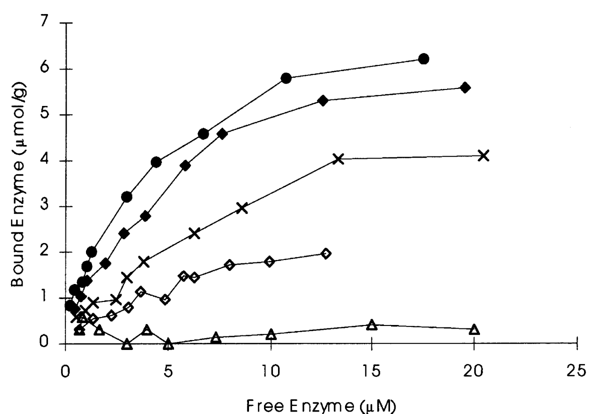

Fig. 2. Equilibrium binding isotherms for wild-type CALB and four CBD–CALB fusion proteins to BMCC. (●) CBD–CALB 1; (◆) CBD–CALB 4; (×) CBD–CALB 6; (◇) FLAG-CBD–CALB 4; (△) CALB.

on soluble ethyl butyrate (100 mM) were measured with the enzymes free in solution and as anchored to cellulose via the CBD, with no apparent difference between the fusion proteins (Table II). Active site titration confirmed that there was no loss in active sites in CBD–CALB preparations in which more than half of the enzyme was anchored to cellulose. The fusion proteins were shown to remain attached to BMCC during measurements.

Cellulose-binding studies

The equilibrium binding isotherms for wild-type CALB and three of the CBD–CALB fusion proteins representing different linker lengths are shown in Figure 2. A comparison between CBD–CALB 4 constructs with and without an N-terminal FLAG peptide is also shown. As can be seen in Figure 2, the FLAG peptide containing protein FLAG-CBD–CALB 4 displayed a lower affinity towards BMCC compared with CBD–CALB 4.

Only a slight decrease in binding capacity was seen for CBD–CALB 4 containing a linker of 13 amino acids compared with CBD–CALB 1 with its 40 amino acid long linker. The construct CBD–CALB 6 with a linker of four amino acids had a lower binding capacity than the two fusion enzymes containing longer linkers. The difference became significant at enzyme concentrations approaching saturation of the cellulose surface. Wild-type CALB did not show any binding to BMCC.

Discussion

Our strategy for designing new proteolytically stable linkers for the CBD–CALB fusion proteins was to choose candidates with an amino acid composition that resembled different naturally occurring linkers. Western blot analysis of the original fusion protein, CBD–CALB 1, revealed proteolytic cleavage at the linker region during production in *P.pastoris* (Figure 1A). This fusion enzyme is similar to the protein described previously (Rotticci-Mulder *et al.*, 2001) but lacks the N-terminal FLAG peptide. N-Terminal sequencing of the CBD–CALB 1 degradation product, which was equal in size to wild-type CALB, showed that only four amino acids of linker 1 remained attached to the lipase. This could be the primary cleavage site. However, Markaryan *et al.* have found that proteins containing a Gly–Ser site are more sensitive to proteolytic cleavage during expression in *P.pastoris* (Markaryan *et al.*, 1999). As can be seen in Table I, a Gly–Ser site is present in linker 1, upstream of the amino acids that remained fused to the degradation product. It is therefore also possible that the linker is first cleaved at the Gly–Ser site and then further degraded towards the C-terminus until the protease is stopped by the proximity of the bulky lipase at the amino acids identified. CBD–CALB 2 contains a linker based on that of the *N.patriciarum* Cel6A. It was chosen since it is the natural linker for the CBD used in this study. Linker 2 is similar in length but differs in amino acid composition from linker 1 and was cleaved by proteolysis to an equal extent during expression (data not shown).

CBD–CALB 3 contains a shorter variant of linker 2, rendering the fusion protein less susceptible to proteolysis (Figure 1B). Linkers 2 and 3 are similar in amino acid composition but different in length, suggesting that length may be a factor for linker stability. Alternatively, the sequences preceding the NNN-repeat in linker 1 and 2 are susceptible to proteolysis.

CBD–CALB 4 has a 13 amino acid long linker composed of only prolines and threonines. Such linkers are commonly found in bacterial cellulases (Gilkes *et al.*, 1991) and have been suggested as good linker candidates for general gene fusions (Argos, 1990). Linker 4 is a shorter variant of the natural linker of *Cellulomonas fimi* endoglucanase A, which consists of 23 amino acids $[(PT)_4T(PT)_7]$ in an extended, kinked and rigid conformation (Shen *et al.*, 1991). Only a minimal degree of proteolysis in the linker region could be observed for CBD–CALB 4 (Figure 1C).

Studies with *C.fimi* cellulases produced by the native host or in *E.coli* indicate increased linker stability upon *O*-glycosylation (Langsford *et al.*, 1987; MacLeod *et al.*, 1992; Ong *et al.*, 1994). Even *N*-glycosylation may play a role in protein stability (Imperiali and O'Connor, 1999; Pratap *et al.*, 2000). In order to test the role of glycosylation in linker stability, we designed a variant of linker 4 with one *N*-glycosylation site. However, no difference in stability was observed between the CBD–CALB 4 and 5 (data not shown), probably because linker 4 already was stable. To study the

effect of glycosylation on linker stability, glycosylated versions of longer linkers are needed. Finally, a linker composed of only four residues was designed. This very short linker construct CBD–CALB 6 was as stable during expression as CBD–CALB 4 and 5 (data not shown).

The lipase hydrolytic activity towards tributyrin for CBD–CALB variants free in solution was shown to be the same for all fusion proteins and equal to the activity of wild-type CALB (Table II). Close proximity to the CBD therefore does not seem to disturb the activity of CALB, even when only four amino acids are separating the modules. Furthermore, anchoring the CBD–CALB on to cellulose did not alter the hydrolytic activity of any of the fusion proteins as compared with the same free in solution (Table II). The results suggest that the catalytic module is not in any way impaired by the immobilization. Active site titration confirmed that the concentration of available active sites was unaltered after immobilization.

The CBD used in this study occurs naturally as an N-terminal CBD. Fusion of a highly charged FLAG peptide (DYKDDDDK) to the N-terminus of the CBD decreased the cellulose-binding capacity of the CBD–CALB (Figure 2). This may be due to disruption of the three-dimensional structure of the CBD and/or prevention of close packing of CBD–CALB molecules on to the cellulose surface due to electrostatic repulsion. The result would in both cases be a lower affinity of the CBD to cellulose.

The binding capacity of CBD–CALB towards cellulose decreased with decreasing linker length (Figure 2). The decrease in binding capacity for constructs with shorter linkers could be due to steric hindrance caused by the lipase catalytic module as it approaches closer to the cellulose surface, thereby making it difficult for the CBD to reach all available binding sites. However, the difference in cellulose-binding capacity between CBD–CALB fusion proteins with different linker lengths was small and comparable to the differences obtained on linker peptide deletions of the fungal cellobiohydrolase Cel7A (Srisodsuk *et al.*, 1993).

It is likely that the flexibility between the two functional domains decreases with decreasing linker length. Unless an application is dependent on high flexibility, this should not become a problem as shown here for the CBD–CALB fusion proteins. Among the different fusion proteins investigated here, CBD–CALB with linker 4 is the most attractive for future studies since it is stable during protein production and reproduces the good cellulose-binding capacity observed with CBD–CALB 1.

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