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ORIGINAL PAPER

Effect of the cellulose-binding domain on the catalytic activity of a β-glucosidase from *Saccharomycopsis fibuligera*

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Abstract Enzyme engineering was performed to link the β-glucosidase enzyme (BGL1) from *Saccharomycopsis fibuligera* to the cellulose-binding domain (CBD2) of *Trichoderma reesei* cellobiohydrolase (CBHII) to investigate the effect of a fungal CBD on the enzymatic characteristics of this non-cellulolytic yeast enzyme. Recombinant enzymes were constructed with single and double copies of CBD2 fused at the N-terminus of BGL1 to mimic the two-domain organization displayed by cellulolytic enzymes in nature. The engineered *S. fibuligera* β-glucosidases were expressed in *Saccharomyces cerevisiae* under the control of phosphoglycerate-kinase-1 promoter ($PGK1_P$) and terminator ($PGK1_T$) and yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$). The secreted enzymes were purified and characterized using a range of cellulosic and non-cellulosic substrates to illustrate the effect of the CBD on their enzymatic activity. The results indicated that the recombinant enzymes of BGL1 displayed a 2–4-fold increase in their hydrolytic activity toward cellulosic substrates like avicel, amorphous cellulose, bacterial microcrystalline cellulose, and carboxy methyl cellulose in comparison with the native enzyme. The organization of the CBD in these recombinant enzymes also resulted in enhanced substrate affinity, molecular flexibility and synergistic activity, thereby improving the ability of the enzymes to act on and hydrolyze cellulosic substrates, as characterized by adsorption, kinetics, thermal stability, and scanning electron microscopic analyses.

Keywords Cellulose-binding domain · Cellulose hydrolysis · β-glucosidase · BGL1 · *Saccharomycopsis fibuligera* · *Saccharomyces cerevisiae*

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Introduction

Cellulolytic enzymes belong to the family of enzymes that are capable of degrading cellulose. Cellulolytic micro-organisms produce at least three major types of enzymes: endoglucanases, exoglucanases, and β-glucosidases. Endo- and exoglucanases have a conserved tripartite structure with a large catalytic core domain linked by an O-glycosylated peptide to a cellulose-binding domain (CBD), which is required for interaction with crystalline cellulose. The CBD mediates the binding of the cellulolytic enzymes to the substrate, thereby playing a fundamental role in its hydrolysis [19]. It has been suggested that the CBD enhances the enzymatic activity of cellulolytic enzymes simply by reducing the dilution effect of the enzyme at the substrate surface [16], by promoting the solubilization of single glucan chains off the cellulose surface [20], or by loosening individual cellulose chains from the cellulose surface prior to its actual hydrolysis [18].

Most information on the role of CBDs has been obtained from the removal [1], domain exchange [13] and site-directed mutagenesis of the CBDs [15], or by artificial addition of the CBD [9]. It therefore seems that the CBDs are interchangeable to a certain degree, but much more data are needed on different catalytic domain-CBD combinations to elucidate the exact functional role of the CBDs. Since there are possibly many factors at work in the interaction between the CBD and the catalytic domain, it would also be interesting to study the role of CBDs in the context of a non-cellulolytic catalytic domain, like β-glucosidase. In nature, the presence of an N-terminal CBD has been found in a β-glucosidase enzyme from the wood-degrading fungus, *Phanerochaete chrysosporium* [11]. Further, results of our previous study in which the non-cellulolytic catalytic domains of *Saccharomyces cerevisiae* exoglucanases, like EXG1 and SSG1, were used have demonstrated that the CBD has the ability to impart a catalytic role to these domains, to act on cellulosic substrates [6].

In the present study, domain engineering was performed to link the non-cellulolytic β-glucosidase domain from the *S. fibuligera* BGL1 enzyme [12] to the CBD2 of *Trichoderma reesei* cellobiohydrolase (CBHII) to investigate whether the CBDs would be able to modulate these non-cellulolytic domains to function in cellulose hydrolysis. Recombinant enzymes were constructed with single and double copies of CBD2 fused at the N-terminus of BGL1, displaying the two-domain organization, which is shown by many cellulolytic enzymes. The recombinant enzymes were expressed in *S. cerevisiae* and characterized for their catalytic and physicochemical properties using various cellulose and non-cellulose substrates.

Materials and methods

Strains and plasmids

Escherichia coli strain DH5α (F-α80d*lac*ZΔM15 Δ(*lac*-ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rK-, mK_+) *pho*A *sup*E44 *λ- thi*-1 *gyr*A96 *rel*A1) was used as an intermediate host for the cloning of the recombinant β-glucosidase gene constructs and for the propagation of all the plasmids. The *S. cerevisiae* strain CENPK42 (*MATα, leu2-3,112 ura3-52 trp1-289 his3-Δ1*) was used as a host for recombinant enzyme expression. The 2 μ multi-copy expression vector pCEL15 [6], a derivative of YEp352 [22] containing the sequences required for replication in *E. coli* and yeast, the ampicillin resistance gene, the yeast *URA3* gene, the mating pheromone α-factor secretion signal ($MF\alpha1_S$) secretion signal, and the phosphoglycerate-kinase-1 promoter ($PGK1_P$) and terminator ($PGK1_T$) were used for expression. Plasmid pEFB19 [12] was used as the source for gene coding the β-glucosidase (*BGL1*) from *S. fibuligera*. The DNA fragment coding for the CBD2 of *T. reesei* CBHII was kindly provided by Dr. P. van Rensburg, Institute for Wine Biotechnology at Stellenbosch University. Sequencing of the recombinant constructs was performed using pGEM®-T Easy plasmid (Promega, Madison, WI, USA).

Cloning, construction and transformation of the recombinant forms of BGL1

The various genetic constructs that led to the production of native and recombinant forms of the *BGL1*-encoded enzyme are described. The region of the *CBH2* gene of *T. reesei* between nucleotides 73 and 186, which encodes the CBD2, was amplified by PCR with primers containing *Hin*dIII site (underlined) at the 5′-termini of both CBD-F (5′-AAGCTTAGCGT CTGGGGCCAA-3′) and CBD-R (5′-AAGCTTAA GACACTGGGAGTAATA-3′) to facilitate multi-copy insertion into pCEL15. The amplification reaction contained standard PCR buffer (provided by Roche), 1.25 mM dNTPs, 1.0 mM $MgCl_2$, 0.3 μM of each primer, 2 ng μl^{-1} of template, and 3.5 U DNA polymerase (Roche) in a total volume of 100 μl. PCR was carried out in a PCR Express thermal cycler for 15 cycles: denaturation, 2 min at 94°C; primer annealing, 30 s at 48°C; and primer extension, 1 min at 68°C. The plasmids with a single copy and a double copy of CBD2 with the same orientation were chosen and named pCEL16 and pCEL41, respectively. The fragment of the *BGL1* gene (nucleotides 159–2,535) that encodes the mature BGL1 was amplified using the primers BGL1-F (5′-CCATCGATGTCCCAATTCA AAACTATAC-3′), containing a *Cla*I site (underlined), and BGL1-R (5′-CCGCTCGAGTCAAATA GTAAACAGGACAG-3′) containing an *Xho*I site (underlined). This PCR product was cloned into the *Cla*I and *Xho*I sites of pCEL15, pCEL16 and pCEL41, thereby generating the plasmids pCEL29, pCEL30, and pCEL31, respectively. The PCR mix and cycler program were the same as mentioned earlier, except that the annealing temperature was changed to 55°C. Standard methods were used for the isolation, restriction, purification, and ligation of DNA, the plasmid transformation into *E. coli* and agarose gel electrophoresis. Both the coding and non-coding strands were sequenced to ensure the reliable identification of all constructs (PE/Applied Biosystems, Foster City, CA,

USA). The transformation of the *S. cerevisiae* CENPK42 strain was carried out by the lithium acetate method [5] and selected on synthetic complete dextrose medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with tryptophan, leucine, and histidine.

Purification of native and recombinant β-glucosidases

Purification of the native and recombinant enzymes was performed as described by Suzuki et al. [17]. *S. cerevisiae* transformants carrying either native or recombinant BGL1 were grown in synthetic complete dextrose medium (0.67% yeast nitrogen base without amino acids, 2% glucose) at 30°C for 24 h in a rotary shaker (200 rpm). The pre-culture was used to inoculate 500 ml of YPD (1% yeast extract, 2% peptone, and 2% glucose) at an optical density (OD) of 0.1 (measured at a wavelength of 600 nm). After incubation for 60 h, the enzymes were purified from the supernatant. The culture fluid was obtained by centrifugation at 2,300 *g* for 10 min and then brought to 80% saturation by the addition of pre-chilled, saturated ammonium sulphate solution, and left overnight at 4°C. Precipitates were collected by centrifugation and dissolved in 50 mM citrate buffer at pH 6.0. The solutions were subsequently diafiltered with a membrane a 50 kDa cut-off (Amicon, TX, USA), with 50 mM citrate buffer at pH 6.0. The proteins were then separated by anion exchange chromatography, on a Bio-Rad Automated Econo system equipped with a DEAE sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ, USA), equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl and the monitored fractions showing activity were pooled. The purified enzyme was dialyzed against 50 mM citrate buffer at pH 4.8 and concentrated by ultrafiltration (100 kDa cut-off membrane, Amicon). The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), with purified bovine serum albumin (Promega, WI, USA) as the standard.

Antibody preparation

Antibodies to almond β-glucosidase (Sigma, MO, USA) were obtained, essentially as described by Bellstedt et al. [2]. Purified β-glucosidase was used for the primary immunization of the rabbit (1 ml of 1 mg ml^{-1}). Antigen boosts were performed at 3 and 5 weeks after primary immunization, and the antiserum was collected on the 28th and 42nd days.

Immunochemical identification and quantification of proteins

The purified samples of the native and recombinant β-glucosidase were concentrated using Amicon micron-10 microconcentrators. Samples were subjected to electrophoresis in tris–glycine–SDS gels and then transferred to a polyvinylidene fluoride (PVDF) microporous membrane (ImmobilonTM-P transfer membrane, Millipore, Bedford, MA, USA) by means of a standard procedure [21]. Western blot analysis was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech, NJ, USA). Blots were sequentially treated with rabbit anti-β-glucosidase (1:30,000) and donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:20,000). The HRP/hydrogen peroxide-catalyzed oxidation of luminol in alkaline conditions emits enhanced chemiluminescence. This was detected by short exposure to a blue light-sensitive autoradiography film (Hyperfilm ECL, Amersham Biosciences). Microtiter plates were coated with enzyme samples overnight at 4°C, after which the unbound material was removed and the wells were washed twice with PBS solution. The plates were blocked with 4.5% skimmed milk powder and incubated at room temperature for 1 h. The residual blocking buffer was removed and the wells were washed four times with PBS solution. The wells were subsequently loaded with PAb in blocking buffer at a dilution of 1:30,000 and incubated at room temperature for 1 h. Following incubation, the wells were washed four times with PBS. An anti-rabbit IgG whole molecule alkaline phosphatase conjugate was used to amplify the signal generated by the bound antibodies of PAbs at an optimal working dilution of 1:20,000 in the blocking buffer. The plates were incubated at room temperature for 1 h, followed by the washing steps mentioned previously. *p*-Nitrophenyl phosphate substrate (Sigma) suspended in 1 M diethanolamine buffer at pH 9.8, and containing 0.5 mM $MgCl_2$, was added to each of the wells. Absorbance at 405 nm was determined with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA). Pre-immune serum was probed with horseradish peroxidase-conjugated anti-rabbit IgG as the negative control. Standards were prepared using linear concentrations of purified β-glucosidase from almonds (Sigma).

Enzyme assays

β-Glucosidase activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenol-β-glucoside (PNPG) [11]. The enzyme solution was incubated with 1 mM PNPG in 50 mM phosphate buffer,

pH 4.5, at room temperature. One unit of β-glucosidase activity is defined as that amount of enzyme that will hydrolyze 1 μmol of PNPG per minute. Enzyme equivalent to 10 units of PNPG activity was used further in the hydrolytic activity assays. The β-glucosidase activity toward the cellobiose substrate was determined by assaying the amount of free glucose released. The reaction mixture contained 50 μl of 1% cellobiose (Sigma) stock solution in 50 mM citrate buffer at pH 4.8 and the enzyme preparation. After 1 h of incubation at 37°C, the free glucose released was measured using the Trinder glucose assay kit (Sigma).

The hydrolytic activity on various cellulosic substrates was assayed using the two-step standard assay with little modification [3]. Reactions were carried out in 2-ml, O-ringed screw-cap, micro-centrifuge tubes containing the enzyme preparations and the commercial enzyme endoglucanase EGII (*Trichoerma longibrachiatum*) in a reaction volume of 750 μl, with 1.0 g l^{-1} of substrate [Avicel: amorphous, bacterial micro-crystal cellulose (BMCC) or carboxy methyl cellulose (CMC)] in 50 mM sodium citrate buffer (pH 4.8). The reaction mixtures were incubated at 50°C with end-over-end rotation at 40 rpm for 16 h. The samples were filtered through 0.45 μm cellulose acetate filters (Amicon, TX, USA) and analyzed for reducing sugar. Each reaction was done in triplicate. One unit of hydrolytic activity was expressed as 1 μmol of reducing sugar released per minute. The hydrolytic assays on cellooligosaccharide and cellopentose were performed under similar conditions as mentioned above, except that the hydrolytic mixtures contained only the native or the recombinant enzymes.

Preparation of substrates

Avicel and amorphous cellulose were purchased from FMC Co., Philadelphia, USA. CMC, laminarin and barley β-glucan were obtained from Sigma. BMCC was obtained from Cellulon (Weyerhaeuser, WA, USA). Avicel, amorphous cellulose and BMCC were weighed, washed with distilled water and filtered as described by the supplier. Cellooligosaccharides were purchased from Sigma.

HPLC analysis of hydrolysis products

The HPLC analysis of the products of hydrolysis was performed using a refractive index detector (Agilent Technologies, Santa Clara, CA, USA). The column used for separation was an Aminex HPX-87H column (Bio-Rad). Analyses were carried out at 30°C using 6 mM of sulphuric acid as the mobile phase, at a flow rate of 0.8 ml min^{-1}.

Adsorption assays

Adsorption assays were performed in a similar manner as described by Gal et al. [4]. Enzyme preparations of native and recombinant enzymes were incubated with 1 g l^{-1} of Avicel in 50 mM sodium citrate buffer (pH 4.8), in a final reaction volume of 750 μl, in an end-over-end rotation at 40 rpm. The incubation temperature was maintained at 4°C for 16 h and the preparations were then centrifuged at 5,000 *g* for 15 min. In the binding assays, the free protein fraction was estimated from the residual enzymatic activity in the supernatant. This was subtracted from the initial enzyme activity to calculate, approximately, the substrate-associated fraction.

Kinetic parameters

The Michaelis–Menten parameters, V_{max} and K_m, were determined for all the recombinant enzymes from Michaelis–Menten plots of specific activities for 6–10 concentrations of Avicel. The rates were measured in triplicate, and ranged from 0.2 to 5 times the value of K_m. The values of V_{max} and K_m were determined by non-linear regression analysis using the graph pad prism program.

Temperature and pH profiles

The temperature profiles for the native and recombinant enzymes were measured at temperatures between 30 and 60°C at pH 4.8, using 1 g l^{-1} cellobiose in 50 mM of sodium citrate buffer. The effect of pH on the activity of the native and recombinant enzymes was investigated in the pH range 2–7 at 50°C, under similar assay conditions.

Thermostability

The native and recombinant enzymes were incubated in 50 mM of sodium citrate buffer (pH 4.8) at temperatures between 30 and 70°C for 30 min, and snap-cooled immediately in ice. The remaining activity was measured at 50°C, using cellobiose as substrate.

Sample preparation for scanning electron microscopy (SEM)

The electron micrographs of the Avicel particles were taken after 16 h of digestion at 50°C at a pH of 6, with the native or recombinant enzymes added together with the commercial preparation of the *T. longibrachiatum* endocellulase. The preparation of the Avicel samples

treated with the recombinant enzyme preparations was performed as described previously [3]. The specimens were examined using a fully analytical S440 Leo Scanning Electron Microscope (SEM) operated at an accelerating voltage of 10 kV. All the images were visualized at a magnification of 1.00 k×.

Results

Construction and expression of recombinant enzymes

All the recombinant molecules that were constructed were cloned into the yeast expression plasmid and transformed into the *S. cerevisiae* CENPK42 strain. The native and recombinant enzymes of BGL1 were successfully expressed in *S. cerevisiae* and purified from the culture supernatant. The purified enzymes were analyzed and confirmed by SDS-PAGE gel and immunoblotting (Fig. 1). The following molecular weights were determined: 220 kDa for the native enzyme [12] and approximately 225 and 230 kDa (theoretical values) for the recombinant enzymes (Fig. 1).

Hydrolytic activity of the recombinant enzymes

The recombinant BGL1 enzymes were assayed for their ability to hydrolyze β-1.4 linkage-specific cellulosic substrates (Table 1). It was observed that the recombinant CBD2-BGL1 enzyme exhibited the highest rate of hydrolysis on all the celluloses—approximately fourfold higher than the mix containing the native enzyme. The recombinant CBD2-CBD2-BGL1 enzyme showed an activity that was approximately twofold higher than that of the native enzymes. Interestingly, on native cellobiose substrate, the native BGL1 enzyme and the recombinant CBD2-BGL1 exhibited comparable hydrolytic activity, but the activity of CBD2-CBD2-BGL1 was decreased more than twofold. No significant background activity was measured for the CBD2 and CBD2-CBD2 constructs.

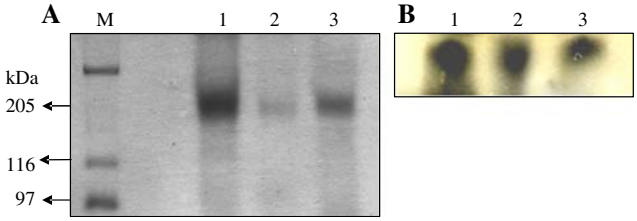

Fig. 1 Analysis of purified native and recombinant β-glucosidase enzymes by SDS–PAGE (**a**) and Western blot (**b**). *Lane M* broad range protein marker, *Lane 1* native BGL1, *lane 2* CBD2-BGL1 and l*ane 3* CBD2-CBD2-BGL1

The native and recombinant enzymes of BGL1 displayed different hydrolytic product profiles on the cellooligosaccharide mix [Table 2 (part a)]. The percentage of soluble product released summarizes the improved performance of hydrolysis by the recombinant enzymes CBD2-BGL1 and CBD2-CBD2-BGL1 to act on longer chain, β-1.4-specific substrates. They showed threefold and twofold-higher activity, respectively. Although the native BGL1 enzyme released glucose as its major hydrolytic product, most of glucose was derived from the hydrolysis of cellobiose in the mix. However, the wild-type BGL1 showed less significant activity on the cellotriose substrate, which corresponds with the data published by Machida et al. [12]. The pattern of hydrolysis shown by the native and recombinant enzymes on cellopentose was comparable with the profile shown in cellooligosaccharides [Table 2 (part b)]. Glucose is the major hydrolysis product of hydrolysis for CBD2-BGL1, and CBD2-CBD2-BGL1 generated cellobiose as the major hydrolytic product. The profiles of the recombinant enzymes also revealed a sequence in which cellopentose was hydrolyzed into cellotriose, cellobiose, and glucose, respectively. The sequential hydrolysis of cellooligosaccharides, leading to the release of glucose as the major hydrolytic product, illustrates the bi-functional β-glucosidase, and β-1.4-exoglucanase activity by the recombinant enzymes.

Table 1 Hydrolytic activity of the recombinant enzymes on different substrates

Enzyme	Reducing sugars released[a] (μmol min^{-1} U^{-1})				
	Cellobiose	Avicel	Amorphous	BMCC	CMC
BGL1	0.70 ± 0.049	0.015 ± 0.001	0.055 ± 0.005	ND	ND
CBD2-BGL1	0.70 ± 0.068	0.068 ± 0.007	0.230 ± 0.015	0.051 ± 0.004	0.044 ± 0.003
CBD2-CBD2-BGL1	0.26 ± 0.023	0.032 ± 0.003	0.170 ± 0.014	0.019 ± 0.001	0.027 ± 0.002
CBD2	ND	ND	0.021 ± 0.001	ND	ND
CBD2-CBD2	ND	ND	0.019 ± 0.001	ND	ND

ND not detected

[a] One unit of enzyme is equivalent to the amount of purified protein preparation required to hydrolyze 1 μmol of PNPG per minute under the assay conditions mentioned

Table 2 HPLC analysis of the hydrolysis products using cellooligosaccharide mix (part a) and cellopentose (part b) as substrate

Enzyme	Hydrolysis products (g l^{-1})				Soluble products[a]
	Glucose	Cellobiose	Cellotriose	Cellopentose	
Part a					
BGL1	0.086	0.009	0.031	0.091	21
CBD2-BGL1	0.290	0.095	0.006	0.220	61
CBD2-CBD2-BGL1	0.140	0.150	0.008	0.120	42
Substrate control	ND	0.016	0.064	0.100	18
Part b					
BGL1	0.11	0.015	ND	0.88	
CBD2-BGL1	0.32	0.170	0.02	0.52	
CBD2-CBD2-BGL1	0.14	0.240	0.10	0.59	
Substrate control	ND	ND	ND	1.00	

The presented values are the average of two independent measurements, which varied by less than 15%

ND not detected

[a] Percentage of soluble products is measured only in relation to the total soluble products (18%) analyzed above, from the mix of cellooligosaccharides of 1 g l^{-1}

Adsorption as a function of hydrolysis

Table 3 summarizes the measured binding capability of the different peptides. The data showed that the percentage of adsorption of the recombinant enzymes, CBD2-BGL1 and CBD2-CBD2-BGL1, toward Avicel increased 10- and 16-fold, respectively, compared with the wild-type BGL1. In addition, the net adsorption of the recombinant enzyme, CBD2-BGL1, was noticed to increase until 16 h, after which a total desorption was observed (data not shown). However, the chimera CBD2-CBD2-BGL1 did not show any desorption at that point.

Kinetic properties of the recombinant enzymes

The recombinant enzymes CBD2-BGL1 and CBD2-CBD2-BGL1 actively catalyzed the hydrolysis of Avicel (Table 3). CBD2-BGL1 yielded approximately twofold higher V_{max} values for the Avicel substrate than the recombinant enzyme CBD-CBD2-BGL1, although it displayed a similar K_m. As for the wild type BGL1, the values for both K_m and V_{max} were significantly poorer than the recombinant enzymes.

Table 3 Adsorption and kinetic parameters of the recombinant enzymes analyzed using Avicel as substrate

Enzyme	Adsorption (%)	Kinetic parameters	
		V_{max} (μmol min^{-1})	K_m (g l^{-1})
BGL1	3	0.09 ± 0.01	1.11 ± 0.15
CBD2-BGL1	29	0.30 ± 0.04	0.22 ± 0.03
CBD2-CBD2-BGL1	51	0.17 ± 0.01	0.25 ± 0.03

Experiments were performed in triplicate and the standard deviation was less than 10%

Temperature and pH profiles

To investigate the pH- and temperature-dependent activity profiles of the recombinant enzymes, the profiles were investigated at pH 2–7 and temperatures of 30–60°C, respectively (Fig. 2a, b). The data illustrates a diverse trend of hydrolytic pattern between the native enzyme BGL1, and the chimeric enzymes CBD2-BGL1 and CBD2-CBD2-BGL1. The native enzyme BGL1, tested for activity showed a quick increase in activity through pH 3–5 reaching the highest activity at pH 5 followed by a very slow decline between pH 5–7. While the chimeric enzymes with single and double CBD's at the N-terminus displayed a diverse trend in their hydrolytic performance attaining their optimum activity at a range between 5 and 6, followed by a sharp decline in their activity from pH 6–7.

The temperature profile shows that all the chimeric enzymes exhibit a similar activity profile at all the temperatures with the highest activity measured at 50°C. The hydrolytic activity data of the native and hybrid enzymes showed in common, a rapid increase of ~40–50% in activity from 30 to 50°C, followed by a rapid decrease from 50 to 60°C accounting to ~30–40% of the maximum obtained.

Thermostability

An investigation of the thermal denaturation kinetics of the enzymes showed that, while the native BGL1 enzyme showed a steady loss of activity after incubation at 40°C, the recombinant enzymes, CBD2-BGL1

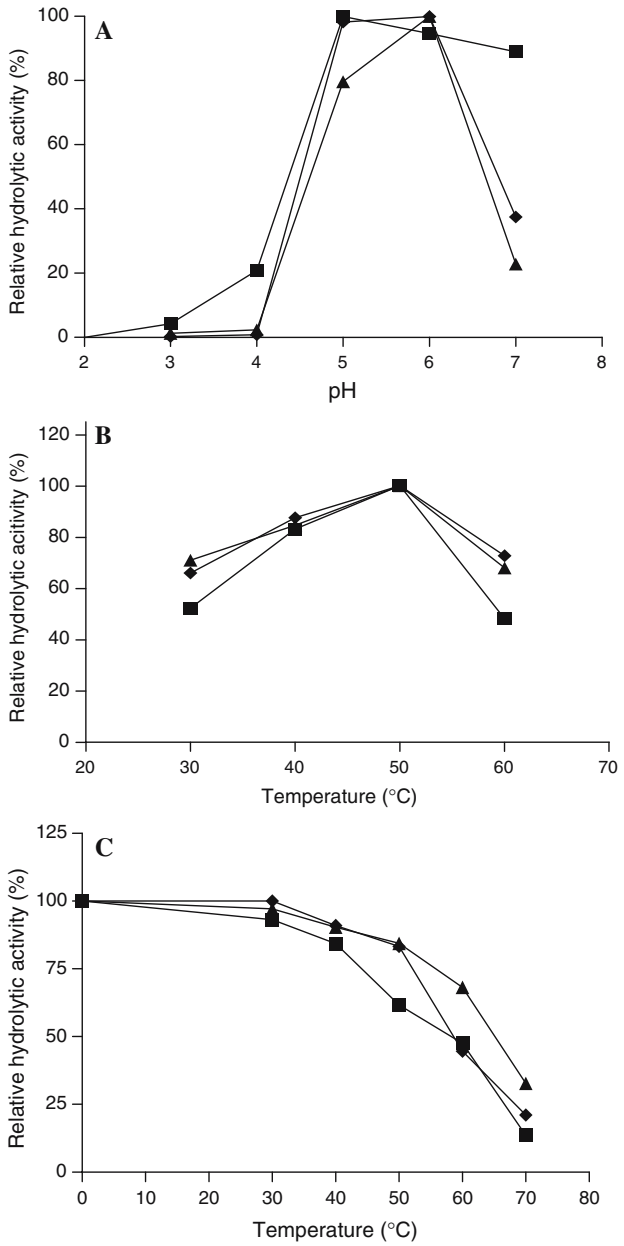

Fig. 2 The pH (**a**) and the temperature optimum (**b**), and the thermal stability (**c**). BGL1, *filled square*; CBD2-BGL1, *filled diamond*; CBD2-CBD2-BGL1, *filled triangle*. Average values from at least three independent experiments are presented and the standard deviation was less than 10%

and CBD2-CBD2-BGL1, were stable until 50°C (Fig. 2c). It was also noted that the stability of CBD2-BGL decreased significantly, to ~50% of its maximum activity, from 50 to 70°C, whereas the stability of the CBD2-CBD2-BGL1 decreased gradually.

Scanning electron microscopy (SEM)

The morphological modification imparted to Avicel cellulose particles by the recombinant enzymes in comparison with that of the native forms is illustrated in the series of electron micrographs displayed in Fig. 3. Figure 3b is the result of digestion with the native BGL1 enzyme and EGII mixture, where the surface of the cellulose particle is clearly distorted in comparison with that of the untreated sample (Fig. 3a). In Fig. 3c, d, corresponding to the hydrolytic data of the CBD2-BGL1 and CBD2-CBD2-BGL1 enzymes, a dramatic increase in digestion is clearly noticed on the surface, as reflected by meshy, loosened and tiny, micron-long particles in the case of CBD2-BGL1 and by more brittle and larger particles in the case of CBD2-CBD2-BGL1. A significant morphological difference is also noticed between the samples, with the particles in Fig. 3d solubilized more at the ends than on the surface, unlike the samples treated with CBD2-BGL1 where Avicel was completely digested.

Discussion

The results reported in this article suggest that the CBD coupled to a non-cellulolytic β-glucosidase enzyme modulates its ability to act and hydrolyze cellulosic substrates. The ability of the CBD to modify the role of non-cellulolytic catalytic domains to act on cellulosic substrates has previously been demonstrated using *S. cerevisiae* exoglucanases like EXG1 and SSG1 [6]. The data from this study also clearly suggest that the degree of hydrolysis and mode of action of these recombinant enzymes differ with regard to the number of copies of the CBD. The ability of these enzymes to sequentially hydrolyze the cellooligosaccharides to free glucose demonstrates their bi-functional activity. The presence of the CBD in the recombinant enzymes facilitated their binding to the surface of the substrates, thereby promoting better stability and hydrolysis [8]. The increased affinity of the recombinant enzymes toward the cellulosic substrate clearly demonstrates this aspect. The CBD, as has been reported previously, would also have enhanced the hydrolysis simply by reducing the dilution effect of the enzyme at the substrate surface, thus promoting the solubilization of single glucan chains from the cellulose surface [16, 20]. The lower rate of hydrolysis observed for the double-copy CBD enzyme probably reflects the fact that, although they exhibited the greatest adsorption ability, the fusion of multiple CBDs might have translated themselves into misfolding of the recombinant molecule and consequent distortion of the 3D conformation. In support of this, the kinetic data show that the maximum hydrolytic activity of this recombinant enzyme is significantly reduced in comparison with

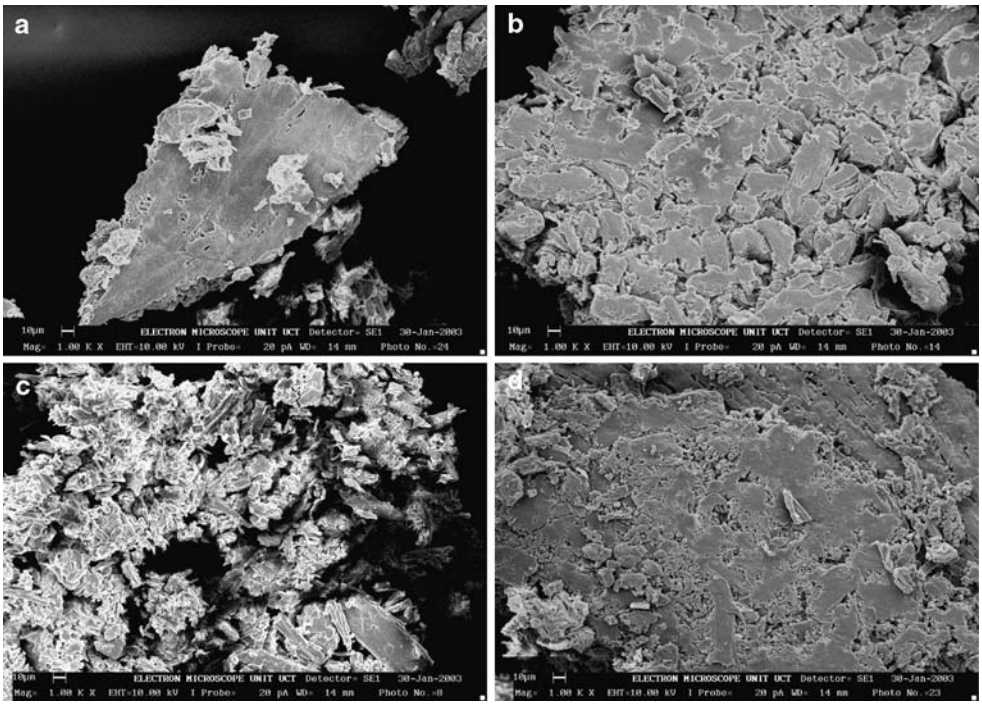

Fig. 3 SEM images of the Avicel cellulose particles after 16 h digestion by the recombinant enzymes of BGL1. Untreated (**a**), BGL1 (**b**), CBD2-BGL1 (**c**), and CBD2-CBD2-BGL1 (**d**)

that of its counterpart with single-copy CBD. Further, the reduced activity of the double CBD recombinant enzyme could also be attributed to the lack of processing of this enzyme in *S. cerevisiae* in comparison with the single CBD enzyme. This would also explain the reduced native cellobiase activity of the double CBD enzyme. Since the isolated CBDs did not contribute toward the hydrolysis of cellulose, it could be suggested that the ability of the recombinant molecules to act on cellulose was rather a co-operative interaction between the catalytic domain and the CBD. Such interactions between the domains have previously been demonstrated in the cellulolytic enzymes from *T. reesei* [14].

The release of glucose as the major hydrolytic product of cellooligosaccharides by the recombinant enzymes suggests that, while the hydrolysis product of the modulated exo-cellulase activity was cellobiose, the native activity of β-glucosidase subsequently converted the cellobiose to glucose. The presence of trace amounts of cellotriose as a transient by-product in the hydrolysis of both cellooligosaccharide and cellopentose suggests that the presence of the CBD increased the affinity of the recombinant enzymes for this substrate. The increase in the concentration of cellopentose in the case of cellooligosaccharide hydrolysis also places it alongside cellotriose as a transient product, although the affinity toward this substrate is lower.

The recombinant enzymes, such as the thermophilic enzymes, gained sufficient molecular flexibility for improved thermostability [10]. Our data suggest that the CBD endowed the recombinant enzymes with this thermostable property, and this reiterates the designation of CBDs as "thermostabilising domains" [7]. Indeed, the characterization of a novel cellulose-binding β-glucosidase from cellulose-degrading cultures of *P. chrysosporium* assigned a thermostabilising role to the CBD [11].

The substantial reduction in the size and morphology of the hydrolyzed Avicel particles, as shown by SEM, indicates synergistic activity between the recombinant enzyme and EGII enzyme present in the hydrolytic mix. The contribution of the CBD to synergistic activity has already been reported for free cellulolytic enzymes from *T. reesei* and *Humicola insulans* [14, 3]. This finding also illustrates that recombinant enzymes primarily engage in a dominant exo-processing mode of action, as shown by the pattern in hydrolysis. As demonstrated, the engineered enzymes should have undergone a dynamic process of binding and desorption, allowing successive hydrolysis and/or relocation to new, enzymatically accessible sites on the Avicel cellulose surface, as has been suggested for the cellulolytic enzymes boarding different CBD modules [8]. The structure of these enzymes might thus be balanced between the requirement for stability and that for

dynamics. The fragmentation observed on the surface of the particles can be clearly attributed to the endocellulase action of the EGII enzyme in the mix.

In conclusion, results of the present study suggest that the engineered β-glucosidase enzymes would have gained sufficient stability and affinity for cellulosic substrates, thus achieving significant hydrolytic efficiency in optimized enzymatic mixtures. It is also clear that the CBD sequence plays the role of a thermostabilising domain in the engineered enzymes. A more detailed understanding of these recombinant enzymes, such as the elucidation of the 3-D structure, will enable us to illustrate the specific role and function of the CBD in these enzymes. Further, this should facilitate the development of efficient engineered enzymes adapted to bioprocess-efficient microbial systems.

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