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A simple method to separate cellulose-binding domains of fungal cellulases after digestion by a protease

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Abstract

Core-binding domains of fungal cellulases from *Trichoderma reesei* were purified using a new and simple technique. Cellulases were hydrolysed with papain and the binding domains were then separated from the digested mixture by ultrafiltration. The enzymatic digestion process was monitored using capillary electrophoresis. This methodology produced a yield of 85% of binding domains.

Introduction

The purification and immobilisation of biologically active proteins is an area of great importance in both research and industry. Most protein immobilisation methods require a chemical modification of the matrix, which at times can result in the introduction of toxic compounds. Moreover most of the matrices employed are expensive materials. The choice of cellulose as an immobilisation matrix is attractive as it offers an inexpensive and inert alternative. This explains the recent attention on promoting the use of cellulose-binding domains (CBDs), which have a natural ability to bind to cellulose, as important 'tools' in biotechnology. In relation to protein-based application areas, the CBD genes can be expressed alone or in combination with other genes of interest to produce recombinant or fusion proteins. These proteins can be purified or immobilised on cellulose matrices. Many CBD fusion proteins have been produced, such as CBD-alkaline phosphatase (Greenwood *et al.* 1989), CBD-β-glucosidase (Ong *et al.* 1991), CBD-proteinA (Ramirez *et al.* 1993) and more recently CBD-heparinase I (Shpigel *et al.* 1999). Furthermore, the use of CBDs in textile and paper industry can be advantageous as CBDs may have potential applications in the modification of polysaccharide fibres, for instance in cotton, wood or paper (Din *et al.* 1991). The majority of the information concerning the role of CBDs was gathered by the use of domain exchange (Srisodsuk *et al.* 1997), domain removal (Van Tilbeurgh *et al.* 1986), or site-directed mutagenesis (Reinikainen *et al.* 1992, 1995, Mattinen *et al.* 1997).

The fungus *Trichoderma reesei* produces several cellulases (Knowles *et al.* 1987), which show a similar structural organisation, i.e., a catalytic domain (or core domain), which is connected by a glycosylated linker to a core-binding domain (CBD) located at either the N or C terminal of the protein (Van Tilbeurgh *et al.* 1986, Abuja *et al.* 1988). All these CBDs consist of 36–40 amino acids with very closed related sequences (Linder *et al.* 1995). Most of the CBDs investigated in recent years were obtained by genetic engineering, however the separation of the two domains has also been achieved using proteolysis (Van Tilbeurgh *et al.* 1986, Tomme *et al.* 1988, Woodward *et al.* 1992, 1994). In these cases the interest in proteolysis was more directed to the isolation of catalytic cores of cellulases.

In this work we have developed a simple method to separate and isolate the binding domains from cellulases (in this case *Trichoderma reesei*) using proteolysis.

Materials and methods

Enzymes

A crude cellulase preparation (Celluclast, Novo Nordisk) was diluted (4×) in sodium acetate buffer (50 mM, pH 5), washed in an ultrafiltration cell (Amicon) with a 10-kDa nominal weight cut-off, polysulphone membrane to remove low molecular weight compounds.

Proteolysis was done with papain (papaya latex, Sigma). The protein concentration in the cellulase preparation was determined using BCA Protein Assay Kit (Pierce).

Digestion conditions

Different cellulase to papain (w/w) ratios were taken, as well as duplicates for each experiment. The digestion was performed at room temperature (∼20 °C) for a maximum of 4 h. At intervals samples were checked for enzymatic activity and analysed using capillary electrophoresis (CE). Two digestions were performed (ratios 300:1 and 50:1) with the digested mixture ultrafiltrated through both 10-kDa and 30-kDa nominal cut-off membranes to assure the separation of the cellulose-binding domains (CBDs) from the digested mixture.

Enzymatic measurements

Carboxymethylcellulose-CMC (1% w/v) was used as soluble substrate and filter paper (Whatman no. 1, 3.9 mg) as insoluble substrate. The amount of sugar released was measured by the dinitrosalicylic acid method. Papain activity was assessed by QuantiCleave Protease Assay Kit (Pierce).

Capillary electrophoresis

Protein separations were performed using a BioRad, BioFocus 2000 with an uncoated capillary (total length 37 cm and 50 μm internal diameter). Electrophoretic injection was applied to the sample at 10 kV for 5 s, from negative to positive polarity. The applied voltage was 15 kV and the temperature was 20 °C. Protein fractions were separated by molecular weight and detected by monitoring the absorbance at 220 nm.

For peptide analysis a coated capillary (total length 24 cm and 25 μm internal diameter) was used. Injection of the samples (filtrates) was done by pressure. The run voltage was 10 kV and the absorbance was measured at 200 nm.

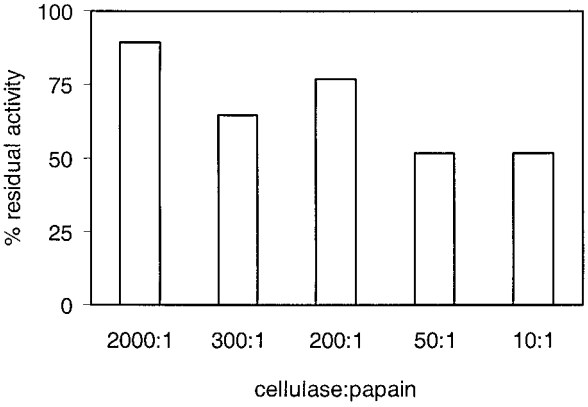

Fig. 1. Effect of hydrolysis on the FPase activity after 4 h digestion for different ratios cellulase:papain (w/w). Residual activities are given as percentages of the original.

Adsorption assays

Purified CBDs were incubated at room temperature (∼20 °C) for 1 h with agitation. Samples were taken to evaluate the percentage of protein adsorbed into cellulose (Sigmacel, type 101–30 mg ml^{-1}). The depletion of the protein was analysed by monitoring the decrease in absorbancy at 280 nm, after the centrifugation of the suspensions.

Results and discussion

Optimisation of digestion conditions

The effect of proteolysis on the enzyme activity and on the electrophoretic profiles is described below. Initially the best enzyme:cellulase ratio was determined.

Effect of proteolysis on the enzyme activity

The papain-digested solution was found to maintain its activity against carboxymethycellulose in all the papain:cellulase ratios studied indicating that the catalytic activity was preserved.

A decrease of activity was observed when an insoluble substrate (filter paper) was used. The FPase activity decreased with incubation time when papain was employed, with a greater decrease at higher papain concentrations (Figure 1). These results suggest that the protein cannot adsorb to the solid substrate surface and therefore the cellulases were split into

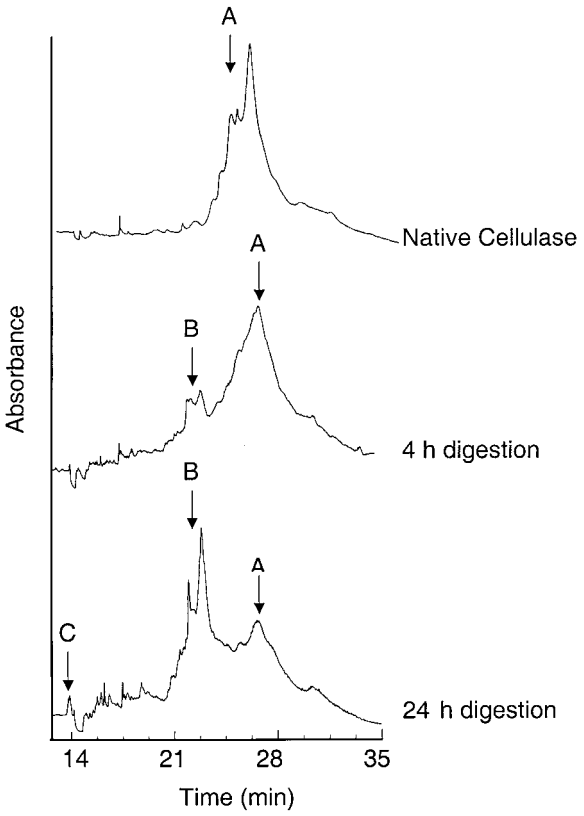

Fig. 2. Evolution of the digestion followed by capillary electrophoresis, using the uncoated capillary (20 °C), for the digestion 300:1. The native cellulase, band A, is hydrolysed by papain giving rise to catalytic domains, band B, and the binding domain, band C.

both the CBD and catalytic domain. This is in agreement with other published results (Van Tilbeurgh *et al.* 1986, Tomme *et al.* 1988).

Electrophoretic profiles of cellulases and digested mixtures

The protein fractions were separated by molecular weight and an internal standard was used to correct migration times. When the high ratio cellulase:papain (2000:1) was used the papain concentration was too low to attack the cellulases, as the cellulase profile pattern did not change. However, for the lower ratios, it was evident that the cellulases were split in protein fractions with smaller molecular weights, as a result of proteolysis. It is interesting to compare the different electropherograms obtained with the digestion ratio of 300:1, where it is possible to follow the digestion process with time (Figure 2). On the first electropherogram there is one main broad band (A). Part of the protein corresponding to this band is broken (because of the hydrolysis by the papain) leading the appearance of a second band (B) after 4 h of digestion. It can be seen that this 'new' protein has lower molecular weight (as the migration time is proportional to molecular weight). After digestion for 24 h a further increase in B with a concomitant decrease in A was observed and a third band (C) can also be seen in the electropherogram. This is explained by the fact that the cellulases (A) were split in the catalytic domains (B) and binding domains (C). The latter fact is confirmed in the forthcoming section.

Generally, if we look at the effect of the papain on the enzyme activity we can distinguish three main types of digestion: (I) corresponding at very light digestion (2000:1), (II) medium light digestion (300:1 and 200:1), and (III) heavy digestion (50:1 and 10:1). In the very light digestion the effect of papain on the enzyme activity is not noticeable even after several days. The medium and heavy digestions seem to be the optimal ratios in order to obtain the CBDs. In these conditions the papain activity led to a decrease of the enzyme activity towards filter paper, meaning that the two cores were split. Considering this, and in order to separate the CBDs from the digested mixture, two digestions in medium and heavy conditions were performed and the CBD separation by ultrafiltration was assayed.

Separation of CBDs by ultrafiltration

The digested mixtures were ultrafiltrated using membranes with nominal cut offs of 10 kDa (PM10) and 30 kDa (PM30). The same mixture was ultrafiltrated several times to obtain the maximum yield of CBDs. Enzymatic measurements, electrophoresis analysis and adsorption tests were performed to confirm the presence of CBDs and to evaluate the extent of the proteolysis.

Enzymatic activity

CMCase and FPase activities were determined for the digested mixture and filtrates. In the digested mixture the CMCase activity was maintained, while a decrease of FPase activity was observed (as seen previously). The filtrates obtained with the PM10 membrane did not show any hydrolytic activity, however CMCase activity was detected in the filtrates obtained with PM30 membrane showing that some catalytic cores managed to pass through the PM30 membrane.

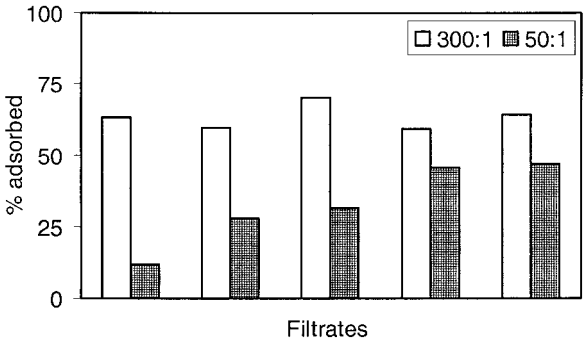

Fig. 3. Adsorption of the different filtrates obtained sequentially from the 300:1 and 50:1 digestions (using the PM10 membrane) into cellulose. The % adsorbed was obtained by the difference in absorbancy (280 nm) in the filtrate and the supernatant (obtained by centrifugation after incubation of the filtrate with cellulose, 30 mg ml^{-1}, for 1 h at ~20 °C).

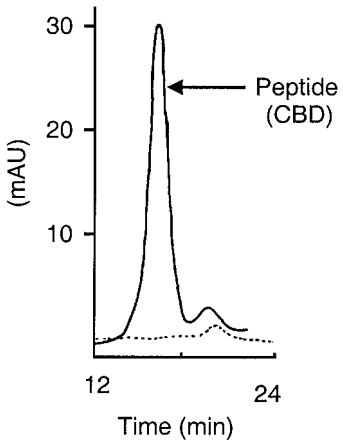

Fig. 4. Comparison of electropherogram profiles of a filtrate (—) and supernatant (- - -), obtained by the peptide analysis with coated capillary. The supernatant was obtained by centrifugation after incubation of the filtrate with cellulose (30 mg ml^{-1}) for 1 h at ~20 °C.

Electrophoretic profiles (filtrates)

The purity of the filtrates was checked using CE. The results obtained displayed one peak corresponding to a protein with a molecular weight close to 9 kDa. The retention time obtained confirms that peak C in Figure 2 is likely to correspond to this peptide. The molecular weight of the core-binding domain of fungal cellulases is close to 5 kDa, however the peptide released can be heavily glycosylated thereby increasing the peptide molecular weight to about 10 kDa (Tomme *et al.* 1988). Thus, it appears that we have obtained a glycosylated CBD.

Adsorption tests

The affinity of the native cellulase and the proteolysed mixture for cellulose was evaluated. A significant reduction of affinity towards cellulose occurred when the enzyme was submitted to proteolysis. Native enzyme showed an adsorption of 71%, while when using the digested mixture at different ratios it was found that the lowest ratio (50:1) gave the lowest adsorption (41%, cf. 51%, for the ratio 300:1).

The filtrates obtained showed an absorbance at 280 nm, indicating that proteic material was present. In order to prove the presence of CBDs adsorption tests were performed. Figure 3 shows the percentage of adsorption for the filtrates, obtained with a PM10 membrane, from the digestion ratios 300:1 and 50:1. Adsorption studies of the filtrates obtained with the PM30 membrane were not performed since a residual catalytic activity was found in these filtrates.

The filtrates obtained from the 300:1 ratio adsorbed in a higher percentage than the ones from the 50:1 digestion. This can be explained by the fact that the higher concentration of papain can lead to the destruction and/or damage of the peptide chain giving rise to a loss of amino acids which, as proved by Reinikainen *et al.* (1992) and Linder *et al.* (1995), are important in the binding of the CBDs to cellulose.

To further ascertain the purity of the filtrate, another capillary electrophoresis method, in which the migration time depends on both the charge and molecular weight, was performed. A typical electropherogram is shown in Figure 4 displaying a dominant peak, thus the peptide appears reasonably pure. To confirm that this peak represents the peptide corresponding to CBDs, microcrystalline cellulose was added to CBDs solutions. The supernatant was analysed by the same peptide analysis method and, as a reduction in the main peak was observed (Figure 4), we suggest that this peak corresponds to the CBD peptide.

Conclusion

This simple technique can be used to isolate these CBDs, which can have further application in the paper and textile industries. A ratio of 300:1 (cellulase:papain) was found to be the best ratio to perform the proteolysis. By considering that CBDs contribute for 10% of initial mass and calculating the concentration of the CBDs in the filtrates using the molar extinction coefficient 5545 cm^{-1} m^{-1} (Linder *et al.*

1995) we may conclude that these conditions give rise to yield 85% of CBDs. This methodology can be extended to quickly obtain CBDs from different microbial sources.

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