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Exo- and endo-glucanolytic activity of cellulases purified from *Trichoderma reesei*

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Four cellulases, produced by *Trichoderma reesei*, have been purified by preparative isoelectric focusing (Rotofor), size exclusion (Sephacryl 100 HR), anionic (Mono Q) and cationic (Mono S) chromatography and chromatofocusing (Mono P). Enzymatic activity with a large number of substrates allowed the proteins to be classified as: cellobiohydrolase I, cellobiohydrolase II, endoglucanase I and endoglucanase II. The exo- or endo-glucanase character of these enzymes was analysed by using a technique based on the measurement of the Avicel insoluble fibres reducing power.

Introduction

Cellulases are enzymes which breakdown cellulose to glucose. This hydrolytic process involves several steps requiring different enzymes, which work together synergistically. The presence of minor contaminants may influence significantly the reaction kinetics (Reinikainen *et al.*, 1995). Furthermore, the potential use of cellulases for oligosaccharide synthesis requires highly purified cellulases (Gama and Mota, 1998). Therefore, highly purified enzymes are necessary for biochemical work. Considerable work has been done on the purification of cellulases, and particularly on the purification of cellulases from *Trichoderma reesei*. Conventional downstream processing techniques have been used and cellulase genes have been cloned and expressed in yeasts. In the first case, the protein microheterogeneity, aggregation of the proteins in solution, difficulties in effectively distinguishing the enzymes by their specific activity, and the similarity of the physical properties of some of the proteins, make it difficult to obtain homogeneous enzymes (Reinikainen *et al.*, 1995). On the other hand, with the use of molecular biology, homogeneous proteins may be obtained, but their molecular weight, level of glycosilation and specific activity do not always compare with the native ones (Arsdell *et al.*, 1987).

The purification strategy tested in this work consisted of two first fractionation steps, which led to low grade purification of four cellulases. Since high loads are possible in the first step (preparative isoelectric focusing), the purification may be achieved with good yields at this stage. A third purification step provides highly purified enzymes.

The methodologies utilised for the measurement and characterisation of the cellulolytic enzymes evolved over the past 30 years from the utilisation of complex substrates, like filter paper, cotton and carboxymethylcellulose (CMC), to more homogeneous and simple substrates like bacterial cellulose and chromogenic soluble compounds (Nidetzky and Claeyssens, 1994). However, the exo/endo character of these enzymes is still a matter of controversy. With the objective of identifying more realistically the endoglucanolytic character of a cellulase, a new method was used to measure the degree of polymerisation, based on the measurement of the reducing power of the insoluble fibres (Gama *et al.*, 1991; 1993) which has also been used by other authors (Irwin *et al.*, 1993; Stahlberg, *et al.*, 1993; Sineiro *et al.*, 1995). This technique was applied in the current work to characterise the purified enzymes.

Materials and methods

Purification of cellulases

The commercial enzyme prepared from *Trichoderma reesei*, Celluclast (Novo), was purified in an isoelectric preparative system, Rotofor (BioRad). The enzyme was diluted (10 ml:110 ml) and dialysed against distilled water (final concentration: 450 mg protein/55 ml). Afterwards, 1.2 ml ampholyte was added (pH range: 4–6). A constant power of 12 W was applied to the Rotofor cell for 3.5 h.

The automatic collection system of Rotofor allowed the separation of 20 fractions which were frozen at −70 °C till

the next purification step. The fractions were pooled and, after pH and enzymatic activities analysis as described in the Results and Discussion section, were further fractionated using Fast Protein Liquid Chromatography (see Figure 1).

Size exclusion chromatography
A sample of 2.5 ml was injected in a XK 26 chromatography column (Pharmacia) containing Sephacryl 100 HR. The eluent was 25 mM TrisHCl, pH 8.0.

Anionic chromatography
Samples were previously equilibrated with 25 mM TrisHCl with pH 8.0 buffer, which was also the eluent used at 0.75 ml/min. The proteins were eluted through a Mono-Q HR5/5 column, with a linear gradient of 1 M NaCl (0–40% in 40 minutes).

Cationic chromatography
A Mono-S HR5/5 column was used with 20 mM sodium dimethylglutarate (pH 3.0) as eluent at 0.75 ml/min. The proteins were eluted with a linear gradient of 1 M NaCl (0–40% in 40 minutes).

Chromatofocusing
A Mono-P HR 5/20 column was equilibrated with 25 mM TrisHCl (pH 8.0) buffer. The eluent was a solution of Polybuffer 74 (Pharmacia), diluted 1:10.

Isoelectric point and molecular weight determination

To determine isoelectric points, molecular weights and simultaneously access the purification effectiveness, analytical isoelectric focusing and electrophoresis with polyacrylamide gel (SDS-PAGE) were executed in a Phast-System, according to standard procedures.

Assays for the characterisation of the enzymatic activity

All the assays were done in acetate buffer 50 mM, pH 5.0. One unit of enzymatic activity (U) corresponds to the release of 1 μmol of glucose equivalents per minute. The analytical procedures are described below.

CMCase
The enzyme was added to 0.5 ml of CMC 10.0 g/l solution, in a test tube. The reaction medium was then incubated for 1 h at 50 °C and the reducing sugars were measured by the DNS method.

Avicelase
Suspensions of Avicel (50 mg) in 1 ml of buffer were prepared in 2.2 ml eppendorfs. The enzyme was added and the reaction medium was then incubated for 1 h at 50 °C in a orbital incubator, at 100 rpm. After centrifugation, the supernatant was analysed by the DNS method.

Fpase
A circle of filter paper Whatman no. 1 (3.9 mg) was suspended in 0.5 ml of acetate buffer. After the addition of the purified cellulase, the reaction medium was incubated for 20 h at 50 °C. Finally, the reducing sugars were measured by the DNS method. Additionally, assays were conducted to study the synergism between the purified enzymes. Mixtures of the cellulases were used. The degree of synergism was calculated as the ratio between the activity of each enzyme mixture and half of the value given by the addition of their activities when acting alone.

Xylanase
A solution with 0.5 g insoluble xylan/l was prepared. The enzyme was added to 0.5 ml of this solution, and the reaction medium was incubated for 50 °C for 20 h. Afterwards, the reducing sugars were measured by the DNS method.

Laminarinase
A laminarine (β-1,3-β-1,6-glucan) solution with 1.0 g/l concentration was prepared. The enzyme was added to 0.5 ml of this solution, and the reaction medium was incubated at 50 °C for 20 h. Afterwards, the reducing sugars were measured by the DNS method.

Lichenase
A solution of lichenane (β-1,3-β-1,4-glucan) with 1.0 g/l concentration was prepared. The enzyme was added to 0.5 ml of this solution and the reaction medium was incubated at 50 °C for 3 h. The released reducing sugars were measured by the DNS method.

Endoglucanase
1 ml of a 10.0 g/l suspension of amorphous Avicel (prepared by reprecipitation in water of H_3PO_4 dissolved Avicel), was incubated with the purified enzyme at 50 °C for 15 minutes. The reaction medium was boiled for 10 minutes to inactivate the enzyme. The insoluble fibres were then washed and analysed for the reducing power by the Nelson-Somogyi method, in order to calculate the degree of polymerisation.

Activity upon 4-methylumbelliferyl-β-glucoside cellobioside (MUBC)
A 0.5 mM solution was prepared. The enzyme was added to 1.5 ml of this solution, and the reaction medium was incubated at 40 °C for 0.5 h. The released umbelliferone was estimated spectrophotometrically at 347 nm, after addition of 0.5 ml of sodium carbonate 1 M.

Results and discussion

Celluclast was fractionated in the Rotofor isoelectric focusing cell. The twenty fractions obtained from the Rotofor automatic collection system were analysed for pH, Avicelase and CMCase activity. Considering the isoelectric points of the main cellulases from *Trichoderma reesei* (Kubicek, 1992), the fractions 3 to 8 and 13 to 15 obtained from the Rotofor where pooled for further purification, respectively by size exclusion in Sephacryl 100 HR and anionic chromatography in a Mono Q column (Figure 1).

The isoelectric point and the molecular weight of the purified proteins were determined by analytical IEF and SDS-PAGE in a Phast System. The comparison of the obtained isoelectric points and molecular weights with the values in the literature (Kubicek, 1992) allowed the identification of each protein: cellobiohydrolase I (Mr = 63 kDa, Ip = 4.2) cellobiohydrolase II (Mr = 58 kDa, Ip = 5.9), endoglucanase I (Mr = 46 kDa, Ip = 5.0) and endoglucanase II (Mr = 48 kDa, Ip = 6.1). Considering the proposed composition of the *Trichoderma reesei* cellulase (Kubicek, 1992), the following purification yields were obtained (all values given in % (w/w)): CBH I – 7.4; CBH II – 1.8; EG I – 2.4 and EG II – 8.9.

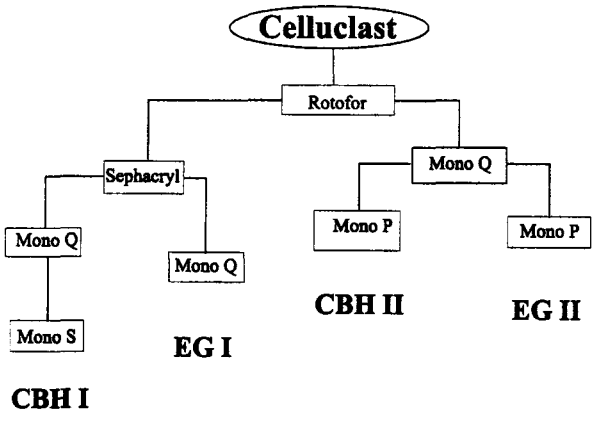

Figure 1 Flow sheet of cellulases purification. CBH = cellobiohydrolase; EG = endoglucanase.

The enzymatic activity of each purified cellulase is shown on Table 1.

As expected, the results show that endoglucanases are more active upon CMC than cellobiohydrolases. To simplify the purification procedure, attempts were made to omit one of the chromatographic steps in CBH I purification. In each assayed situation, however, much higher CMCase activities were detected. Therefore, to obtain a homogeneous preparation of CBH I, the four successive purification steps were proven to be necessary.

The xylanase activity is very low, and is present mainly in the EG I preparation. Bailey *et al.* (1993) showed, by using CBH II and EG I obtained from recombinant strains of *Saccharomyces cereviseae*, that these enzymes are active on insoluble xylan, the measured activity being comparable to the activity detected in this work (EG I activity > CBH II activity).

In the same work, Bailey *et al.* showed that although EG I and CBH II are both active on β-glucan, EG I is the more active one. Similar results were obtained in this work, using lichenan, which, like β-glucan, is a β-1,3-β-1,4-glucan. CBH II hydrolyses β-1,4 covalent bonds only. In contrast with CMC, β-glucan has up to ten successive β-1,4 covalent bonds, which explains the fact that CBH II is more active on lichenan than on CMC (Henriksson *et al.*, 1995).

Concerning the activity on laminarin (β-1,3-β-1,6-glucan), the results on the literature are scarce. The CBH II and EG I used by Bailey *et al.* (1993) have low activity. These results were confirmed here in what concerns CBH II, but the same was not verified for EG I. Since, according to Henriksson *et al.* (1995), CBH II is not active upon β-1,3 covalent bonds we may conclude that some cellulases are active on β-1,6 covalent bonds.

With respect to the chromophoric substrates, only CBH II is not able to hydrolyse the heterosidic bond of MUFC. The more active enzyme upon this substrate is EG I.

These results are in good agreement with other found in the literature (Kubicek, 1992), and confirm the classification of the four purified proteins. Summing up:

Table 1 Enzymatic activities (U/mg) of the purified enzymes.

Enzyme	*Xylanase*	*CMCase*	*Lichenase*	*Laminarase*	*MUBCase**
CBH I	0.003	0.06	0.03	0	1.600
CBH II	0.004	0.21	0.13	0.0016	0.013
EG I	0.014	2.56	0.45	0	2.322
EG II	0	2.82	0.55	0.0004	0.960

* Absorbancy of MUBC solution after incubation with the enzymes.
EG = endoglucanase; CBH = cellobiohydrolase.

Table 2 Enzymatic filter paper conversion to soluble sugars (%(w/w), and degree of synergism and enzyme mixtures.

Enzyme	CBH I	CBH II	EG I	EG II	CBH I CBH II	CBH I EG I	CBH I EG II	CBH II EG I	CBH II EG II	EG I EG II	CBH I CBH II EG I EG II
FP conversion	6.07	8.33	7.65	6.20	29.79	23.21	17.14	23.46	19.87	7.69	42.56
Degree of synergism					4.1	3.4	2.8	3.0	2.8	1.1	6.0

FP = filter paper; EG = endoglucanase; CBH = cellobiohydrolase.

- EG I is an endoglucanase of low specificity, since it can also breakdown xylan;
- EG II can hydrolyse β-1,6 covalent bonds;
- The cellobiohydrolases are also non-specific, since they can act upon xylan;
- There is a major difference between the two CBH's. CBH II is more active than CBH I upon polymers with substituted carboxymethyl groups and upon substrates where β-1,4 bonds alternate with β-1,3 bonds (lichenan) or β-1,6 (laminarin). This demonstrates that CBH II acts on soluble compounds in the same way as an endoglucanase. However, to be active, it requires a larger number of contiguous β-1,4 covalent bonds, comparatively to EG's.

The synergism of the purified enzymes on the hydrolysis of filter paper is shown in Table 2. The degree of conversion of filter paper by the several enzymes was within the same order of magnitude, CBH II and EG I displaying the higher activity. When the enzymes were mixed, while conserving the total amount of protein, an important increase on the enzyme activity could be observed. The degree of synergism reached the value of six when the four enzymes were mixed together.

The effect of the enzymes on the degree of polymerisation of an insoluble substrate was analysed, by measuring the variation on the insoluble fibres reducing power. Table 3 shows the obtained results.

Table 3 Effect of enzymatic treatment of amorphous Avicel: soluble sugars and degree of polymerisation of the residual cellulose.

	Soluble sugars (mg/l)	Degree of polymerisation
Non-treated	-	188
CBH I	58	195
CBH II	292	177
EG I	650	103
EG II	773	94

EG = endoglucanase; CBH = cellobiohydrolase.

A clear difference is detectable between EG's and CBH's. EG's action produces a drop in the degree of polymerisation from 190 to approximately 100, simultaneously solubilising the substrate to a considerable extent. It is interesting to note that CBH II, which was the more active upon filter paper (20 h reaction period), has a relatively low initial activity upon the amorphous Avicel (15 minutes reaction period). Also, CBH II seems not to be able to reduce the degree of polymerisation of this substrate. These results confirm that a contamination of any EG on this preparation is unlikely. CBH I has a low capability to hydrolyse amorphous Avicel under the conditions of the enzymatic assay, and a reduction on the degree of polymerisation is undetectable.

In conclusion, the measurement of the insoluble reducing power of acid swollen Avicel provides a sensitive and expeditious methodology for the detection of endoglucanolytic activity. Moreover, the use of different reaction periods, and of substrates with different properties, can provide information regarding the detection of the subtle differences on the exo/endo character of the several β-glycanases.

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