New methodology for the characterization of endoglucanase activity and its application on the *Trichoderma longibrachiatum* cellulolytic complex

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The cellulolytic complex of Trichoderma longibrachiatum *was separated in nine fractions using FPLC. The avicelase, cellobiase, carboxymethylcellulase, and endoglucanase activities of these fractions were characterized. The endoglucanase activity was measured by a method that allows the determination of the variation in the degree of polymerization of the insoluble celluloses. This method, which is based on the measurement of the reducing power of the insoluble fibers, is proposed as a tool for the identification and characterization of the endoglucanases. Using this technique and H_3PO_4-swollen cotton as substrate, the kinetic parameters of two proteins that showed high specific endoglucanase activity (pI = 5.25; M_r = 55 kDa and pI = 4.70; M_r = 70 kDa) were determined. The measurement of the degree of polymerization variation during digestion of Sigmacell gives evidence that the endoglucanase activity is located at the beginning of the reaction.*

Keywords: Endoglucanase; *Trichoderma longibrachiatum*; cellulase mechanism

Introduction

Cellulose is a natural product with a wide range of applications. However, neither the structure of cellulose nor the cellulolytic mechanism is clearly understood. This is due to a number of factors, the most important of which are the complex heterogeneity of enzymes and substrates[1] and the technical problems related to the determination of the several kinds of cellulase activities.[2]

Endoglucanase (EG) activity on insoluble substrates is usually assessed with the help of several methods, viz. turbidimetry,[3] electron microscopy,[4] resistence to tensile strength,[5] and short fiber formation,[6] each presenting one or more inconveniences. Indeed, they are inaccurate, indirect, or nonquantitative.

The substrates usually applied for EG identification and characterization, such as carboxymethylcellulose (CMCellulose), are water-soluble and stereochemically different from native cellulose.[2] Also, the electrostatic interactions of the negatively charged CMCellulose with the active site of the enzyme may influence the reaction, as was already suggested to be the case with cellobiohydrolase (CBH).[7] Consequently, the classification of enzymes based on their activity on CMCellulose (characteristic of CMCase or EG: EC 3.2.1.4) and Avicel (characteristic of Avicelase or CBH: EC 3.2.1.91) is rather arguable. Apparently, there are some EGs that do not act on CMCellulose but hydrolyze Avicel.[8] As a result, some confusion arises from the use of this nomenclature.[9]

Materials and methods

Enzyme

The commercial cellulase enzyme from *Trichoderma longibrachiatum*, Multifect L-250, was obtained from Finnish Sugar Co, Ltd, Helsinki, Finland.

Substrates

Sigmacell type 100, cellobiose, and low-viscosity sodium-CMCellulose were purchased from Sigma Chemical Co. Avicel PH101 was obtained from

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Honeywell and Stein, London W1, UK. Commercial cotton free of loose fibers, Competência™, was purchased from Bastos e Viegas, Porto, Portugal. Amorphous cotton was prepared from this cotton by solubilization in 85% H_3PO_4, water reprecipitation, and solvent drying according to Lee *et al.*[10]

Determination of the degree of polymerization (DP) of cellulose

Cellulose DP was calculated by the following equation:

$$DP = TC * 1.1/(IFRP)$$

where:

TC = total cellulose dry weight, determined by weighing after overnight drying at 100°C;
1.1 = correction factor for expressing cellulose as glucose equivalents;
IFRP = insoluble fibers reducing power, in glucose equivalents.

The IFRP value was determined by applying the Nelson–Somogyi method[11] to cellulose suspensions, according to the method described by Gama *et al.*[12] The determination of the IFRP of the digested celluloses was made in the same way,[12] after washing the fibers three times with cold water (4°C) in a refrigerated centrifuge (5,000 rev min^{-1}, 5 min). Between washings the supernatant was carefully removed with a Pasteur pipette. The total residual cellulose was calculated by the difference between initial cellulose and solubilized glucose and cellobiose equivalents, as measured by HPLC.

Analytical methods

The reducing sugar concentration was measured with the help of two different methods: the Nelson–Somogyi[11] method for IFRP determination and the dinitrosalicylic acid (DNS) assay[13] in all other situations. D-glucose was used as a standard.

The analysis of solubilized glucose and cellobiose was done by HPLC, on a Jasco chromatograph (880-PV pump and 830-RI refractometer), equipped with a Chrompack-Pb carbohydrate column operating at 80°C and using helium degassed water as eluent. The eluent flowrate was 0.4 ml min^{-1}, corresponding to a 24 psi pressure. Prior to injection, all samples were filtered through a 0.45-μm filter.

Protein was determined by the method of Katzenellenbogen and Dobryszyck.[14] The calibration curve was made using bovine serum albumin as standard.

Proteins isolated by FPLC were analyzed for isoelectric point (pI) and molecular weight in a Phastsystem. Isoelectric focusing (IEF) was done on an IEF 3–9 gel. The gel was developed by staining with silver nitrate.[15] The protein molecular weight determination was performed in a Homogeneous 20 gel. Prior to silver staining, this gel was developed with the Coomassie Blue R method. Electrophoresis, calibration, and gel staining were executed according to Pharmacia instructions.

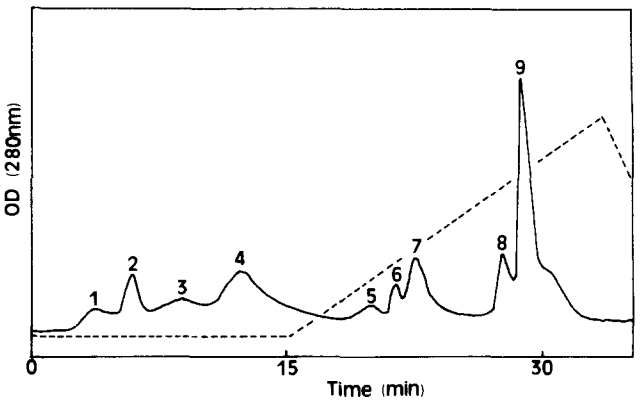

Figure 1 FPLC chromatography of the enzyme complex (------ ionic strength gradient). The eluent (50 mM Tris-HCl pH 7.8) flow rate was 0.30 ml min^{-1}. After 5 ml elution, a linear ionic strength gradient (0 to 0.4 M NaCl), at a flow rate of 0.75 ml min^{-1}, was imposed in the system during 15 ml elution

Separation of the cellulolytic fractions

The enzymatic solution was first desalted and equilibrated with a 50 mM Tris–HCl pH 7.8 using a Phast desalting column (FPLC). To avoid any enzymatic action on dextran, this operation had to be done at 4°C. The flowrate was 3 ml min^{-1}. The obtained enzymatic solution was fractioned in a Mono Q HR5/5 column, as described in the caption to *Figure 1*. The separated fractions were concentrated by ultrafiltration using the Immersible Millipore system, with filters having a nominal molecular weight cutoff of 10,000. After concentration, the sample volume was 1.5 ml; 0.5 ml of 100 mM sodium 3,3′-dimethylglutarate (DMGS) buffer pH 4.75 was added to these concentrated enzymatic fractions.

Enzymatic characterization

The enzymatic activity is expressed according to the following definitions of activity for each enzyme.

One International Unit (IU) of cellobiase and CMCase activity corresponds to the release of 1 μmol min^{-1} of glucose equivalents. One IU of Avicelase corresponds to the release of 1 μmol min^{-1} of cellobiose. One IU of EG corresponds to the variation of 1 μmol min^{-1} of glucose equivalents in the IFRP of amorphous cotton. All experiments were performed at 50°C and 120 rev min^{-1} in an orbital incubator.

Characterization of the fractions isolated by FPLC. The determination of the predominant enzymatic activity was done using four substrates: cellobiose, CMCellulose, Avicel, and amorphous cotton.

(a) *Cellobiase activity*: 0.5 ml of a 3 g l^{-1} cellobiose solution with 1 ml of 100 mM DMGS buffer (pH 4.5) was incubated in a test tube with variable volumes of enzymatic solution (between 55 and 260 μl, in order to have similar enzyme concentration in each

assay) for 90 min. The reaction medium was analyzed by HPLC.

(b) *CMCase activity*: The enzymatic solution (15–50 μl) was added to 1 ml of a 1% CMCellulose solution in 100 mM DMGS buffer pH 4.7 and incubated for 45 min. The released reducing sugars were measured afterwards.

(c) *Enzymatic activity on amorphous cotton (EG activity)*: 25 mg of amorphous cotton was suspended in 3 ml of 100 mM DMGS buffer pH 4.7, in a centrifuge tube. After blending in a vortex, the suspension was incubated with the enzymatic solution (45–150 μl) for 10 min and it was centrifuged. No significant amount of reducing sugars was detected in the supernatant. Finally, the IFRP was determined.

(d) *Avicelase activity*: 30 mg of Avicel was suspended in 1.5 ml of 100 mM DMGS buffer pH 3.9. The Avicel suspensions were incubated with enzymatic solution (65–170 μl) for 5 h. The suspensions were centrifuged at 5,000 rev min^{-1} for 5 min and the supernatant was analyzed by HPLC.

Analysis of the DP variation with enzymatic digestion time. The DP variation with hydrolysis time was determined in the following way: In 50-ml conical flasks, 20 mg of Sigmacell was incubated with 0.5 ml diluted (1 : 125) enzymatic complex and 1.5 ml 100 mM DMGS buffer pH 5.0, for periods ranging from 10 min to 4 h. After pouring 5 ml of cold water into the sample, the IFRP was determined and the solubilized sugars were measured by HPLC. All the experiments were carried out in duplicate.

Results and discussion

Separation of the cellulase fractions

Initially *Trichoderma longibrachiatum* cellulase (Multifect L-250) was purified by chromatography in order to isolate several fractions, each having a different level of endoglucanase activity. A very effective separation—nine fractions—was obtained in a single chromatographic step (*Figure 1*). The degree of resolution of the first four fractions is highly dependent on the pH of the buffer used and on low eluent flow rates, since no ionic strength gradient is imposed in the system during this period.

Characterization of the isolated fractions

The relative enzymatic activities of the isolated fractions on the four tested substrates are plotted in *Figure 2*. The same data quantified on a specific activity basis are represented in *Table 1*. It may be seen that almost all fractions have several types of activity, which is most probably caused by contamination of adjacent fractions.

Fraction 1 has the highest cellobiase activity and a much higher specific activity than the initial complex. A protein with a pI of 8.70 was detected by isoelectric focusing (*Figure 3*). Both fractions 2 and 6 exhibit high CMCase and EG specific activities. The pI of the predominant protein of each fraction is 5.25 and 4.70, respectively. As fraction 4 presents a high Avicelase specific activity and a low CMCase activity, it is probably a CBH. The Avicelase activity of fraction 8 is also significant. The presence of many proteins lowered its specific activity. It must be noted that the protein with a pI of 3.90 is probably the same CBH purified by Shpanchenko *et al.*[16] The enzymatic activities of fractions 3 and 7 are not clearly defined. Isoelectric focusing detected proteins belonging to adjacent endoglucanolytic (2/6) and cellobiohydrolytic (4/8) fractions. *Table 2* shows the preceding results.

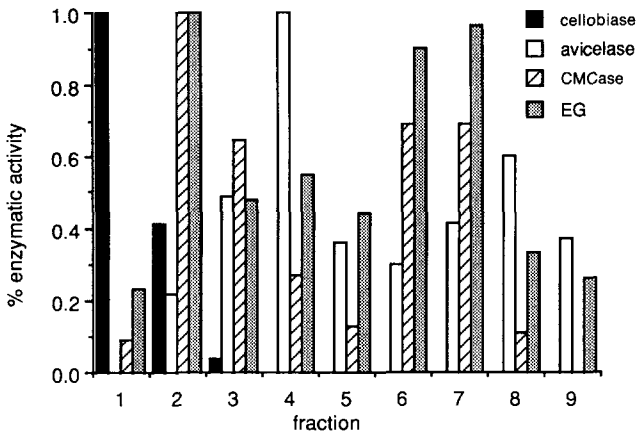

Figure 2 Enzymatic activity of the fractions obtained in FPLC, expressed as percentage of the more active fraction

The measurement of EG activity on amorphous cotton (see Materials and methods) confirmed the results obtained using CMCellulose as a substrate. Both methods seem to have the same degree of accuracy, since the measured relative activities are the same for all but fraction 3. In this case, the CMCase specific activity is 70% of the activity of fraction 2, while EG activity is only 20%. These results suggest that the enzyme isolated in fraction 3 has a higher affinity for CMCellulose than for amorphous cotton. Such a behavior was suggested by Poulsen *et al.*[17] to depend on the glycosylation level of the enzyme. Despite the use of a similar cellulose concentration in both assays, a lower EG activity on amorphous cotton was observed. This may be explained by a lower number of active sites accessible to EG action due to the structure of cellulose and/or to diffusional limitations.

This set of results provides evidence of the effectiveness of the described assay procedure. Its application may be extremely useful for the classification of the proteins referred to by Chanzy *et al.*[4] (CBH I from *T. reesei*, with EG activity on *Valonia* crystals), by Bronnembeier and Staudenbauer[18] (CMCase from *Clostridium stercorarium* with Avicelase activity), and in other cases where there is no well-defined enzymatic activity.[19] It must be stressed that it is possible to use this methodology to measure the EG activity on any insoluble purified substrate and not only on amorphous cotton.[12]

Table 1 Protein concentration and specific enzymatic activity of the enzyme complex and of the ultrafiltered fractions (1–9) obtained by FPLC

Sample	Protein ($g\ l^{-1}$)	Specific activity ($IU\ mg^{-1}$)			
		Cellobiase	EG	CMCase	Avicelase ($\times 10^3$)
Multifect	75.0	0.19	1.90	7.60	0.21
1	0.059	5.20	0.84	16.90	–
2	0.097	1.60	3.11	63.90	0.18
3	0.100	0.10	0.80	44.80	0.40
4	0.200	–	0.97	16.80	0.51
5	0.070	–	1.10	14.90	0.34
6	0.107	–	2.96	44.20	0.22
7	0.333	–	1.93	29.60	0.17
8	0.376	–	0.35	6.30	0.23
9	0.615	–	0.25	1.10	0.14

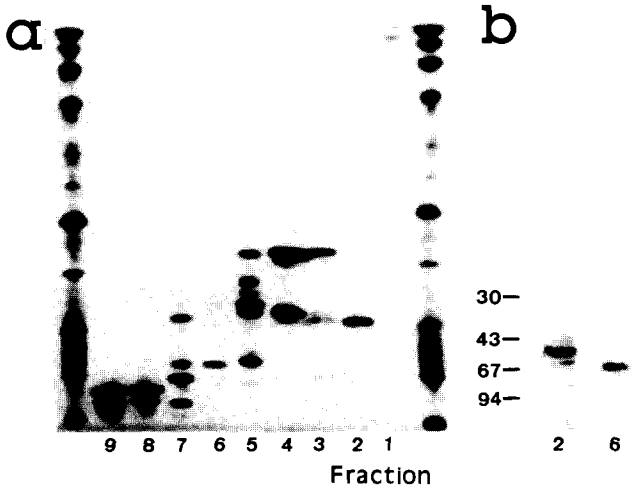

Figure 3 (a) Isoelectric focusing of the fractions obtained in FPLC, and (b) molecular weight determination of the proteins in fractions 2 and 6; the positions of migration of molecular weight markers ($\times 10^3$) are indicated

Table 2 Enzymatic activity and enzyme pI on the fractions obtained by FPLC

Fraction	Enzymatic activity	pI
1	Cellobiase	8.70
2	CMCase-EG	5.25
3	CMCase/avicelase	5.25/5.95
4	Avicelase (+)/CMCase-EG	5.95/5.25
5	–	–
6	CMCase-EG	4.70
7	CMCase-EG (+)/Avicelase	4.70/3.90
8	Avicelase	3.90
9	–	–

(+) Indicates the predominant activity

Determination of kinetic parameters

As previously stated, fractions 2 (pI = 5.25; M_r = 55 kDa) and 6 (pI = 4.70; M_r = 70 kDa) have a high degree of purification (*Figure 3*) and high specific EG and CMCase activities. The kinetic parameters of the EG activity of these enzymes were determined using amorphous cotton as substrate. This substrate was selected because of its similarity to the amorphous zones of natural celluloses (although the molecules are antiparallel[20]), since EGs act preferentially on these sites. The main advantage of this substrate when compared with CMC lies in its insolubility, allowing for the determination of the kinetic parameters on a more realistic basis, i.e., reflecting substrate structure and diffusional limitations. Furthermore, the reduced reaction time is another important feature of this procedure, since the interference by contaminating CBH on DP is reduced. As the DP value remains high throughout the reaction, there will not be a reduction in the affinity of the enzyme for the substrate; the initial DP of the utilized amorphous cotton was 2,700 and 300 after 10 min digestion with fractions 2 and 6.

A Lineweaver–Burk plot was used to calculate the following kinetic parameters:

Fraction 2: $K_M = 4.6 \pm 1.4\ g\ l^{-1}$;

$V_{max} = 4.5 \pm 0.8\ \mu mol\ eq\ gluc\ min^{-1}\ mg^{-1}\ prot$

Fraction 6: $K_M = 19.6 \pm 6.2\ g\ l^{-1}$;

$V_{max} = 10.5 \pm 2.7\ \mu mol\ eq\ gluc\ min^{-1}\ mg^{-1}\ prot$

(eq = equivalent; gluc = glucose; prot = protein)

These parameters may be used in kinetic models that describe the mechanism of cellulase action.[21]

Analysis of DP variation with enzymatic digestion time

In agreement with the data reported by Wood,[5] using a viscosimetric technique, a sharp reduction in the medium length of cellulose molecules was observed in the

early stage of the reaction. After 10 min reaction time, the DP of Sigmacell was reduced from 391 to 93. However, at the end of the reaction, the DP was close to its original value, i.e., DP = 310. These results are understood as a tandem action of EG and CBH: the smaller molecules released by the EG action are subsequently solubilized by CBH, thereby allowing for a recovery in DP.

There is some discrepancy between these results and the generally accepted mechanism of hydrolysis of crystalline celluloses.[22,23] Since the DPs are similar at the beginning and the end of digestion, the residual cellulose fibrils must still have crystalline and amorphous zones in the same proportions as in the beginning. The former hypothesis is confirmed by the verification that successive hydrolyses of crystalline celluloses have identical initial reaction rates.[24,25] This result indicates that there is no modification in the structural properties of cellulose that control the rate of hydrolysis, leading to the conclusion that EGs probably do not act on all of the transverse section of the amorphous zones, possibly because of accessibility limitations.

On the other hand, it was observed that after 4 h of enzymatic digestion, only a 40% fraction of Sigmacell was solubilized. By considering an average DP of 310 for the residual cellulose after a 10 min reaction period, a simple mass balance gives, for the solubilized fraction, a DP value of 45. This result agrees with published values for the leveling-off DP of crystalline celluloses.[26] The experiments reported in the present work suggest, therefore, that the EG activity takes place mainly in the beginning of the hydrolysis.

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