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Studies on the chromatographic fractionation of *Trichoderma reesei* cellulases by hydrophobic interaction

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Abstract

This work reports new studies on cellulases fractionation by hydrophobic interaction chromatography. The purification procedure for the *Trichoderma reesei* cellulase complex consists of gel permeation chromatography on Sephadex G-25M followed by an ultrafiltration step. The concentrated enzyme solution was then fractionated on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether. The influence of the mobile phase composition on the chromatographic behaviour of the *T. reesei* cellulase complex was investigated. By using 13% (w/v) ammonium sulphate in eluent buffer, a selective separation of β-glucosidase with a two-fold increase in specific activity and a recovery of 60% cellobiase activity were obtained. Other commercial hydrophobic supports (octyl- and phenyl-Sepharose) were also tested and compared under the same conditions.

Keywords: *Trichoderma reesei*; Hydrophobic interaction chromatography; Cellulases; Enzymes

1. Introduction

The filamentous fungus *Trichoderma reesei* is one of the most potent cellulose-degrading organisms known. It produces a complete cellulase system with three types of enzymes: cellobiohydrolases (CBH I and II; EC 3.2.1.91), endoglucanases (EG I, II, III and V; EC 3.2.1.4) and a β-D-glucosidase (EC 3.2.1.21) [1,2]. Cellobiohydrolases act as exoglucanases, initiating their action from the ends of cellulose chains and releasing cellobiose as the main product. Endoglucanases cleave bonds along the length of cellulose chains, thus producing new sites for the cellobiohydrolases. β-Glucosidase hydrolyses cellobiose to glucose, removing a strong inhibitor of cellobiohydrolases from the reaction mixture [3]. The enzymes appear in multiple isoforms [2,4] and act in synergism during the hydrolysis of cellulose [5–10], i.e. the action of a mixture of two or more individual cellulases is greater than the sum of the action of each enzyme. The cooperative action between endoglucanases and cellobiohydrolases is called endo–exo synergism [6,7]. Synergy is rarely found between two different endoglucanases, but is clearly observed between certain cellobiohydrolases [6,8]. This so-called exo–exo synergism has been proposed to be due to different stereospecificity for CBH I and CBH II in the attack on nonreducing end groups in the cellulose chains [11].

Complex cellulase systems present considerable purification problems. The use of affinity chromatography [12] is limited due to difficulties in elution of enzymes from the supports. Ion-exchange chromatographic methods have most often been applied [2,13–15]. Recently, Medve et al. [16] described a purification technique based on ion-exchange chro-

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matography using new materials with high resolution power. Fast protein liquid chromatography (FPLC) was used to purify three major cellulases and to quantitate these enzymes in reconstituted mixtures during cellulose hydrolysis studies. The purification procedure consists of three different chromatographic steps and yields three pure enzymes: CBH I, CBH II and EG II.

Hydrophobic interaction chromatography (HIC) has become a popular technique for the fractionation of biomolecules. This technique provides a powerful additional means of separation which is applicable to the purification of most proteins. The hydrophobic interaction is used for the binding of nonpolar regions, on the surface of the proteins, to adsorbents with hydrophobic ligands [17]. The adsorption increases with high salt concentration in the media and elution (and separation) is carried out by decreasing the salt concentration of the eluent. The diversity of potential eluting conditions can enable the resolution of complex mixtures of proteins which would be difficult to separate using other chromatographic techniques. The type of ligand and matrix, the salt and its concentration, pH, temperature and additives are the most important factors to consider in HIC [18–20].

This work describes the cellulase complex fractionation of *T. reesei* crude extract using a HIC step on a Sepharose CL-6B column modified by covalent immobilization of 1,4-butanediol diglycidyl ether [21]. The purification of a crude preparation of *Chromobacterium viscosum* lipase using this derivatized gel was reported previously [22].

2. Experimental

2.1. Materials

Sepharose CL-6B was obtained from Pharmacia (Uppsala, Sweden) and 1,4-butanediol diglycidyl ether from Sigma (St. Louis, MO, USA). The filter paper was Whatman No.1 (Maidstone, UK). Hydroxyethylcellulose (HEC, medium viscosity) was from Fluka (Bucks, Switzerland). Microcrystalline cellulose (powder 20 μm) and D-cellobiose (98%, predominantly β) were purchased from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade.

2.2. Enzyme preparation

A cellulolytic enzyme complex of *Trichoderma reesei* (Celluclast 1.5 L) from Novo Nordisk (Copenhagen, Denmark) was used.

2.3. Protein and enzymatic activity assays

The concentration of protein in the samples was determined by the method of Bradford, with crystalline bovine serum albumin as standard [23].

Total cellulase activity was determined by the filter paper activity (FPA) method. It was carried out at pH 4.8, 50°C on 50 mg filter paper (Whatman

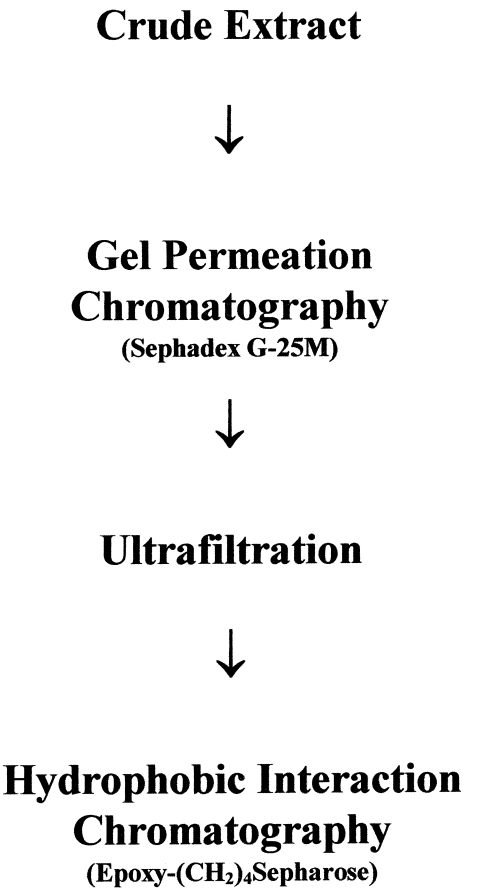

Fig. 1. Purification procedure for *T. reesei* cellulases.

No.1) strips for 1 h [24]. The reaction was stopped by placing the tubes in a boiling water bath for 10 min and, after centrifugation, the reducing sugars were measured by the dinitrosalicylic acid (DNS) method, using glucose as standard [25].

Cellobiohydrolase and endoglucanase activities were quantified using, respectively, 1% microcrystalline cellulose [26] and 1% HEC [27]. The reducing sugars were also determined by the DNS method [25].

Cellobiase activity was determined using cellobiose as substrate and the glucose released was measured by the glucose oxidase method [28].

2.4. Chromatographic method

The cellulase crude extract was fractionated, at room temperature, in a standard chromatographic system (Pharmacia, Uppsala, Sweden).

The gel permeation chromatography first step was carried out on a Sephadex G-25M gel (35×1.6 cm I.D column) and equilibrated with acetate buffer, 25 m*M*, pH 4.8, at a flow-rate of 39.0 ml/h. After an ultrafiltration step using an Ultrafree 10 000 filter (Millipore, France), the concentrated enzyme solution was fractionated by HIC on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether. The gel was packed in a column (1.6×1.6 cm I.D.) and equilibrated with the desired mobile phase (acetate buffer, 0.1 *M*, pH 4.8, with different ammonium sulphate concentrations) at a flow-rate of 9.6 ml/h. The enzyme solution (300 μl) was applied and the elution profile obtained by continuous measurement of the absorbance at 280 nm. Fractions of 1 ml were collected. The protein concentration and the activity towards filter paper, microcrystalline cellulose, HEC and cellobiose were determined.

3. Results and discussion

Cellulases fractionation was carried out using several chromatographic steps, at room temperature, in a standard chromatographic system. The purification procedure is summarized in Fig. 1.

The first step of the purification procedure of the crude extract of *T. reesei* was a gel permeation chromatography on Sephadex G-25M. Two major peaks were obtained (Fig. 2), but only fractions 2 to 5 had cellulase activity, with a recovery of 94% FPA. The cellulase-active fractions were pooled and concentrated by ultrafiltration. The enzyme solution thus obtained had a high retention of total cellulase activity and was injected on the hydrophobic column

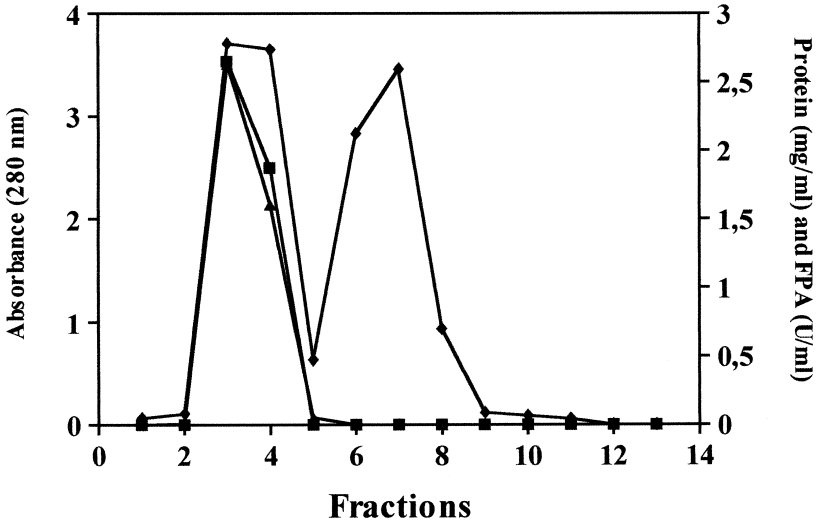

Fig. 2. Chromatographic profile of the *T. reesei* cellulases crude extract on a Sephadex G-25M column. Elution with 25 m*M* acetate buffer, pH 4.8. Flow-rate, 39.0 ml/h; fractions, 5 ml. (♦) Absorbance (280 nm); (▲) protein; (■) cellulase activity (FPA).

(Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether).

The effect of salt concentration in the eluent buffer on the retention of cellulases was studied using ammonium sulphate in the mobile phase. This salt was selected because it presents high increments of molal surface tension, promoting to a great extent cellulase retention on the support [29]. In fact, the progressive augmentation of ammonium sulphate concentration in the eluent buffer leads to a simultaneous increase in the amount of retained cellulases. Thus, for 0% (w/v) salt in the buffer no cellulases remain bound to the gel but, on increasing the salt concentration to 20% (w/v), total retention of cellulases in the column was obtained. Hydrophobic interactions are known to increase upon increasing the ionic strength of the medium [30] and the strategy for cellulases fractionation by HIC implies careful selection of this parameter.

Fig. 3 shows the chromatograms obtained using, respectively, 11 and 15% (w/v) ammonium sulphate in the buffer. For 11% (w/v) ammonium sulphate, cellobiase activity was found only in the first peak (unbounded protein), FPA in the second (very low), but cellobiohydrolase and endoglucanase activities were present in both peaks. Using 15% (w/v)

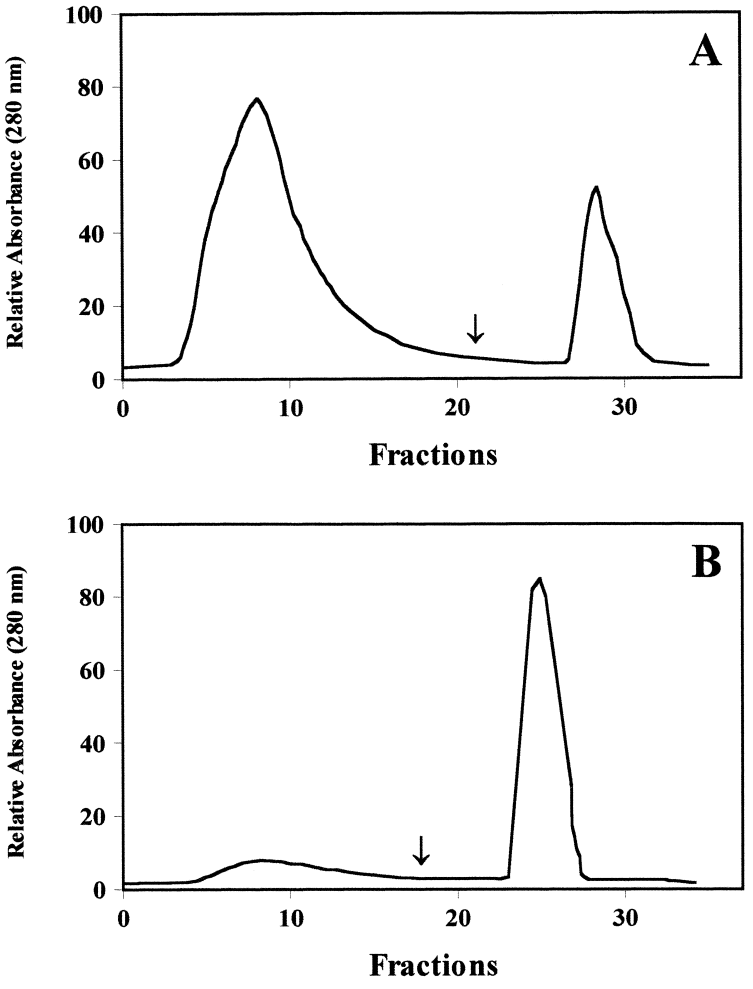

Fig. 3. Hydrophobic interaction chromatography on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether. Buffer: 0.1 *M* acetate, pH 4.8, containing 11% (A) and 15% (B) (w/v) ammonium sulphate. Desorption (↓) was obtained with 0.1 *M* acetate buffer, pH 4.8.

ammonium sulphate, only a small amount of protein, without any cellulase activity, was not retained on the column. The main fraction, obtained after elution with acetate buffer, 0.1 *M*, pH 4.8, had the same enzymatic activity as the injected fractions. In both cases (11 and 15%) no increase in specific activity was detected.

For 13% (w/v) ammonium sulphate, three peaks were obtained (Fig. 4), suggesting a selective fractionation. The fractions of peaks I and II correspond to unadsorbed protein: no cellulase activity was detected in I, but II contained the main β-glucosidase with a two-fold increase in specific activity and a recovery of 60% of cellobiase activity. Therefore, a selective separation of β-glucosidase from a crude cellulase mixture was obtained. Endoglucanase activity was also detected in a very low proportion in II, but no other form of cellulase activity was observed. After elution with 0.1 *M* acetate buffer, pH 4.8, the fractions of peak III were obtained. No β-glucosidase activity was found in this peak, but all the other cellulase activities were detected. There was a two-fold increase in specific endoglucanase activity and a decrease in the other activities (filter paper and microcrystalline cellulose) compared with the injected fractions. This can be explained based on cellulase synergism [4–9]: if the cellulases are fractionated, no cooperative action between them is possible and total cellulolytic activity decreases. In addition, in peak III fractions there is no β-glucosidase to hydrolyse cellobiose (a strong inhibitor of cellobiohydrolases) to glucose, therefore the activities against filter paper and microcrystalline cellulose are low.

The measurement of the total activity of nonfractionated systems, usually determined by the method of the cleavage of filter paper, provides no information on the levels of individual enzymes or on their role in the hydrolysis of cellulose substrates. In the present study, the methods conventionally used for the quantification of cellulases (activity measurements against filter paper, microcrystalline cellulose and hydroxyethylcellulose) were useful since we were looking at the qualitative aspects of the enzymes and the cellulase synergism in mixtures.

Control experiments using unmodified Sepharose CL-6B did not result in any retention of cellulase on the column, even using 13% (w/v) ammonium sulphate in the eluent buffer. Other commercial hydrophobic supports (octyl- and phenyl-Sepharose) were also tested and compared under the same conditions. Using an octyl-Sepharose column (pure hydrophobic character [31]), three peaks were obtained, as above with our modified gel (Fig. 4), but

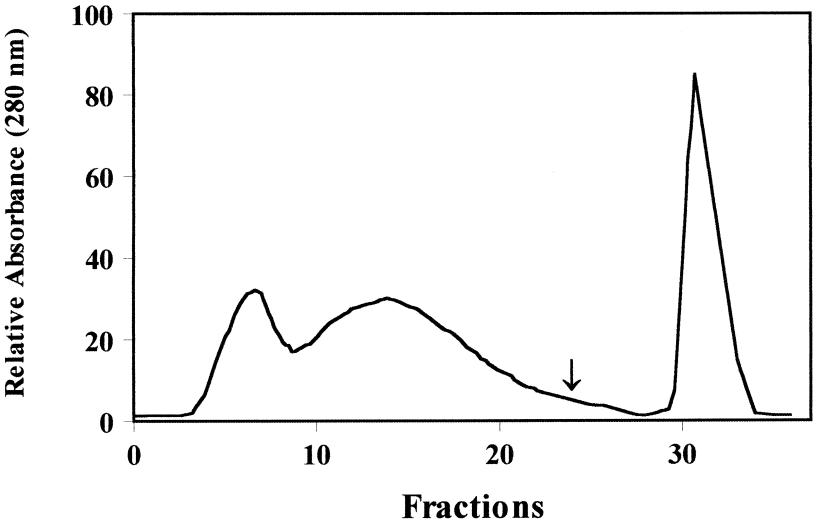

Fig. 4. Hydrophobic interaction chromatography on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether. Buffer: 0.1 *M* acetate, pH 4.8, containing 13% (w/v) ammonium sulphate. Desorption (↓) was obtained with 0.1 *M* acetate buffer, pH 4.8.

only retained protein (peak III) presents total celullase activity (FPA). For the phenyl-Sepharose support (aromatic and hydrophobic character [31]), all protein was retained on the column (results not shown). In both cases a selective cellulases fractionation was not obtained.

In conclusion, the experimental results obtained suggest that the properties of the chromatographic support used and the ionic strength play an important role in the separation of a cellulases complex based on their hydrophobic properties. By using 13% (w/v) ammonium sulphate in the eluent buffer a selective separation of β-glucosidase with a two-fold increase in specific activity and a recovery of 60% of cellobiase activity were obtained. Cellulases are most often purified by ion-exchange chromatography [13–16]. However, the fractionation of *T. reesei* cellulases by HIC with a gel prepared by coupling 1,4-butanediol diglycidyl ether to Sepharose CL-6B has not been reported. The present purification procedure appears to represent a new fractionation method for cellulases.

References

[1] J. Knowles, P. Lehtovaara, T. Teeri, Trends Biotechnol. 5 (1987) 255.
[2] A. Goyal, B. Ghosh, D. Eveleigh, Bioresource Technol. 36 (1991) 37.
[3] P. Béguin, J.-P. Aubert, FEMS Microbiol. Rev. 13 (1994) 15.
[4] T.-M. Enari, M.-L. Niku-Paavola, Crit. Rev. Biotechnol. 5 (1987) 67.
[5] K.M. Kleman-Leyer, M. Siika-Aho, T.T. Teeri, T.K. Kirk, Appl. Environ. Microbiol. 62 (1996) 2883.
[6] B. Henrissat, H. Driguez, C. Viet, M. Schülein, Bio/Technology 3 (1985) 722.
[7] B. Nidetzky, W. Steiner, M. Hayn, M. Claeyssens, Biochem. J. 298 (1994) 705.
[8] J. Medve, J. Stählberg, F. Tjerneld, Biotechnol. Bioeng. 44 (1994) 1064.
[9] P. Tomme, R.A.J. Warren, N.R. Gilkes, Adv. Microb. Physiol. 37 (1995) 1.
[10] J. Woodward, Bioresource Technol. 36 (1994) 67.
[11] T.M. Wood, S.I. McCrae, Biochem. J. 234 (1986) 93.
[12] H. Tilbeurgh, R. Bhikhabhai, G. Pettersson, M. Claeyssens, FEBS Lett. 16 (1984) 215.
[13] A. Fliess, K. Schügerl, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 314.
[14] R. Bhikhabhai, G. Johansson, G.J. Pettersson, J. Appl. Biochem. 6 (1984) 336.
[15] S. Ellouz, H. Durand, G. Tiraby, J. Chromatogr. 396 (1987) 307.
[16] J. Medve, D. Lee, F. Tjerneld, J. Chromatogr. A 808 (1998) 153.
[17] S. Ree, in: E.L.V. Harris, S. Angal (Eds.), Protein Purification Methods: A Practical Approach, IRL Press, Oxford, 1989, p. 221, Ch. 4.
[18] J.L. Ochoa, Biochimie 60 (1978) 1.
[19] G. Rippel, L. Szepesy, J. Chromatogr. A 664 (1994) 27.
[20] S. Oscarsson, P. Kärsnäs, J. Chromatogr. A 803 (1998) 83.
[21] J.A. Queiroz, F.A.P. Garcia, J.M.S. Cabral, J. Chromatogr. A 707 (1995) 137.
[22] J.A. Queiroz, F.A.P. Garcia, J.M.S. Cabral, Isolat. Purif. 3 (1999) 1.
[23] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[24] M. Mandels, R. Andreotti, C. Roche, Biotechnol. Bioeng. Symp. 6 (1976) 21.
[25] G.L. Miller, Anal. Chem. 31 (1959) 426.
[26] T.M. Wood, K.M. Bhat, Methods Enzymol. 160 (1988) 87.
[27] T.K. Ghose, Pure Appl. Chem. 59 (1987) 257.
[28] J.B. Lloyd, W.J. Whelan, Anal. Biochem. 30 (1969) 467.
[29] W. Melander, D. Corradini, Cs. Horváth, J. Chromatogr. 317 (1984) 67.
[30] P.P. Berna, N. Berna, J. Porath, S. Oscarsson, J. Chromatogr. A 800 (1998) 151.
[31] B.H. Hofstee, N.F. Otillio, J. Chromatogr. 159 (1978) 57.