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Comparative study on the fractionation of cellulases on some hydrophobic interaction chromatography adsorbents

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Abstract

In this work, a comparative study for the fractionation of *Trichoderma reesei* cellulases on five different hydrophobic interaction chromatography adsorbents (Butyl–Sepharose 4 FF, Phenyl–Sepharose 6 FF, Octyl–Sepharose 4 FF, Epoxy–Sepharose CL-6B and Polypropylene glycol–Sepharose CL-6B) is shown. The influence of the mobile phase composition on the chromatographic behaviour of *T. reesei* cellulases complex was evaluated using different concentrations of ammonium sulphate in the eluent buffer. A selective separation of β-glucosidase with two-fold increase in specific activity and good recoveries of cellobiase activity were obtained with Butyl–Sepharose 4 FF and Phenyl–Sepharose 6 FF using 7% (w/v) ammonium sulphate in the eluent buffer. A β-glucosidase fractionation was also obtained with Epoxy–Sepharose CL-6B, using 13% (w/v) of the salt in the mobile phase.

Keywords: Hydrophobic interaction chromatography; *Trichoderma reesei*; Stationary phases, LC; Cellulases; Enzymes

1. Introduction

Hydrophobic interaction chromatography (HIC) is a powerful technique for purification of biomolecules at both small scale laboratory level, as well as in process scale applications in industry.

HIC takes advantage of the hydrophobicity of proteins, promoting its separation based on hydrophobic interactions between immobilized hydrophobic ligands on the gel and non polar regions on the surface of proteins. In HIC, the adsorption (and retention) of the proteins is carried out in the presence of an aqueous mobile phase, at a high salt concentration and the elution is performed by decreasing the salt concentration of the eluent. The change in the elution conditions can be used for separation of complex mixtures of proteins. In fact, HIC has been widely used for separation processes, because it exhibits binding characteristics complementary to other protein chromatographic techniques [1]. In HIC, the interaction between ligand and protein is weaker than in affinity, ion-exchange or reversed-phase chromatography, thus minimizing the structure damage of the protein and preserving its biological activity [2,3].

Two major parameters have to be considered in a HIC process: the stationary phase and the fluid mobile phase. The different stationary phases can differ on the type of ligand, the ligand chain length, ligand density and on the type of the matrix or support. Linear chain alkanes (e.g. butyl or octyl) and aromatic groups (e.g. phenyl) are the most widely used ligands in HIC. The hydrophobicity and the strength of the interaction increase with the increase in *n*-alkyl chain length, but the adsorption selectivity may decrease [4]. The protein binding capacity of a stationary phase in HIC can also be

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improved by increasing the degree of substitution of the immobilized ligand due to the formation of multi-point attachment between the protein and the ligand [5]. However, it can promote an irreversible adsorption of the proteins or a denaturation during the elution with strong conditions (chaotropic agents, organic solvents and detergents) [6].

The use of the ligands with intermediate hydrophobic character has been useful in HIC processes, since they promote a mild binding strength and an elution by simple decreasing the ionic strength of the eluent. These mild hydrophobic stationary phases have been synthesized by immobilization of polymer ligands [e.g. polyethylene glycol (PEG) or polypropylene glycol (PPG)] on an agarose matrix [7]. In fact, these stationary phases have been used successfully in protein purification [8,9].

The protein retention in HIC also depends on characteristics of the mobile phase, such as the type and concentration of the salt, pH, temperature and additives. The effect of salt type on protein adsorption follows the order of the salts in lyotropic (Hofmeister) series for the precipitation of proteins [10,11]. The change of salt type and salt concentration in the eluent buffer affects not only the retention of the proteins but also the selectivity of the separations [12,13].

In this work, an investigation was undertaken for cellulases fractionation with different HIC stationary phases (Butyl–Sepharose 4 FF, Phenyl–Sepharose 6 FF, Octyl–Sepharose 4 FF, Epoxy–Sepharose CL-6B and PPG–Sepharose CL-6B) using different concentrations of ammonium sulphate in the eluent buffer. The cellulase complex from *Trichoderma reesei* have three different enzyme types, cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and a β-D-glucosidase (EC 3.2.1.21), that act in synergism during the hydrolysis of cellulose [14–16]. Endoglucanases cleave internal glucosidic bonds of the cellulose producing free chain ends. Cellobiohydrolases act as exoglucanases initiating their action from the ends of cellulose chains and releasing cellobiose as the main product. β-Glucosidase hydrolyses cellobiose to glucose, thus removing a strong inhibitor of cellobiohydrolases from the media [14].

HIC does not been widely used in cellulases purification. Recently, Mawadza et al. [17] have reported the purification of cellulases produced by two *Bacillus* strains (CH43 and HR68) using a Phenyl–Sepharose column. We have also reported a selective fractionation of β-glucosidase from *T. reesei* crude extract, using a Sepharose CL-6B column modified by covalent immobilization of 1,4-butanediol diglycidyl ether [18]. The PEG polymer was used on β-glucosidase fractionation of *T. reesei* by partitioning in aqueous two-phase systems [19].

In the present paper, a comparison between the chromatographic profiles obtained at different concentrations of ammonium sulphate with cellulolytic enzyme complex, on the different HIC adsorbents mentionated above, will be provided.

2. Experimental

2.1. Materials

Sephadex G-25M, Sepharose CL-6B, Phenyl–Sepharose 6 FF and Octyl–Sepharose 4 FF were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Butyl–Sepharose 4 FF was a kindly gift from Amersham Pharmacia Biotech (Portugal). PPG diglycidyl ether (average number-average molecular mass, M_n, ca. 380) was purchased from Aldrich (Milwaukee, WI, USA) and sodium borohydride was from Merck (Darmstadt, Germany). The filter paper was Whatman No.1 (Maidstone, UK). Hydroxyethylcellulose [HEC, medium viscosity, degree of substitution (DS) 0.8] was from Fluka (Bucks, Switzerland). D-cellobiose (98%, predominantly β) was purchased from Aldrich. All other reagents were of analytical grade.

2.2. Enzyme preparation

A cellulolytic enzyme complex of *Trichoderma reesei* (Celluclast 1,5L) from Novo Nordisk (Denmark) was used.

2.3. Protein and Enzymatic activity assays

Protein concentration was determined by the Bradford method, using bovine serum albumin as standard [20].

Total cellulase activity was determined by the filter paper activity method. It was carried out at pH

4.8, 50°C on 50 mg filter paper strips for 1 h [21]. The reaction was stopped by placing the tubes in a boiling water bath for 10 min and after centrifugation, the reducing sugars were measured with dinitrosalicylic acid (DNS) reagent, using glucose as standard, as described by Miller [22].

Endoglucanase activity was quantified using 1% HEC [23]. The reducing sugars were determined by the DNS method [22].

Cellobiase (β-glucosidase) activity was determined using cellobiose as substrate [24] and the glucose released was measured by the glucose oxidase method [25].

2.4. Chromatographic method

The chromatographic processes were performed, at room temperature, using a Pharmacia (Uppsala, Sweden) Fast Protein Liquid Chromatography (FPLC) system consisting of two P500 pumps, an MV 7 injection valve, a fraction collector FRAC 100, a dual path monitor UV-1 and a recorder 112, controlled by a LCC 501 Plus controller. The gel filtration chromatography 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 a Amicon cell with a polyethersulphone membrane (Millipore) of 5000 Da nominal molecular mass cut-off, the concentrated enzyme solution was fractionated by HIC, on different hydrophobic adsorbents (Butyl–, Phenyl–, Octyl–, Epoxy– and PPG–Sepharose). The Epoxy–Sepharose CL-6B was prepared by covalent immobilization of 1,4-butanediol diglycidyl ether on Sepharose CL-6B according to Sundberg and Porath [26]. The PPG–Sepharose was obtained by coupling PPG diglicidyl ether to Sepharose CL-6B as previously reported by Diogo et al. [9]. The gel was packed in a column (2.5×1.6 cm I.D.) and equilibrated with 0.1 *M* acetate buffer, pH 4.8 with different ammonium sulphate concentrations, at a flow-rate of 24.0 ml/h. The enzyme solution (300 μl) was loaded onto the column and isocratic elution was performed with the same concentration of ammonium sulphate. After elution of unretained species, the ionic strength of the buffer was decreased (25 m*M* acetate buffer, pH 4.8) to promote the elution of retained species. The elution profile was obtained by continuous measurement of the absorbance at 280 nm. Fractions of 1 ml were collected and the protein concentration and the activity towards filter paper, hydroxyethylcellulose and cellobiose were determined.

3. Results and discussion

The HIC experiments were performed after a gel filtration chromatography of the *T. reesei* crude extract and an ultrafiltration step, as described previously [18]. Fig. 1 summarizes the purification procedure.

In this work, a comparison between chromatographic profiles obtained with *T. reesei* cellulases on five different HIC stationary phases (Butyl–Sepharose 4 FF, Phenyl–Sepharose 6 FF, Octyl–Sepharose 4 FF, Epoxy–Sepharose CL-6B and PPG–Sepharose CL-6B) (Table 1) is shown.

The effect of the salt concentration on the cellulases retention, on the above HIC adsorbents was performed using different percentages (0 to 20%, w/v) of ammonium sulphate in the eluent buffer. This salt presents a high molal surface tension increment, therefore it can promote the cellulases adsorption on the support in a great extent [10,11].

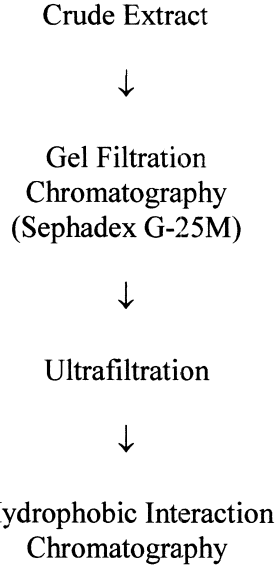

Fig. 1. Purification procedure for the *T. reesei* cellulases.

Table 1
Filter paper activity (%) and cellobiase activity (%) for *T. reesei* cellulases obtained by HIC on five different adsorbents[a]

HIC adsorbent	Ligand	Salt concentration for fractionation (%, w/v)	Filter paper activity (%)	Cellobiase activity (%)
Butyl–Sepharose 4 FF	$-OCH_2\underset{OH}{C}HCH_2O-(CH_2)_3-CH_3$	7	24	43
Phenyl–Sepharose 6 FF	$-OCH_2\underset{OH}{C}HCH_2O-C_6H_5$	7	17	47
Octyl–Sepharose 4 FF	$-OCH_2\underset{OH}{C}HCH_2O-(CH_2)_7-CH_3$	10	19	50
Epoxy–Sepharose CL–6B	$-OCH_2\underset{OH}{C}HCH_2O-(CH_2)_4-OCH_2\underset{OH}{C}HCH_2OH$	13	32	60
PPG–Sepharose CL-6B	$-OCH_2\underset{OH}{C}HCH_2O-(CH_2\underset{CH_3}{C}HO)_3-CH_2\underset{OH}{C}HCH_2OH$	16	44	36*

[a] The filter paper activity recovery was in peak III and the cellobiase one in peak II (*except for PPG-Sepharose, peak III).

The elution of the retained enzymes was effected by decreasing the salt concentration in the mobile phase.

The chromatographic profiles of the *T. reesei* cellulases fractionation using the different adsorbents are shown in Figs. 2–4. For 0% (w/v) ammonium sulphate in the eluent buffer, no cellulases were adsorbed to any of the adsorbents, but with an increase in the salt concentration to 15% (w/v) a total retention of the enzymes on all of the above columns was obtained. In this case, it was not observed any increment in total cellulolytic activity or endoglucanases and cellobiase activities, comparing to the injected fractions. The progressive increase in ammonium sulphate concentration leads, in all cases, to a simultaneous increase in the amount of adsorbed protein. However, the selective fractionation of the cellulases in three peaks by HIC was only

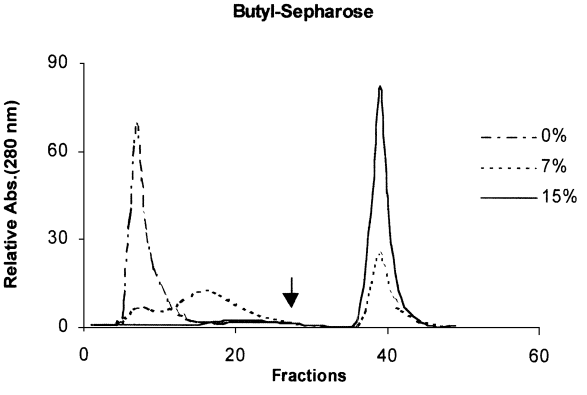

Fig. 2. Hydrophobic interaction chromatography on Butyl–Sepharose 4 FF. Buffer: 25 m*M* acetate, pH 4.8 containing 0, 7 and 15% (w/v) ammonium sulphate. Desorption (↓) was performed with 25 m*M* acetate buffer, pH 4.8.

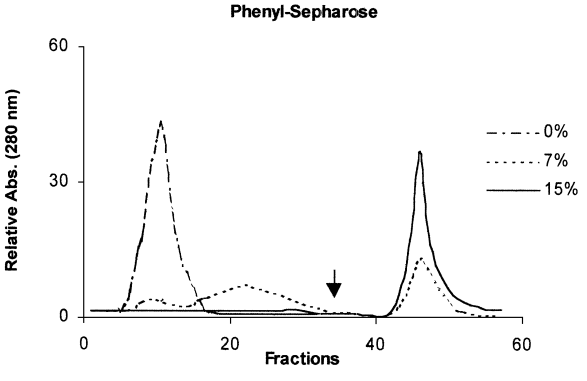

Fig. 3. Hydrophobic interaction chromatography on Phenyl–Sepharose 6 FF. Buffer: 25 m*M* acetate, pH 4.8 containing 0, 7 and 15% (w/v) ammonium sulphate. Desorption (↓) was performed with 25 m*M* acetate buffer, pH 4.8.

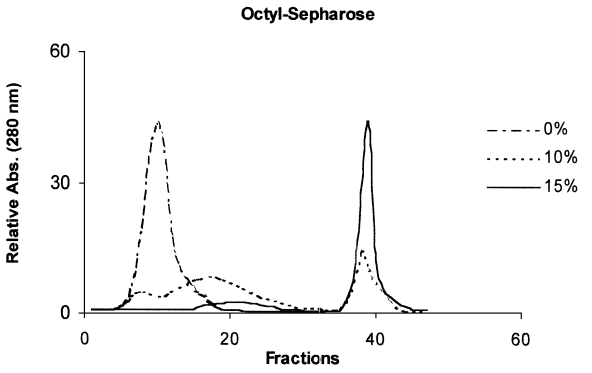

Fig. 4. Hydrophobic interaction chromatography on Octyl–Sepharose 4 FF. Buffer: 25 m*M* acetate, pH 4.8 containing 0, 10 and 15% (w/v) ammonium sulphate. Desorption (↓) was performed with 25 m*M* acetate buffer, pH 4.8.

obtained at an appropriate ammonium sulphate concentration in the eluent buffer: 7% (w/v) ammonium sulphate for Butyl–Sepharose (Fig. 2) and Phenyl–Sepharose (Fig. 3), 10% (w/v) ammonium sulphate for Octyl–Sepharose (Fig. 4), 13% (w/v) ammonium sulphate for Epoxy–Sepharose [18] and 16% (w/v) ammonium sulphate for PPG–Sepharose [27]. With the mild hydrophobic adsorbents Epoxy–Sepharose and PPG–Sepharose, a high salt concentration (13 and 16% (w/v), respectively) has to be used to the fractionation of the cellulolytic complex. On the other hand, it was observed that Octyl–Sepharose, despite having a long alkyl chain, needs a higher concentration of ammonium sulphate than Butyl–Sepharose (10 versus 7%, w/v, respectively) to obtain the above fractionation of cellulases. In fact, the ligand density of Octyl–Sepharose gel is lower than the one of Butyl–Sepharose (5 μmol/ml gel versus 50 μmol/ml gel). In general, an increase in the degree of substitution leads to an increase in protein retention capacities of HIC gels, due to the higher probability of forming multi-point attachment between the protein and the ligand [5]. The fractionation obtained with Phenyl–Sepharose was performed using only 7% (w/v) ammonium sulphate in the eluent buffer, due probably to the mixed aromatic and hydrophobic character of the ligand.

Thus, as expected, the amount of adsorbed cellulases depends not only on the salt concentration but also on the type and density of the ligand. Despite the similar chromatographic fractionation profiles obtaining when using the above salt concentrations, the selective separation of the cellulases in each case is quite different.

In fact, using 7% (w/v) ammonium sulphate with Butyl–Sepharose or Phenyl–Sepharose (Figs. 2 and 3) it was observed a selective fractionation of β-glucosidase in peak II (unretained enzymes), with a two-fold increase in specific activity and a recovery of 43% of cellobiase activity for Butyl-Sepharose and 47% for Phenyl–Sepharose (Table 1). Endoglucanases activity was detected with good recoveries in peak III (retained enzymes) showing also an increase in the specific activity (two-fold for Butyl–Sepharose and three-fold for Phenyl–Sepharose). In both peaks there was a decrease in the total cellulolytic activity compared to injected fractions. This can be explained by the fractionation of the different type of cellulases, that prevents enzyme synergism [14–16]. The methods used for measurement of the cellulase activities were used looking at the qualitative aspects of the cellulases and the synergism in mixtures, because they provide no information about the role of the individual cellulase in the hydrolysis. However, these methods can be useful to measure the variation of the hydrolysis ratio due to the presence or absence of certain type of cellulases and to give general information about the cellulases' activities [28].

The results obtained with Butyl–Sepharose and Phenyl–Sepharose were according to previous work with Epoxy–Sepharose [18]. In the peak II, 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% cellobiase activity was obtained (Table 1). Endoglucanase was also detected mainly in peak III with 50% activity recovery and a two-fold increase in specific endoglucanase activity. Total cellulolytic activity was decreased in both peaks compared with injected fractions. In the peak I it was not observed any type of cellulolytic activity.

When we tested a less hydrophobic adsorbent (PPG–Sepharose) [27], a higher concentration of ammonium sulphate had to be used to obtain cellulases fractionation. In this case, a selective separation of β-glucosidase was only partially obtained in peak II. The endoglucanases activity and total cellulolytic activity were mainly detected in peak III, with high

specific activities compared to injected fractions. Contrarily to the above cases, in this peak the cellulases were not fractionated and the total cellulolytic activity was increased due to the cellulases synergism. The experiments performed with Octyl–Sepharose (Fig. 4) using 10% (w/v) ammonium sulphate in the eluent buffer did not promote a selective fractionation of the cellulases. The β-glucosidase activity was detected in peaks II and III (50 and 39% activity recovery, respectively) despite showing an increase in specific activity compared to injected fractions. The endoglucanases activity was observed mainly in peak III and the total cellulolytic activity was too low in both peaks. As in above cases, it was not detected any type of cellulolytic activity in peak I.

In conclusion, the results obtained suggest that any of the HIC adsorbents are suitable for cellulases fractionation by HIC but this implies a correct selection of the properties not only of the chromatographic support but also of the ionic strength of the mobile phase. Using an appropriate salt concentration for each adsorbent and depending on the desired purpose, a selective fractionation of the different cellulases could be obtained. A selective β-glucosidase fractionation was obtained with Butyl–Sepharose, Phenyl–Sepharose and Epoxy–Sepharose using, respectively, 7 and 13% (w/v) ammonium sulphate in the eluent buffer with an increase in cellobiase specific activity.

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