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Cândida T. Tomaz[a]; Ana S. Rocha[a]; João A. Queiroz[a]
[a] Department of Chemistry, University of Beira Interior, Covilhã, Portugal

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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF *TRICHODERMA REESEI* CELLULASES ON POLYPROPYLENE GLYCOL–SEPHAROSE

Cândida T. Tomaz, Ana S. Rocha, and João A. Queiroz*

Department of Chemistry, University of Beira Interior,
6201-001 Covilhã, Portugal

ABSTRACT

The fractionation of *Trichoderma reesei* cellulases complex by hydrophobic interaction chromatography was investigated using a mild hydrophobic ligand—polypropylene glycol immobilized on Sepharose CL-6B. The influence of different salt type (sodium chloride, sodium sulfate, and ammonium sulfate) and their concentration in the mobile phase, on the chromatographic behavior of *T. reesei* cellulases was evaluated. A partial fractionation of β-glucosidase was obtained using 13 and 16% (w/v) sodium sulfate and ammonium sulfate in eluent buffer, respectively.

Key Words: Cellulases; Fractionation; Hydrophobic interaction chromatography; *Trichoderma reesei*

*Corresponding author. Fax: 351 275 319730; E-mail: jqueiroz@alpha2.ubi.pt

INTRODUCTION

The cellulase system of the fungus *Trichoderma reesei* has been found to be one of the most effective systems for hydrolysis of cellulosic materials. Cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and a cellobiase (β-D-glucosidase, EC 3.2.1.21) are the three different enzyme types of *T. reesei*. The enzymes act synergistically during hydrolysis of the cellulose, i.e., the action of a mixture of two or more individual cellulases is greater than the sum of the action of each enzyme (1–4). Endoglucanases cleave internal glucosidic bonds of the cellulose fibrils, increasing the number of free chain ends that can be attacked by the cellobiohydrolases. These enzymes act as exoglucanases promoting the cleavage of the ends of cellulose chains and releasing cellobiose as the main product. β-Glucosidase converts cellobiose to glucose, eliminating a strong inhibitor of cellobiohydrolases from the media (5).

Purification procedures for complex cellulase systems were usually based on chromatographic techniques, such as affinity chromatography (6) and ion exchange chromatography (7,8). Although hydrophobic interaction chromatography (HIC) is not widely used in cellulases purification, recently we have reported a selective fractionation of β-glucosidase from the *T. reesei* crude extract by this chromatographic technique (9). Hydrophobic interaction chromatography has become a new alternative for protein purification since it has the advantage of surface protein hydrophobicity. In HIC, proteins are bound to a hydrophobic stationary phase in the presence of salting-out salts, such as ammonium sulfate or sodium sulfate. The adsorption increases with high-salt concentration in the mobile phase and the elution is promoted by decreasing the salt concentration of the eluent (10,11).

However, in many cases, the usual hydrophobic ligands (e.g., *n*-alkyl or phenyl) may promote strong hydrophobic interactions that can result in an irreversible adsorption of the proteins or denaturation due to severe elution conditions (organic solvents, chaotropic agents, detergents). In order to overcome these difficulties, ligands with soft hydrophobicity have been utilized. They promote a mild interaction with the proteins, facilitating the posterior elution by decreasing the salt concentration of the mobile phase.

The mild stationary phases most widely used were polymers such as polyethylene glycol (PEG) or polypropylene glycol (PPG) immobilized on agarose supports (12). These stationary phases already have been used successfully in the purification of other proteins (13–15). The PEG was also utilized on a β-glucosidase fractionation in a *T. reesei* culture liquid by use of aqueous two-phase partitioning (16).

A β-glucosidase fractionation by HIC using an epoxy-activated spacer arm as the ligand with intermediate hydrophobicity (Sepharose CL-6B column modified by covalent immobilization of 1,4-butanediol diglycidyl ether) was

previously reported (9). The purpose of the present work is to study and to compare the influence of mobile phase composition (type of salt and its concentration) on *T. reesei* crude extract fractionation, using another hydrophobic stationary phase (Sepharose CL-6B modified by covalent immobilization of PPG). This mild hydrophobic ligand had been used successfully on *Chromobacterium viscosum* lipase purification (15).

MATERIALS AND METHODS

Materials

Sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). Polypropylene glycol diglycidyl ether (average number-average molecular mass, M_n, ca. 380) from Aldrich (Milwaukee, WI) and sodium borohydride was from Merck (Darmstadt, Germany). The filter paper used was Whatman No.1 (Whatman International, Maidstone, UK). Hydroxyethylcellulose (medium viscosity, degree of substitution 0.8) was from Fluka (Bucks, Switzerland). Microcrystalline cellulose (powder 20 μ) and D-cellobiose (98%, pred.β) were purchased from Aldrich (Milwaukee, WI). All other reagents used were of analytical grade.

The enzyme preparation was a commercial cellulolytic complex of *T. reesei* from Novo Nordisk, Denmark (Celluclast 1,5L).

Protein and Enzymatic Activity Assays

Protein concentration was determined according to the Bradford method (17), with crystalline bovine serum albumin as standard.

Total cellulolytic activity was determined by the method of filter paper activity (FPA). It was carried out at pH 4.8, 50°C on 50 mg filter paper (Whatman No.1) strips for 1 hr (18). 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 dinitrosalicylic acid (DNS) method using glucose as the standard (19).

Cellobiohydrolase and endoglucanase activities were quantified using, microcrystalline cellulose (20) and hydroxyethylcellulose (21) as substrates, respectively. The reducing sugars were determined by the DNS method (19).

β-Glucosidase activity was determined using cellobiose as the substrate. The reaction was performed at 50°C, for 30 min, using 50 *m*M citrate buffer, pH 4.8. The enzyme solution (0.1 mL), in different dilutions, was added to 0.4 mL of a 6.25 *m*M cellobiose solution in citrate buffer and incubated at 50°C. The reaction in the samples was stopped in a boiling water bath (100°C) for 10 min

and then the samples were transferred to cold water. The control samples were not incubated at 50°C, but immediately placed in the boiling water bath to inactivate the enzymes (22). The glucose released was measured by glucose oxidase method (23).

Preparation of the Gel

The gel was obtained by coupling the PPG diglycidyl ether to Sepharose CL-6B according to Sundberg and Porath (24) and previously reported by Diogo et al. (15).

Five milliliters of PPG diglycidyl ether were mixed with 5 g of suction-dried Sepharose CL-6B, previously washed with distilled water on glass filter-funnel. Then 5 mL of 0.6 *M* sodium hydroxide solution containing 10 mg of sodium borohydride was added. After the mixture of the suspension by rotation for 8 hr at 25°C, the reaction was stopped by washing the gel, on a glass filter-funnel with large portions of distilled water. The amount of bound epoxy groups was around 150 μmol/g dry gel. In order to inactivate the free epoxy groups, the PPG–Sepharose CL-6B gel was treated with sodium hydroxide 1 *M* overnight, at room temperature.

Chromatographic Method

The cellulase crude extract was fractionated, at room temperature, in a standard chromatographic system (Pharmacia, Sweden). Initially, the separation was performed by gel filtration on a Sephadex G-25M gel (35 × 1.6 cm I.D. column) equilibrated with 25 *m*M acetate buffer, pH 4.8, at a flow rate of 39.0 mL/hr. After an ultrafiltration step using an Amicon cell with a polyethersulfone membrane (Millipore, Bedford, MA) of 5000 Da nominal weight cut-off, the concentrated enzyme solution was fractionated by HIC, on a PPG–Sepharose CL-6B column. The gel was packed in a column (1.6 × 1.6 cm I.D.) and equilibrated with the desired mobile phase (25 *m*M acetate buffer, pH 4.8 with different concentrations of sodium chloride, ammonium sulfate, or sodium sulfate) at a flow-rate of 9.6 mL/hr. The enzyme solution (300 μL) was applied and the elution profile was obtained by continuous measurement of the absorbance at 280 nm. Fractions of 1 mL were collected. After elution of the unbound species, the ionic strength of the buffer was decreased (25 *m*M acetate buffer, pH 4.8) to promote the elution of bound species. The protein concentration and the activity towards filter paper, microcrystalline cellulose, hydroxyethylcellulose, and cellobiose were determined.

RESULTS AND DISCUSSION

In this work, the HIC experiments were performed after two sequential first purification steps of the *T. reesei* crude extract, as previously reported: a gel filtration on Sephadex G-25M and an ultrafiltration (9). The enzyme fractions thus obtained and then used in the HIC essays, had a cellulolytic activity of 8.0 U/mL with high recoveries of FPA (96%) and protein (73%). The initial crude extract had a FPA of 48.1 U/mL and 43.7 mg/mL of protein.

The global aim of this study was the analysis of the influence of some salts (sodium chloride, ammonium, and sodium sulfate) and its concentration, on HIC fractionation of the cellulolytic enzymes, using PPG–Sepharose CL-6B as the stationary phase (Fig. 1).

The change of the salt type in the eluent is an important parameter, not only in the protein retention, but also in the selectivity of HIC (25). According to Melander et al. (10,26), the effect of salt composition on the protein retention in HIC follows the Hofmeister (lyotropic) series for the precipitation of proteins or for their positive influence in increasing the molal surface tension of water. The experimental results show that using sodium chloride 2 *M*, the cellulases were not retained on the column (Fig. 2A). Even using the highest concentration of the sodium chloride (4 *M*) in the mobile phase, the binding of cellulases to the support was too low (Fig. 2B), because this salt has the smallest surface tension increment (1.64×10^3 dyn g/cm mol) (10). For ammonium and sodium sulfate (Figs. 3 and 4), the total retention of the cellulases was promoted with lower concentration of these salts (20 and 15% w/v, respectively) since they present high increments of molal surface tension (2.16×10^3 dyn g/cm mol for $(NH_4)_2SO_4$ and 2.73×10^3 dyn g/cm mol for Na_2SO_4) (10).

In HIC, an increase in salt concentration in the mobile phase, improves the hydrophobic interactions promoting an increase in the amount of the bound protein. In fact, the ionic strength is an important parameter that has to be selected carefully for cellulases fractionation by HIC. The effect of salt concentration in the eluent on the retention of the cellulases was performed using different percentages of ammonium sulfate and sodium sulfate in the mobile phase (Figs. 3 and 4). In a previous work (9), we already reported a fractionation of β-glucosidase from *T. reesei* extract with 13% (w/v) ammonium sulfate, on a Sepharose CL-6B column

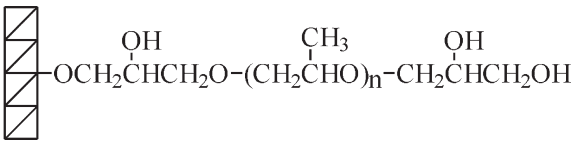

Figure 1. Schematic structure of the PPG–Sepharose CL-6B gel.

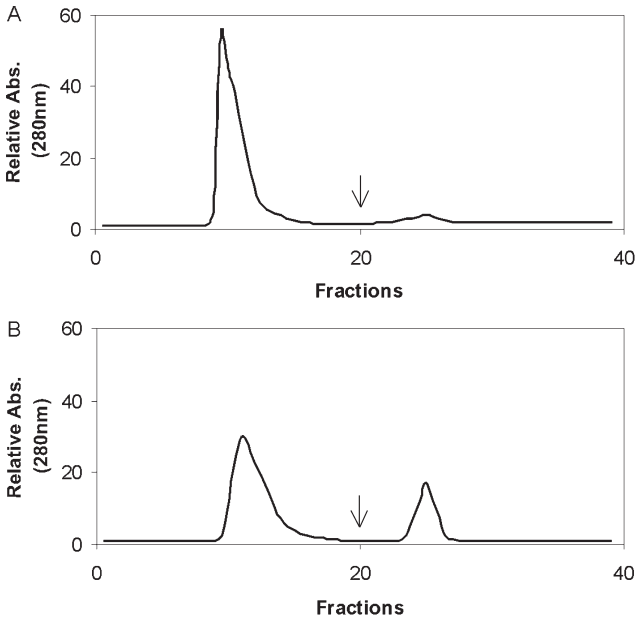

Figure 2. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffer: 25 *m*M acetate, pH 4.8 containing 2 *M* (A) and 4 *M* (B) NaCl. Desorption (↓) was obtained with 25 *m*M acetate buffer, pH 4.8.

modified by covalent immobilization of 1,4-butanediol diglycidyl ether. When we tested the PPG–Sepharose CL-6B support, we have obtained a similar chromatogram profile with three peaks but only using 16% (w/v) ammonium sulfate in the buffer (Fig. 3B). This support is less hydrophobic than the above mentioned and a higher concentration of the salt had to be used to promote the protein binding. The fractions of peaks I and II correspond to unadsorbed protein without any cellulase activity in I and some β-glucosidase activity and filter paper activities in II. Thus, a selective separation of β-glucosidase from the cellulase mixture was only partially obtained on the peak II fractions. After elution with 25 *m*M acetate buffer, pH 4.8, the fractions of bound cellulases (peak III) were obtained. The other cellulase activities were mainly detected in this peak. There was an increase in the specific activities of the endoglucanases, cellobiohydrolases, and also in FPA, compared with injected fraction. Using 15% (w/v) ammonium sulfate in the buffer with PPG–Sepharose CL-6B, we have obtained a cellulase fractionation in only two peaks (chromatogram not shown). The peak I corresponds to unbound proteins and has showed some FPA and most of the β-glucosidase activity. All the other cellulase activities were detected mainly in peak II.

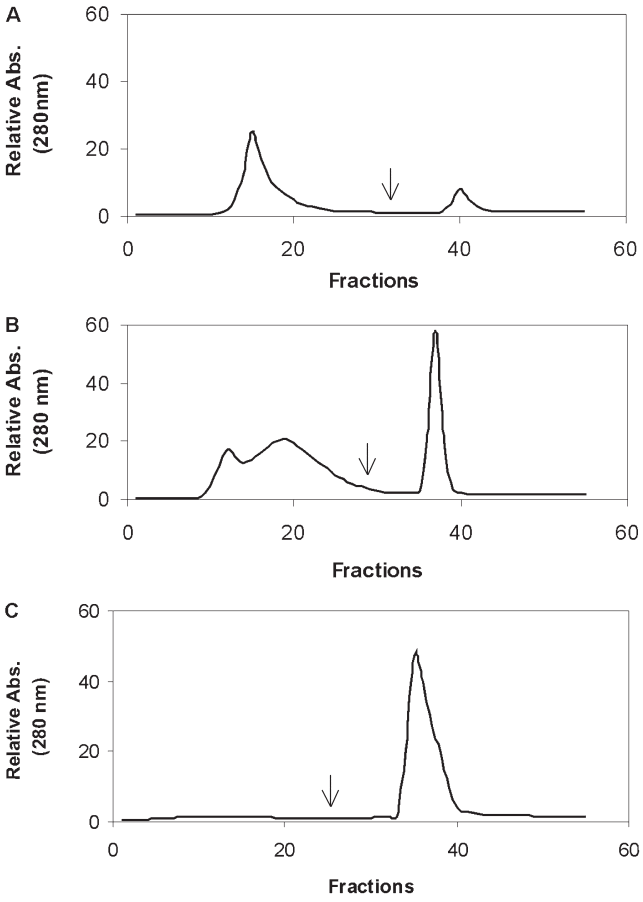

Figure 3. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffer: 25 *m*M acetate, pH 4.8 containing 13% (A), 16% (B), and 20% (C) (w/v) $(NH_4)_2SO_4$. Desorption (↓) was obtained with 25 *m*M acetate buffer, pH 4.8.

With 13% (w/v) ammonium sulfate in the buffer (Fig. 3A), we also obtained a fractionation of the cellulases in two peaks, but only the first (unbound cellulases) has showed cellulase activity. On the other hand, the peak (bound cellulases) obtained using 20% (w/v) ammonium sulfate in the buffer (Fig. 3C) has showed the same cellulase activity as the injected fraction. In both cases (13 and 20% ammonium sulfate), there was no fractionation and no increase in the specific activity of the different cellulases.

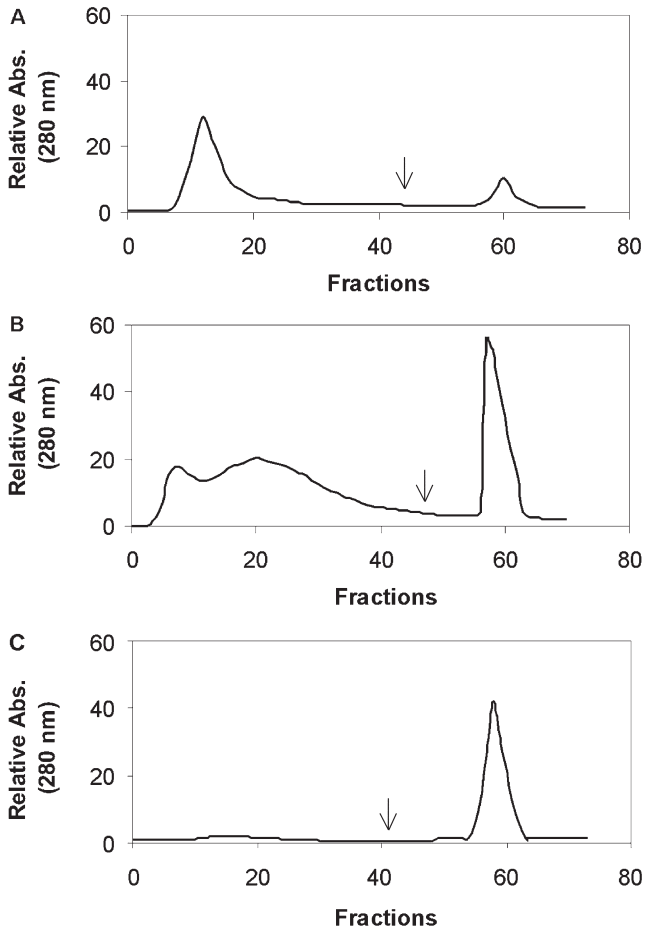

Figure 4. Hydrophobic interaction chromatography on PPG–Sepharose CL-6B column. Buffer: 25 *m*M acetate, pH 4.8 containing 10% (A), 13% (B), and 15% (C) (w/v) Na_2SO_4. Desorption (↓) was obtained with 25 *m*M acetate buffer, pH 4.8.

A change of ammonium sulfate salt in the mobile phase to sodium sulfate, which has a greater molal surface tension increment, results in a chromatogram profile with three peaks, using 13% (w/v) of this salt (Fig. 4B). In this case, like for ammonium sulfate, there was not a selective fractionation of β-glucosidase from cellulase mixture. The measured cellulase activities were similar to that obtained with 16% (w/v) ammonium sulfate, but in this case, we have obtained better recoveries in β-glucosidase activity in peak III (about 67%) and an increase

in the specific activity, when compared with that in the injected fraction. Using 10 and 15% sodium sulfate (Fig. 4A and C, respectively), we have obtained two chromatograms with the profiles and cellulase activities previously described for the ammonium sulfate assays (Fig. 3A and C). In this case, no increase was observed in cellulase activities or specific activities, when compared with that in injected fractions.

In this work, it is an important fact that the methods used for the quantification of cellulases activities were selected on the basis of the qualitative aspects of the individual cellulases and the synergism in mixtures. Although these methods provide no information on the role of the individual enzymes in the hydrolysis, they can prove to be important in the detection of the variation of the hydrolysis ratio due to the presence or absence of certain type of cellulases and to give global information about the cellulases activity of the sample (8).

It is known that the concentration of the salt strongly affects the selectivity in protein adsorption and this influence is dependent both on stationary phase and on the buffer salts (27). The PPG ligand is, in principle, less hydrophobic than 1,4-butanediol diglycidyl ether, since the PPG ligand has an alkane chain with three carbons (Fig. 1), whereas 1,4-butanediol diglycidyl ether has four $-CH_2-$ groups (28). In fact, the use of the mild PPG–Sepharose CL-6B support implies an increase in salt concentration to achieve the cellulases retention comparing to other more hydrophobic stationary phases, thus decreasing the selectivity of the chromatographic separation (27). But even using a low concentration of a salt with a high molal surface increment (e.g., sodium sulfate), the efficiency in the cellulases fractionation does not increase significantly with this mild ligand.

In conclusion, the chromatographic fractionation obtained for *T. reesei* crude extract with HIC, using a PPG–Sepharose CL-6B column, is rather different than the one obtained with the Sepharose CL-6B modified by immobilization of 1,4-butanediol diglycidyl ether. The results obtained suggest that the properties of the chromatographic support used play an important role in the separation of cellulases complex. In fact, it was found that the fractionation of *T. reesei* cellulases with PPG as the ligand is less effective but is also mainly based on their hydrophobic properties, since it is significantly affected by the salt used and by the ionic strength. Using sodium chloride in the eluent buffer, a small percentage of cellulases were retained on the column. On the other hand, ammonium sulfate promotes a partial β-glucosidase fractionation with 16% (w/v) in the eluent buffer whereas with sodium sulfate, these results are obtained using 13% (w/v) of salt in the eluent buffer. Using lower or higher of the above concentrations of any of these salts in the eluent buffer, no fractionation of cellulases were obtained.

In this work, we proved that a selective fractionation of cellulases by HIC is possible and appears to represent a new purification procedure, since a careful selection of the characteristics of the stationary and mobile phases is made.

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