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Purification of cellobiohydrolase I from *Trichoderma reesei* using monoclonal antibodies in an immunomatrix

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SUMMARY

The several components of the fungal cellulase system present practical problems in devising facile and efficient schemes for their purification. We report on a new single-step affinity chromatographic method for purification of cellobiohydrolase I of *Trichoderma reesei* based on its selective absorption and elution using an immunomatrix constructed with CnBr-activated Sepharose 4B and monoclonal antibody specific for the enzyme. Isoenzymes of cellobiohydrolase I were purified directly from crude culture filtrate. The method is fast, simple, and of high resolution.

INTRODUCTION

Fungal cellulase is an enzyme complex comprised of three enzymes, cellobiohydrolase (CBH), endoglucanase, and β-glucosidases, which act cooperatively and synergistically to degrade cellulose [14]. Multiple forms for each enzyme [7,9,18,21] complicate the purification of the individual cellulase components. Indeed, a whole gamut of purification procedures (ion-exchange resins, molecular exclusion, and a variety of electrophoretic protocols) have been used sequentially with only varying degrees of success. We therefore considered the use of specific immobilized antibodies for the selective purification of the cellulase components. Polyclonal antibodies prepared toward purified CBH I have shown cross-reactivity with endoglucanases [19,21]. We therefore developed several hybridomas that secrete monoclonal antibodies against CBH I [19], and now report on the use of immobilized monoclonal antibodies, from one cell line, for the purification of this enzyme.

MATERIALS AND METHODS

Cellulase preparation

Cellulase was prepared by growing *Trichoderma reesei* Rut-C30 (ATCC 56765) in Mandel's medium

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[15] in a 7 l fermentor, for 7 days at 30°C. The culture broth was collected, clarified, and lyophilized. Protein in crude preparations was estimated spectrophotometrically (1.45 (A_{280}) − 0.74 (A_{260}) = mg protein/ml) [4]. The crude enzyme powder, containing 21% protein, was resuspended in 0.1 M phosphate-buffered saline, pH 7.4 (PBS).

Monoclonal antibodies

The preparation and characterization of monoclonal antibodies against CBH I has been described [19]. A single hybridoma cell line was employed for the production of monoclonal antibody used in preparation of the immunomatrices. For ascites production, $1 \cdot 10^6$ cells were injected intraperitoneally into each of several Balb/C mice (Charles River Breeders. Wilmington, MA) which had been previously primed with pristane. Monoclonal antibody was purified from the ascites fluid by passage through a column of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). The bound antibody was eluted with 0.1 M citrate-phosphate buffer, pH 4.3, containing 0.15 M NaCl.

Preparation of immunomatrices

Purified monoclonal antibody was coupled to CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's protocol [1]. Two columns were prepared. For the first column, 25 mg of purified antibody in 15 ml of coupling buffer (0.1 M Na_2HCO_3, pH 8.3, containing 0.5 M NaCl) was added to 10 ml of activated gel in a centrifuge tube. The contents of the tube were mixed gently overnight (4°C) on a roller shaker. The gel was transferred into blocking agent (1 M ethanolamine, pH 8.0) for 2 h, washed to remove excess protein and blocking agent, and packed into a glass column (1 cm diameter). The second column was prepared in the same manner, except 57 mg of purified antibody was used. The two columns contained approximately 24 and 55 mg of bound protein, respectively, from the original 25 and 57 mg.

A monoclonal antibody protein A-agarose (Boehringer Mannheim Biochemicals, Indianapolis, IN) matrix was prepared as outlined by Schneider et al. [20]. 50 mg of monoclonal antibody in 8 ml of 0.1 M borate buffer, pH 7.4, was mixed with 5 ml of protein A-agarose gel. After completion of the coupling, cross-linking, blocking and washing, the gel was packed into a glass column (1 cm diameter). 32 mg of antibody was bound to the gel.

Immunoadsorption

Lyophilized crude enzyme (25 mg protein) was resuspended in 1 ml of PBS and applied to the columns of antibody-Sepharose 4B. These columns were washed extensively with PBS containing 0.1% Tween 20 until the absorbance was less than 0.02 at 280 nm. Bound material was eluted with either 0.15 M NaCl in 0.1 M glycine buffer, pH 2.5, or 0.25 M NaCl in 0.1 M citrate-phosphate buffer, pH 4.0. Eluates in glycine buffer were neutralized immediately with 2 M Tris, pH 10. The protein content of the eluted material was monitored spectrophotometrically at 280 nm. Gum and Brown [13] have established the extinction coefficient ($E^{1\%}_{280}$) for purified CBH I as 14.2.

Polyacrylamide gel electrophoresis (PAGE)

Proteins in crude fungal culture broth and protein eluted from immunoadsorbent columns were subjected to PAGE using the discontinuous buffer system of Brewer et al. [6] the sodium dodecyl sulfate-PAGE (SDS-PAGE) method of Anderson et al. [3] and the isoelectric focusing (IEF) protocol described by Farkas et al. [11]. Gels containing the separated proteins were stained for protein with Coomassie brilliant blue or analyzed for enzymatic activity (see below).

Measurement of enzymatic activities

The presence of exoglucanases and endoglucanases in gels was visualized using the oligosaccharide/ tetrazolium chloride (TTC) and carboxymethyl cellulose (CMC)/Congo red procedures described by Bartley et al. [5]. β-Glucosidase activity was determined using 4-methylumbelliferyl β-glucoside as substrate with detection of the aglycone product via its UV fluorescence [8].

Proteins eluted from the affinity columns were pooled and concentrated by ultrafiltration (PM 10

membrane in an 8200 ultrafiltration cell, Amicon Corp., Danvers, MA) to 1 mg/ml. The hydrolytic activities of these preparations towards carboxymethyl cellulose (7L CMC) (Hercules Corp. Wilmington, DE) and acid-swollen cellulose (CC-41) (Whatman Co. Clifton, NJ) were determined using the dinitrosalicylic (DNS) method of Miller [16] for measuring the release of reducing sugars. β-Glucosidase was assayed either by using *p*-nitrophenyl-β-D-glucoside (pNPG) as the substrate and following the release of *p*-nitrophenol (pNP) [12] or by using cellobiose as the substrate and following glucose production with the Glucostat reagent [12] (Sigma Fine Chemicals, St. Louis, MO). Xylose oligomers produced by xylanases were measured colorimetrically using DNS, with xylose as a standard.

RESULTS

In the first experiment on affinity purification of the cellobiohydrolase I, an excess of lyophilized culture broth (25 mg protein in 1 ml of PBS) was applied to the Sepharose 4B column that contained 24 mg of antibody. The bound protein was eluted with 0.15 M NaCl in glycine buffer, pH 2.5. Samples of the purified protein (30 μg) and culture broth (150 μg) were analyzed by non-denaturing PAGE electrophoresis. At least ten major proteins were visible in the original culture filtrate after Coomassie blue staining (Fig. 1, lane 2), while only two closely separated proteins were detected in the purified material (Fig. 1, lane 1). The two proteins in this purified preparation had migration patterns identical to those found for a CBH I preparation that had been purified by preparative IEF (Frein, M. (1986) Ph.D. Thesis, Rutgers University) (Fig. 1, lane 3). Both proteins in the immunoaffinity purified preparation showed glucanase activity by the TTC assay (Fig. 1, lane 7) and gave negative results for endoglucanase activity in the CMC/Congo red assay (Fig. 1, lane 4). Neither purified protein reacted positively in a gel overlay assay for β-glucosidases (result not shown). The overall results indicate that both purified proteins are exoglucanases, and this was confirmed by their much greater activity towards acid-swollen cellulose than to CM cellulose (Table 1). Exoglucanases (Fig. 1, lane 6), endoglucanases (Fig. 1, lane 5) and β-glucosidases (not shown) were clearly evident in the original culture broth.

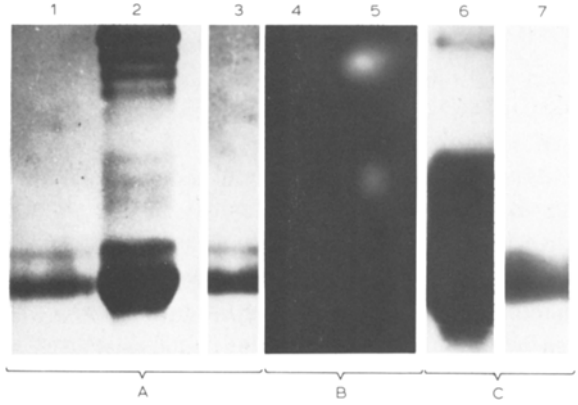

Fig. 1. Electrophoretic characterization of crude cellulase and affinity purified CBH I. Proteins were subjected to electrophoresis under non-denaturing conditions, then stained for protein (Coomassie brilliant blue R-250) and cellulase activities. (A) Protein stain; (B) CMC agar replica of (A); (C) cellulo-oligosaccharide-TTC activity stain. Lanes 1, 4 and 7, affinity purified CBH I; lane 3, CBH I purified by IEF; lanes 2, 5 and 6, crude cellulase.

Unfortunately, the continued use of glycine buffer as an eluent caused a rapid deterioration of the binding capacity of the column. After four successive adsorption/elution cycles less than 50% of the original binding capacity remained (< 1 mg). Gentler desorption methods were explored in an effort to stabilize the binding capacity of the affinity matrix. Using this same column it was found that elution with 0.25 M NaCl in 0.1 M citrate-phosphate buffer, pH 4.0, prevented additional loss in binding capacity after 15 elutions. Although the time required for elution of the bound protein was longer than with 0.1 M glycine buffer, pH 2.5, CBH I could still be purified in less than 1 h.

Routinely (over ten runs) 0.25 M NaCl in citrate-phosphate buffer was used to elute CBH I from the second Sepharose column that contained 55 mg of monoclonal antibody. Consistently, 3.5 mg of purified CBH I was obtained from samples of the crude enzyme containing 25 mg of protein. When the affinity purified material, now lacking the

Table 1

Affinity purification of cellobiohydrolase

The single purification step resulted in a 16.94-fold purification from a crude preparation that initially contained cellobiohydrolase as a major component. The purified fractions lacked activity towards cellobiose and *p*-nitrophenyl-β-D-glucoside, indicating the absence of β-glucosidase.

Enzyme	ASC'ase (specific activity: U/mg)	CMC'ase	ASC'ase/CMC'ase
Crude cellulase	5.7	15.3	0.373
Affinity purified	2.2	0.37	5.946

salts and other components found in crude cellulase, was chromatographed on the Sepharose column, 7 mg of CBH I was obtained, i.e., twice the prior amount bound.

The affinity purified material was further characterized by IEF-PAGE and SDS-PAGE. This preparation yielded a single wide band (M_r between 64000 and 68000) on SDS electrophoresis (Fig. 2, lane 2). Separation by IEF over the pH range 3–10 revealed three proteins. When isoelectrically focussed over the pH range 4.0–6.5 these three proteins are clearly separated and have p*I* values of 4.25, 4.15, and 4.05, respectively (Fig. 2, lane 3). The third protein (p*I* 4.05) was barely detectable even when 50 μg of material was applied.

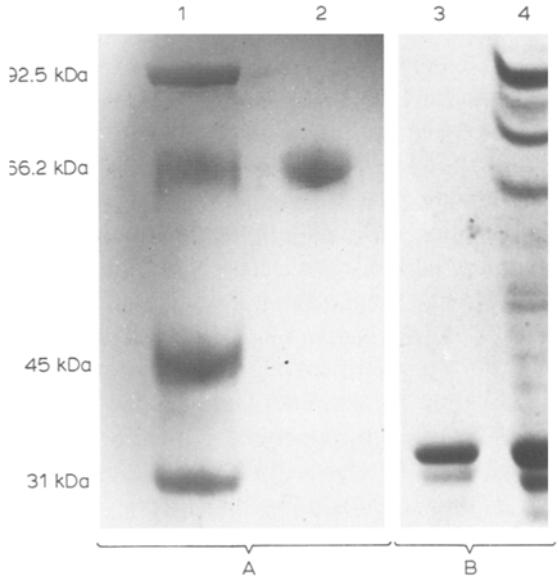

Fig. 2. Electrophoretic characterization of crude cellulase and affinity purified CBH I. Proteins were subjected to SDS electrophoresis (A), and IEF over the pH range 4.0–6.5 (B). Proteins were stained with Coomassie brilliant blue R-250. Lane 1, molecular weight standards; lanes 2 and 3, affinity purified CBH I; lane 4, crude cellulase.

Although good yields of CBH I purified on the antibody-Sepharose column were obtained, it was possible that other immunomatrices would be more efficient. One other support, protein A-agarose, was investigated. It was selected because protein A binds to the Fc (constant fragment) of immunoglobulins, thereby offering optimal spatial orientation of antibody molecules. Initially, 4 mg of enzyme could be purified on a 5 ml column but, unfortunately, after six absorption/elutions cycles (using culture broth as the starting material) the yield of purified CBH I declined to 2 mg.

DISCUSSION

Monoclonal antibody immobilized on Sepharose 4B provided an effective matrix for the affinity purification of CBH I. In a single step, CBH I free from endoglucanases and β-glucosidases was obtained (Fig. 1). The purified enzyme was characterized by its activity on acid-swollen cellulose (ASC), and lack of activity towards other specific substrates (Table 1).

When the binding properties of a protein A immunomatrix were compared to CnBr-Sepharose 4B, the former was found to be more efficient. However, its use is currently unjustified due to a rapid loss of adsorptive capacity and the relatively high cost of the matrix.

Two exoglucanases are present in the immunoaffinity purified preparation. These two proteins were separated by Davis PAGE and identified as exoglucanases by 'in gel' assays (Fig. 1, lane 7). They appear to be isoenzymes of CBH I from their similarities in molecular mass (64–68 kDa), p*I* values (4.05–4.25), and mobility in non-denaturing PAGE. Gum and Brown [13] proposed that there are slightly different forms of the CBH I glycoprotein which have identical roles in cellulose degradation. Such protein variants could arise from proteolytic post-translational changes [9,17 and Kelleher, T.K. (1981) Ph.D. Thesis, Rutgers University]. Alternatively, the multiplicity of enzyme forms, in some part, may reflect the binding of extracellular polysaccharides, derived from cell wall components or reaction products, to CBH I [2,22].

The antibody-Sepharose 4B matrix provides a useful purification method. It is most effective in comparison to ion-exchange chromatography, gel filtration and other routine biochemical protocols which yield a purified product only after a series of sequential steps. Alternatively, CBH can be purified, in a single step, through its adsorption to amorphous cellulose [18]. However, in this approach a great excess of protein was used to eliminate the non-specific adsorption of proteins other than CBH. This resulted in low yields of enzyme in relation to the amount of the absorbent. The Sepharose 4B matrix, in comparison, provides a useful, simple, rapid and very reproducible means for preparing purified CBH I in a single step.

Polyclonal antibodies have been widely employed for the purification and detection of proteins. However, some polyclonal preparations cross-react with proteins that are similar in structure, but functionally different, from the protein used for immunization. Such preparations would be undesirable for the construction of matrices for separating the pure proteins in the complex crude cellulase mixture. Fägerstam and Pettersson [10] found that antiserum toward a homogeneous Avicelase reacted with several proteins in *T. reesei* culture broth, although the activity, such as endoglucanase, β-glucosidase or cellobiohydrolase action, of these proteins was not determined. Recently, the cross-reaction of polyclonal antiserum toward CBH I and a purified endoglucanase [21] and other cellulases [19] has been reported. It is unlikely that immunomatrices constructed with these polyclonal preparations would be useful for the separation of purified CBH I, free from endoglucanases and other proteins, from crude cellulase.

Cross-reactive antibodies can be removed from a polyclonal preparation if purified antigen is available. Nummi et al. [18] used only the antibody fraction which formed an immunoprecipitate with CBH, to construct an immobilized polyclonal antibody matrix for the purification of exoglucanase. However, only 0.5 mg of CBH I was recovered from a matrix which contained 200 mg of antibody immobilized to a 15 ml column of CnBr-Sepharose 4B. Thus, the binding capacity of this matrix was extremely low (0.25%) in relation to the original amount of bound polyclonal antibody. In comparison, our monoclonal immunomatrix had a binding capacity of 6.4%; the matrix retained 3.5 mg of CBH I per 55 mg of immobilized antibody. This 20-times greater binding capacity for CBH I was obtained repeatedly during enzyme purification using the monoclonal immunomatrix. The reasons for this enhanced binding have not been determined.

Monoclonal antibody (to CBH I) is a homogeneous preparation of specific antibodies which can be produced in almost unlimited supply. For these reasons, monoclonal antibodies have been used to construct affinity matrices which are highly selective for purification of CBH I, and can be used repeatedly without a reduction in binding capacity.

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