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Monoclonal Antibody Purification of *Trichoderma reesei* EG I

Scientific Note

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INTRODUCTION

The cellulase system of *Trichoderma reesei* has been studied extensively within the last 30 years owing to the intense interest in applying these enzymes to industrial applications. The enzymes responsible for the degradation of cellulose by this fungus have been identified as cellobiohydrolase (EC 3.2.1.91) (CBH), endoglucanase (EC 3.2.1.4) (EG), and beta-glucosidase (EC 3.2.1.21). These enzymes work in a synergistic manner to accomplish the efficient breakdown of cellulose into utilizable substrates (*1*). The application of cellulases today is quite varied, ranging from food flavoring to the production of organic feedstocks (*2*).

Although cellulase enzymes hold promise for the utilization of renewable resources, such as the lignocellulosics, their application to such systems requires a reduction in the cost of enzyme production. A key feature to reaching such a goal lies in the detailed understanding of the synergism that plays a role in the conversion of cellulose. In order to study this problem, the purified cellulase enzyme components (i.e., CBH I, CBH II, EG I,

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and EG II) must be readily available in milligram to gram quantities. Because these four enzymes are very similar in their physicochemical characteristics (*3,4*), separation and purification of these proteins can be quite difficult. Prior purification efforts of the individual components of cellulase systems in general have been obtained by sequential procedures, such as gel filtration, ion exchange, chromatofocusing, and preparative isoelectric focusing (*3–6*). Despite the high apparent enzyme purity obtained from these procedures, the deleterious effect of such multistep, sequential purifications on the configuration and activity of the native protein is unknown. Therefore, it is advantageous to obtain purified cellulases through limited purification protocols and eventually to compare the activity of these purified cellulases.

Since Kohler and Milstein published their landmark paper on the production of B-lymphocyte hybridomas (*7*), monoclonal antibodies (MAbs) have been utilized in a variety of ways. Affinity purification by MAbs has proven to be very useful in the separation of specific proteins from complex mixtures. In this study, we report our progress in purifying the major endoglucanase of *T. reesei*, EG I, using MAbs specific for this enzyme.

MATERIALS AND METHODS

Purified Enzymes and Reagents

All chemicals, buffers, and detergents used in this study were of analytical grade and obtained from major supply houses. The purified *T. reesi* cellulase enzymes, CBH I and EG I, were a gift from Sharon Shoemaker.

Monoclonal Antibody Production

Six- to eight-week-old female Balb/c mice were inoculated subcutaneously (sc) with approximately 50 μg purified EG I emulsified in 0.5 mL of Freund's complete adjuvant (Difco Lab, Detroit, MI) and 0.5 mL of sterile phosphate-buffered saline (PBS), pH 7.0. After 2 wk, the mice were boosted intraperitoneally (ip) with 50 μg purified EG I emulsified with 0.5 mL of incomplete Freund's adjuvant and 0.5 mL of sterile PBS, pH 7.0. Ten days later, mice were bled for antibody (Ab) titer verification via ELISA. Three days prior to fusion, the mouse to be used for fusion was inoculated with 50 μg purified EG I in PBS, pH 7.0. On the day of fusion, the mouse was sacrificed by neck dislocation and the spleen removed. The splenocytes were collected and diluted 1:40 with PBS and a hemolyzing agent (Curtin Matheson Sci., Houston, TX), then counted on a hemocytometer.

Spleen cells and a nonsecreting myeloma cell line, SP2/O, (*8*) were washed 3× with 30 mL of incomplete RPMI (Sigma Co., St. Louis, MO). Incomplete RPMI (I-RPMI) is tissue culture medium containing penicillin, streptomycin, glutamine, and sodium pyruvate. The cells were mixed

together at a ratio of 1:4 (myelomas:splenocytes) for the last two washes. After the last wash, as much medium as possible was discarded and the cell pellet disrupted by gentle external mixing.

Four grams of polyethylene glycol (PEG) (Merck, Darmstadt, W. Germany) was placed in a sterile vial, capped, and microwaved on high until melted. Four mL of I-RPMI was added to the vial, and this 50% PEG was kept at 37°C. To every 1.6×10^8 spleen cells, 1 mL of 50% PEG was added. PEG fusion was performed by placing the 50 mL conical tube containing the spleen cell–myeloma mixture in a 37°C waterbath. The fused cells were immediately centrifuged at 800*g* for 5 min. The medium was poured off and the cell pellet was gently disrupted by external mixing. For every 1.6×10^8 spleen cells fused, 22 mL of C-RPMI was added to the cells. Complete RPMI (C-RPMI) is I-RPMI containing 10% fetal bovine serum. One drop from a sterile 10 mL pipet was placed in each well of a 96-well plate (Nunclon, Roskilde, Denmark), and the plates were placed in a CO_2 incubator at 37°C. Wells were fed with a C-RPMI-HAT (hypoxanthine-aminopterine-thymidine) medium over the next 7–10 d (*9*). In this time period, wells were examined for a color change in the medium (indicating growth) and assayed via an indirect ELISA.

Indirect ELISA

In order to detect hybridomas producing MAb against EG I, an indirect ELISA (Enzyme-linked immunoadsorbent assay) was performed on all cultured plates (*10*). Wells on polyvinylchloride microtiter plates (Becton-Dickinson, Oxnard, CA) were coated with 100 ng of conventionally purified EG I and tested for reactivity. Hybridomas of interest were brought up to 10 mL for dilution cloning. Hybridomas that were successfully dilution cloned and producing MAb to EG I were tested for specificity by Western blots.

Western Blotting

To verify the specificity of the MAb, conventional Western blots were performed (*11*). Briefly, approximately 5 μg of purified cellulases (EG I, EG II, CBH I, and CBH II) were electrophoresed on a 12.5% SDS-PAGE gel and blotted overnight on nitrocellulose sheets. The next day, the blots were blocked with a 10% nonfat dry milk-Tris-EDTA-NaCl buffer, pH 7.4, and reacted with supernatants from the MAb of interest. Goat antimouse IgG (Cappel, Malvern, PA) antibody at a dilution of 1:1000 was then added. Reactive MAbs were detected by the addition of diaminobenzidene, H_2O_2, and 0.05*M* ammonium acetate, pH 5.0.

Prior to EG I purification by affinity chromatography, it was necessary to verify the presence of EG I in the cellulase preparation to be used for affinity chromatography. A 12.5% SDS-PAGE gel containing 5 μg purified EG I and 10 μg Genencor Cellulase 150 L was prepared and blotted, as described above.

Ascites Production

Mice used for ascites production were injected with 0.5 mL pristane (Sigma, St. Louis, MO) 1–4 wk prior to ip injection of hybridoma cells. One milliliter of growing cells was placed in 9 mL of C-RPMI and grown for 3 d. These 10 mL were then mixed with 20 mL C-RPMI in a 75 cm^2 flask, and cells were grown overnight. For each mouse, 5×10^6 cells were removed from the 75 cm^2 flasks and placed into 15 mL conical tubes. The cells were centrifuged at 800*g*, supernatant discarded, and the cells were then suspended in 1 mL of I-RPMI and injected ip into recipient mice. Ascites fluid was collected 5 d later and purified via protein A affinity chro matography (Bio-Rad, Richmond, CA) (*12*).

Affinity Chromatography of EG I

Eight milliliters of a commerical-activated agarose preparation, Affigel-10 (Bio-Rad, Richmond, CA) was sensitized with 120 mg purified anti-EG I MAb. The gel was poured into a 1×30 cm column and equilibrated with 50 m*M* phosphate buffer–1% Tween 20, pH 7.4. Additionally, 1 mL Affi-gel-10 was sensitized with 10 mg of a MAb specific for CBH I, which was obtained in a procedure similar to that described above for anti-EG I MAb production.

The cellulase to be applied to the immunomatrix for purification of EG I (Genencor Cellulase 150L, Genencor, San Francisco, CA) was dia-filtered with 50 m*M* sodium acetate buffer, pH 4.5. After equilibrating the column containing the anti-EG I MAb, 32 mg of the diafiltered cellulase was diluted 1:4 with 50 m*M* phosphate buffer, pH 7.4, and applied to the column. The column was then extensively washed with application buffer. Elution of bound protein was achieved with 50 m*M* glycine–HCl buffer, pH 2.8. The eluted protein was immediately neutralized using 50 m*M* Tris buffer, pH 8.0.

The eluted protein obtained from the anti-EG I column was then applied to a column containing anti-CBH I MAb. The column was equilibrated and the protein eluted, as mentioned above.

RESULTS

Through various murine fusions, we were able to obtain a MAb that was specific for EG I. When the MAb was tested via Western blots against three other purified enzymes of the *T. reesei* cellulase system, it recognized only EG I (*see* Fig. 1). After ascites production, we were able to harvest approximately 200 mg of this purified antibody. Figure 2 demonstrates the purity of the MAb after Protein A affinity chromatography. Ascites from a previously produced MAb recognizing only CBH I is also shown.

Figure 3 shows a 12.5% SDS-PAGE gel containing 5 μg of the purified EG I that was used for inoculation of mice and 10 μg of the commercial

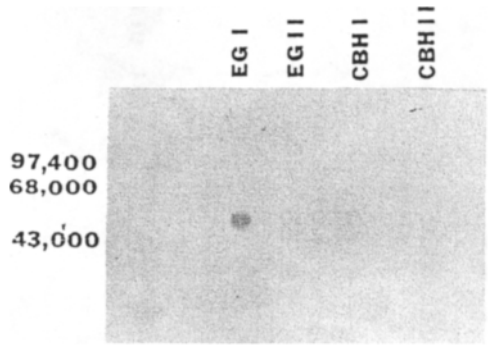

Fig. 1. A conventional Western blot indicating the MAb specificity for EG I. Note no cross-reaction with the other three purified cellulases.

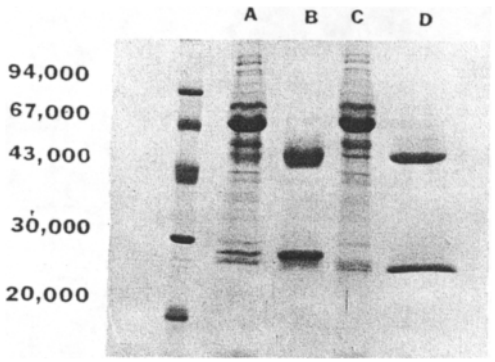

Fig. 2. SDS-PAGE gel demonstrating the purified MAbs and the ascites from which they were obtained. Lane A: 10 μg anti-CBH I ascites; Lane B: 5μg purified anti-CBH I MAb; Lane C: 10 μg anti-EG I ascites; and Lane D: 5 μg purified anti-EG I MAb. Owing to the reduction of the MAbs, the heavy and light chains are seen as two distinct bands on lanes B and D.

cellulase (i.e., Genencor 150L) to be purified. The presence of EG I and other proteins in the cellulase is evident. It was necessary to verify that the MAb specific for EG I would recognize the EG I contained in the crude cellulase sample that was to be chromatographed. Figure 4 shows a Western blot indicating the recognition of EG I in the commercial cellulase along with the purified EG I as a control. It is observed that MAb reacts with only the EG I present in the commercial cellulase. This indicated that purification of EG I from this preparation could be achieved. A heavier

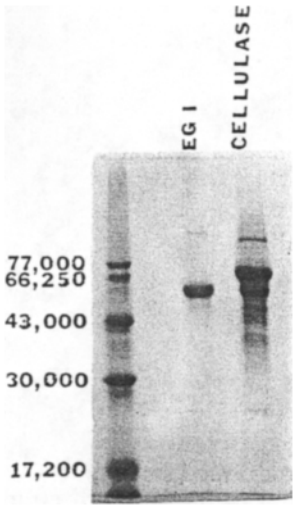

Fig. 3. A 12.5% gel indicating the presence of EG I in the commercial cellulase to be chromatographed.

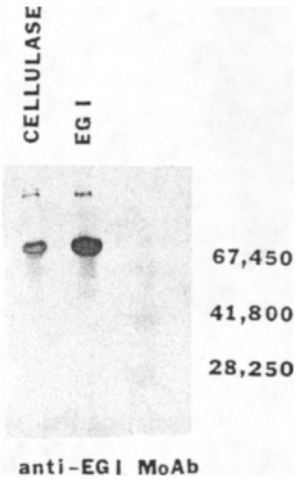

Fig. 4. A Western blot taken from a gel similar to Fig. 3. When this blot was reacted with anti-EG I MAb, only EG I was recognized in the cellulase preparation. Purified EG I was used as a control.

molecular weight band in both lanes (i.e., crude cellulase and conventionally purified EG I) is also detected by the MAb. We speculate this band to be the result of aggregation of EG I, the cause of which is unknown.

Figure 5 illustrates the elution profile of the commercial cellulase using an Affigel-10 column containing anti-EG I MAb. Approximately 400 μg of protein was detected after elution of bound proteins. Initial fractions from affinity chromatography containing protein after elution were

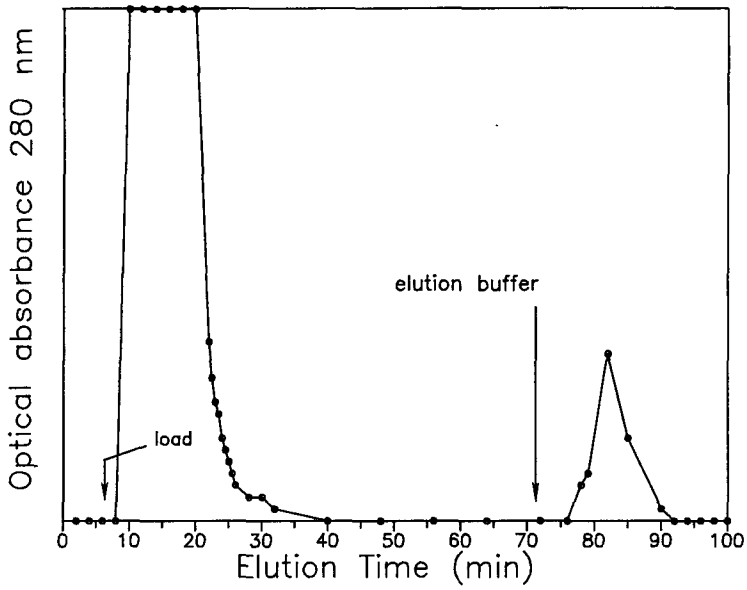

Fig. 5. An elution profile of the commercial cellulase as it was chromatographed through the column containing anti-EG I MAbs. Approximately 400 μg total protein was eluted per run after elution. Eluted protein contained EG I and detectable amounts of CBH I.

analyzed by SDS-PAGE and found to contain nonspecifically bound CBH I (data not shown). The addition of 0.1% Tween-20 to the application buffer did not eliminate CBH I.

In order to remove the nonspecifically bound CBH I, the eluted fractions obtained from the anti-EG I Affigel-10 column were concentrated and applied to an Affigel-10 column containing anti-CBH I MAb for approximately an hour. The void volume was collected, concentrated, and analyzed by SDS-PAGE. Figure 6 shows the purified EG I after removal of CBH I. Protein content of affinity purified EG I was 20–50 μg.

DISCUSSION

When a microorganism such as *T. reesei* is grown in culture and the culture broth is harvested, a vast array of proteins is present. In order to study the enzymes capable of cellulose degradation, it is highly desirable to purify to homogeneity those enzymes of interest for further studies.

The traditional method of cellulase isolation has been one of sequential, conventional purification procedures. It is believed that sequential purification steps are detrimental and could inactivate some of these enzymes (*13*). For this reason, it is advantageous to minimize the number of purification protocols by simpler methods, such as affinity chromatography. With the advent of MAbs, the utilization of these immunoglobulins

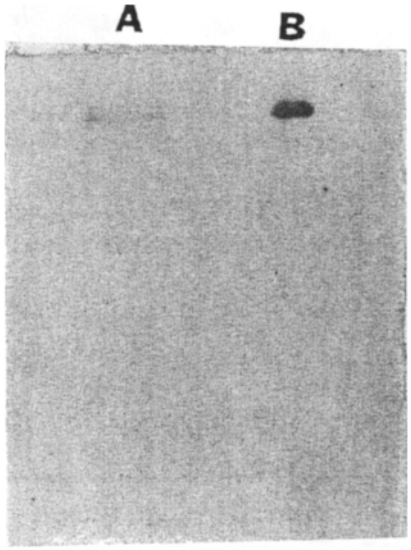

Fig. 6. SDS-PAGE gel demonstrating 6 μg of the affinity purified EG I (Lane A) and 8 μg of the purified EG I used to obtain the hybridoma cells secreting anti-EG I MAbs (Lane B).

for the purification of proteins has shown a great promise in the last decade. Previous reports have dealt with the purification of cellulases by using polyvalent antibodies (*14,15*). Although this was useful, the results of cross-reactivity of the various isoenzymes of endoglucanases and cellobiohydrolases was not eliminated, because these polyvalent antibodies react with epitopes common to all isoenzymes present.

By utilizing MAbs, it was possible to separate and study individual cellulases without the effects of cross-reactivity. MAbs to specific cellulases from *T. koningii* and *T. reesei* (*16,17*), EG I and CBH I, respectively, have been recently reported. Of these, only the anti-CBH I MAb was used for the purification of CBH I (*18*). By producing MAb specific for EG I from *T. reesei,* we have successfully purified the enzyme from diafiltered culture broth.

These results indicate that affinity purification of EG I is possible after elimination of nonspecifically bound proteins. The cause of such protein binding to a matrix and/or ligand can be attributed to ion exchange binding, hydrophobic interaction with matrix or bound material, or conformational occlusion (*19*). Attempts to decrease this phenomenon by utilizing a detergent, such as Tween-20, were not successful. Only when the elution fractions obtained from the anti-EG I MAb column were passed through a column containing anti-CBH I MAb was the CBH I removed. This technique may prove to be useful in larger scales by obtaining both CBH I and EG I in a two-step affinity purification procedure.

At this time, why only microgram amounts of EG I were obtained may only be speculated. The binding of the purified MAb to the maxtrix is through primary amine groups. This type of reaction tends to decrease the total efficiency of the bound MAb by binding the antigen-binding sites of the molecules. In addition, only 30% of the cellulase protein is associated with endo-1,4-β-glucanase, whereas 70% is a 1,4-β-glucan cellobiohydrolase (*20*). Thus, a higher yield can be expected for CBH I than EG I.

It is foreseeable that, in the near future, the various specific components of the *T. reesei* cellulase system can be separated by optimizing these procedures. In order to increase the yields, large scale MAb production and application must be investigated.

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