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Purification of Endoglucanases from Crude Cell Culture Supernatant by Preparative Native PAGE

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Abstract

Microbial cellulases are a source of interest for industrial applications. Thermostable cellulases are especially useful for such purposes. This report describes the use of preparative native PAGE with the Model 491 Prep Cell for purification of 5-6 endoglucanases from a newly discovered thermophilic bacterium. The cellulase-containing culture supernatant from the bacterium was harvested following centrifugation and ultrafiltration. Purification of the cellulases was achieved by applying aliquots of the supernatant directly to a native polyacrylamide gel electrophoresis system in the Model 491 Prep Cell.

This technique separates endoglucanases that were otherwise difficult to purify by such procedures as sequential size exclusion and ion exchange chromatography.

Introduction

Cellulolytic enzymes are continually being investigated because of their potential for industrial exploitation. Uses for cellulases include the conversion of biomass to liquid fuels, the utilization of cellulases for stain removal in the detergent industry, and applications in the food processing industry.[1] The bulk of the cellulases used in industry today are of fungal origin and of these the majority have mesophilic thermal optima. An attractive alternative is the use of thermostable enzymes. These enzymes are of interest because of the reduced cooling costs during fermentation, their apparent resistance to degradation, and the decreased potential for contamination.

Many bacterial cellulase systems exist as complex protein aggregates of very high molecular weight which are largely refractory to conventional protein purification techniques.[2] However, the secreted cellulase system of a newly discovered thermotolerant bacterium isolated from hot springs in Yellowstone National Park presents a different problem. This organism does not possess a cellulolytic protein complex but secretes a multiplicity of endoglucanases that are similar in molecular weight and charge. Because of the apparent physicochemical similarities between some of these enzymes, purification of them by conventional protocols is difficult. Preparative gel electrophoresis offers an attractive alternative purification method for these enzymes.

Materials and Methods

Culture Conditions

Bacterial cells were grown on a low phosphate basal salts medium that contained NH_4Cl (1 g/L), KH_2PO_4 (1 g/L), Na_2HPO_4 (0.1 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.2 g/L). The medium was supplemented with yeast extract (0.5 g/L), cellobiose (0.5 g/L), and Sigmacell-50 (0.5 g/L) and adjusted to pH 5.2. Media in a 150 L fermenter (New Brunswick, Edison, NJ) was inoculated with a 10 L starter culture with cells in mid-exponential growth phase. Growth supernatant from late exponential cultures was harvested by continuous centrifugation using a CEPA Z41 separator (New Brunswick). The clarified supernatant was concentrated 140-fold and exchanged with 50 mM acetate buffer pH 5.0 using an Amicon (Danvers, MA) DC-30 hollow fiber ultraconcentrator fitted with polysulfone membranes (10,000 M_r cutoff).

Electrophoresis and Enzyme Assays

A 6.0% polyacrylamide resolving gel (6 cm) and a 3% polyacrylamide stacking gel (3 cm) were cast in the 37 mm gel tube of the Model 491 Prep Cell. Non-denaturing PAGE was carried out in a discontinuous electrophoresis buffer system.[3] The buffer used to prepare the resolving gel was (236 mM Tris, 0.072 N HCl, pH 8.5). The buffer used in the stacking gel was (39.5 mM Tris, 0.064 N H_3PO_4, pH 6.9). After the gels had polymerized, the apparatus was set up with a native electrophoresis buffer system, which included an upper running buffer (37.6 mM Tris, 40mM glycine pH 8.9) and a lower running buffer (63 mM Tris, 0.50 N HCl, pH 7.5). The elution buffer was the same as the lower buffer.

Approximately 4 ml of crude supernatant, containing 19 mg total protein, 5% glycerol and 0.01% Bromophenol blue tracking dye, was loaded on the upper surface of the stacking gel. Constant current of 40 mA was applied for 14 hours. Collection of fractions started just prior to the elution of the tracking dye. The elution buffer flow rate was 1.0 ml/min. Ten-minute fractions were collected and the fractions were consequently assayed for endoglucanase activity.

Following electrophoresis, the resultant fractions were analyzed for release of reducing sugars from carboxymethylcellulose (CMC). Fractions containing significant endoglucanase activity were pooled and examined by electrophoresis in analytical, native polyacrylamide slab gels. Activity on these gels was visualized using the CMC/Congo red "zymogram" assay. CMC activity was assayed on every other fraction and the results were plotted.

Once endolytic activity was established, fractions of interest from two identical Prep cell electrophoretic runs were pooled and concentrated using an Amicon PM-10 membrane. Additionally, individual fractions from a third Prep cell run were concentrated using Amicon B15 microconcentrators. To verify endolytic activity and to locate the endoglucanases, fractions were electrophoresed and visualized by zymograms. Briefly, the fractions were electrophoresed on mini-gels and immediately thereafter placed on agar media containing CMC. The agar plates were incubated at 65 °C for 15 minutes and endoglucanase activity was visualized after staining with Congo red dye.

Results

After electrophoresis and elution of the protein preparation from the Model 491 Prep Cell, fractions were assayed for CMC activity. Figure 1 demonstrates this activity throughout the 82 fractions collected on one electrophoretic run. Since run to run reproducibility was very good, various fractions from two identical electrophoretic runs exhibiting significant activity were pooled (fractions 4–6, 8–10, 23–32, 46–52, and 56–62). These pooled fractions were concentrated and endoglucanases were visualized with zymograms (Figure 2). The zymograms revealed that numerous endoglucanases were contained in the pooled Model 491 Prep Cell fractions.

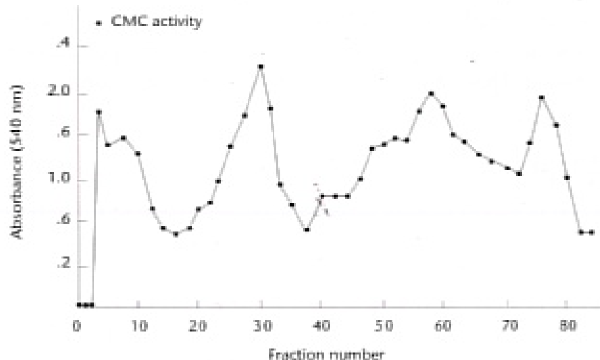

Fig. 1. Carboxymethylcellulose activity (CMC) of individual fractions after electrophoresis and elution on the Model 491 Prep Cell system. Four major CMC peaks were detected throughout the 82 fractions collected.

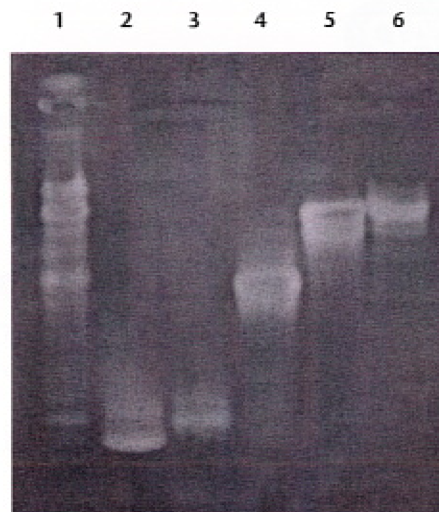

Fig. 2. A zymogram demonstrating the separation of endoglucanases obtained from pooled fractions. Lane 1, crude supernatant; Lane 2, fractions 4–6; Lane 3, fractions 8–10; Lane 4, fractions 23–32; Lane 5, fractions 46–52; Lane 6, fractions 56–62.

In order to determine the purity of specific endoglucanases isolated in individual prep cell fractions, those fractions demonstrating significant CMC activity were concentrated and tested by zymograms as stated above. These individual fractions, obtained from a third electrophoretic run show the distinct separation of 6 endoglucanases from the crude supernatant (Figure 3).

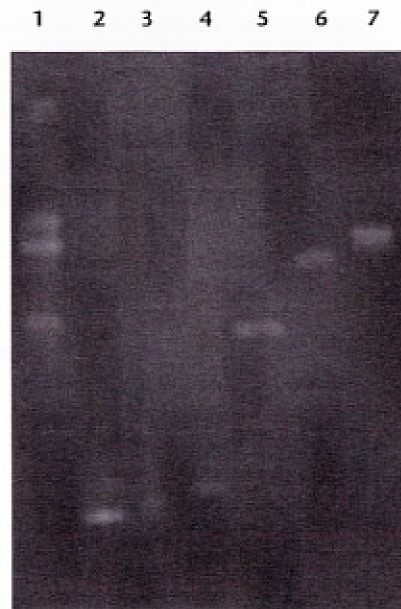

Fig. 3. A zymogram demonstrating endoglucanases obtained from concentrating individual fractions. Lane 1, crude supernatant; Lane 2, fraction 4; Lane 3, fraction 8; Lane 4, fraction 10; Lane 5, fraction 32; Lane 6, fraction 62; Lane 7, fraction 76.

Discussion

The purification of bacterial cellulases has always been a challenge to the protein biochemist. The use of multistep purification leads to loss of enzyme yield.[4] It has also been reported that during purification by chromatography, some cellulases tend to react with the column matrices.[5, 6] Additionally, the existence of partially truncated enzymes in the culture supernatant makes these closely related proteins difficult to purify by conventional methods.

The separation of these cellulases by electrophoresis in a native PAGE system offers the opportunity to bypass multiple purification steps. The Model 491 Prep Cell allowed 6 biologically active endoglucanases from the crude supernatant to be isolated. These enzymes are undergoing further characterization.

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