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PURIFICATION OF HIGH MOLECULAR WEIGHT CELLULOLYTIC ENZYMES FROM *CELLULOMONAS* sp. STRAIN O

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Abstract

Purification of extra-cellular cellulolytic enzymes from *Cellulomonas* sp. strain O, isolated from forest soil in the north of Iran, was studied by using gel filtration and ion-exchange chromatography. Two endo-glucanases (Endo-I & Endo-II) and one exo-glucanase (Exo-I) were purified to homogenity. The purified enzymes had molecular weights of about 39.000, 70.000 and 90.000 daltons for Endo-I, Endo-II and Exo-I, respectively. Heavy metal ions, like Fe^{+2} and Cu^{+2}, inactivated the enzymes by about 80%, while Zn^{+2} and Co^{+2} had no effect on the enzyme activities.

Introduction

Cellulose, an abundant carbohydrate waste, consists of a linear chain of β-1,4-linked glucose and is considered a renewable and clean source of energy. Enzymatic hydorlysis of cellulose requires at least three groups of enzymes, endo-β-1, 4-glucanase (EC 3.2.1.4), exo-β-1, 4-glucanase (EC. 3.2.1.91), and β-glucosidase (EC. 3.2.1.21), differing in substrate specificity, but with synergistic action toward hydrolyzing of crystalline cellulose [1]. Understanding the mechanism of enzymatic cellulose hydrolysis requires the isolation and characterization of individual isozymes of the system. The purification of cellulolytic enzymes in various bacteria such as *Cellulomonas* sp. [2,3,4,5] and *Ruminococcus flavefaciens* [6], and fungi such as *Trichoderma viride* [7,8,9] *Neurospora crassa* [10] and *Aspergillus japanicus* [11] has been widely investigated and reported.

Keywords: Cellulomonas sp.; Cellulolytic enzymes; Purification

Cellulolytic bacteria was isolated from forest soil in the north of Iran and identified as *Cellulomonas* sp. strain O by Malekzadeh *et al.* [12]. It showed good growth on cellulolytic waste material and was capable of complete degradation of cellulose. It might be a useful organism in the conversion of cellulosic waste to single cell protein.

In our previous paper (13), the production, release and thermal stability of *Cellulomonas* sp. strain O was described and it was shown that it could produce and secrete endo- and exo-glucanase while its β-glucosidase remained cell membrane bound. In this paper, the results of enzyme purification and the effect of some metal ions on the activity of the enzymes are presented.

Materials and Methods

Microorganism and Culture Media

All materials were obtained from Sigma (USA). *Cellulomonas* sp. strain O was isolated from forest humus in the soil along the border of the Caspian Sea in Iran and was identified in a published paper by Malekzadeh *et al.*

[12]. It was cultured and maintained on PYG (peptone yeast extract glucose agar) medium containing 2% agar and incubated at 30°C. For the production of cellulase, the strain was grown in 2000 ml flasks containing 500 ml of 0.5% microcrystallin cellulose, 0.2% yeast extract, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, and 0.1% Tween 80, the pH was adjusted to 7.0, and it was incubated at 37°C. After 120 hours of incubation, the supernatant was removed by centrifugation at 8000 rpm at 4°C for 10 minutes and was used as a source of enzyme for further works.

Protein was measured by the method of Bradford [14], with standardization using fraction V bovine serum albumin. Standard curve was plotted for protein concentration between 10-100 µg/ml. During the gel filtration and ion-exchange chromatography steps, protein concentration was estimated by measuring absorbance at 280 nm using Ultrospec III (Pharmacia, LKB).

Carboxymethylcellulose (CMC) of low viscosity served as substrate for measurement of endo-glucanase, and filter paper Whatman No. 1 was used for the estimation of exo-glucanase activity with the procedure described by Yazdi *et al.* [15].

The supernatant was concentrated with 60% w/v of $SO_4(NH_4)_2$ as follows: to 1000 ml of the supernatant, 600 g of $SO_4(NH_4)_2$ was added in three steps with gentle stirring at 4°C. After the salts were, completely dissolved, it was kept at this temperature for four hours. Precipitated enzymes were obtained by centrifugation at 12000 rpm for 15 minutes at 4°C. The precipitated enzymes were dissolved in the minimum amount of phosphate buffer 10 mM, pH 8.0.

Gel filtration was carried out on Sephacryl S-200 (Pharmacia). Column of 140×2 cm was first equilibrated with 10 mM phosphate buffer pH: 8 and proteins were eluted with the same buffer at a flow rate of 0.5 ml/min and 10 minutes for each fraction at 4°C. Protein concentration and endo- and exo-glucanase activity of eluted fractions were estimated. The fractions with the same activity and in the same protein peaks were pooled and used for analysis on SDS-PAGE and further purification by ion-exchange chromatography.

DEAE-Sepharose was used as matrix for ion-exchange chromatography. Thirty grams of DEAE-Sepharose (Pharmacia) was prepared in 10 mM phosphate buffer pH 8 and packed in a column (30×1 cm). Enzyme solution was loaded on the gel and proteins were eluted after washing with 30 ml of the starting buffer with step wise gradient of NaCl ranging from 50 to 500 mM, each step 30 ml. Elution was carried out at a flow rate of 0.5 ml/min and 10 minutes for each fraction. All fractions were analyzed for protein concentration, endo- and exo-glucanase activity. Fractions in the same protein peak and activity were pooled and analyzed by SDS-gel electrophoresis.

The homogenity of the proteins was checked by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Hames *et al.* [16], at pH 8.3 and constant current 3 mA/sample, using bromophenol blue as the tracking dye. Gels

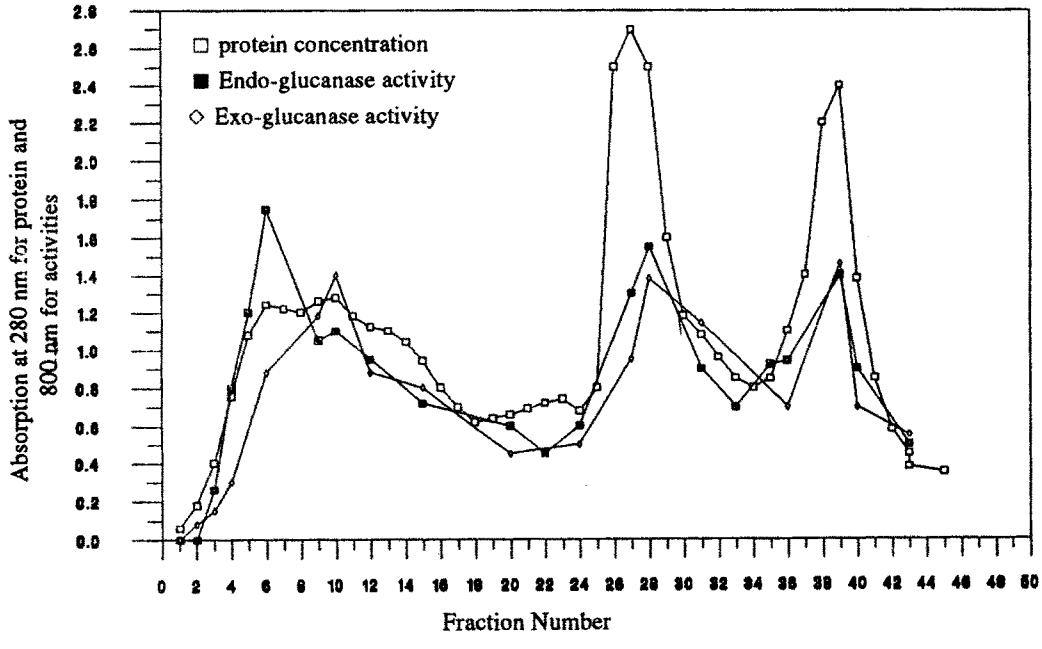

Figure 1. Sephacryl S-200 column chromatography of crude enzymes of *Cellulomonas* sp. strain O

were stained by solution containing 1% of Coomassie brilliant blue in water, methanol and acetic acid (5; 5; 2), for at least three hours. Destaining of the gels was carried out at room temperature for a few hours in a solution of water, methanol and acetic acid (6; 3; 1). Destaining solution was changed every hour.

Molecular weights of the purified enzymes were estimated by comparing their migration on SDS-PAGE against standard low molecular weight proteins (Pharmacia), containing six proteins with the MW of 14,400 to 94,000.

Results

Ninety-five milligrams of powdered crude protein was obtained after precipitation of 1200 ml supernatant with 720 g of $SO_4(NH_4)_2$ as described above. The powder was dissolved in 10 ml of phosphate buffer (10 mM) pH 8 and passed through a column of Sephacryl S-200. A total of 52 fractions were collected, with three distinct protein peaks S1 (tubes 4-15), S2 (25-30) and S3(36-42). All fractions had endo- and exo-glucanase activity (Figure 1). Tubes 4-15 were pooled and used for the next step of purification by DEAE-Sepharose. The results are shown in Figure 2. Four main peaks of protein resulted from this chromatography (S1D1, S1D2, S1D3 and S1D4). Peak S1D1 (tube 5) and S1D3 (tubes 21-23) had only endo-glucanase activity while peaks S1D2 (tubes 7-8) and S1D3 (tubes 14-15) showed both endo- and exo-glucanase activity. Analysis of these peaks on 12.5% SDS-PAGE (Picture 1), showed that S1D1 is a single band with molecular weight about 39,000 daltons, and named Endo-I. Peak S1D4 was also approximately a single band with a molecular weight about 70,000 daltons with a minor impurity, which was further purified by passing through another Sephacryl S-200 and nominated Endo-II. Fraction S1D3 was a mixture of all proteins and was not used for more purification. Fraction S1D2 was composed of two main bands, one of them was at the same position as Endo-I, and the other was probably an exo-glucanase. This fraction was then purified by passing again through DEAE-Sepharose at pH 8.0 and proteins were eluted by step wise decreasing of pH. Results are presented in Figure 3. Two main protein fractions were eluted from this column. The first fraction (S1D2A, tubes 3-6) had both activities and showed two bands on the gel. The second fraction (S1D2B, tube 23) had only exo-glucanase activity and showed on SDS-PAGE a single band with a molecular weight of about 90,000 daltons and was named Exo-I.

The effect of heavy metals such as Cu^{+2}, Fe^{+2}, Zn^{+2}, Co^{+2} and Mn^{+2} at concentrations 1-15 mM on endo- and exo-glucanase activity of crude enzymes inactivated both enzymes up to 90% with 15 mM. Zn^{+2} slightly inactivated enzymes and its inactivation effects were more at higher concentrations. The effect of Co^{+2} on exo-glucanase activity was similar to Zn^{+2}, but it increased endo-glucanase activity about 30% at 7 mM. Mn^{+2} increased the activity of both enzymes up to 50% at concentrations 5-

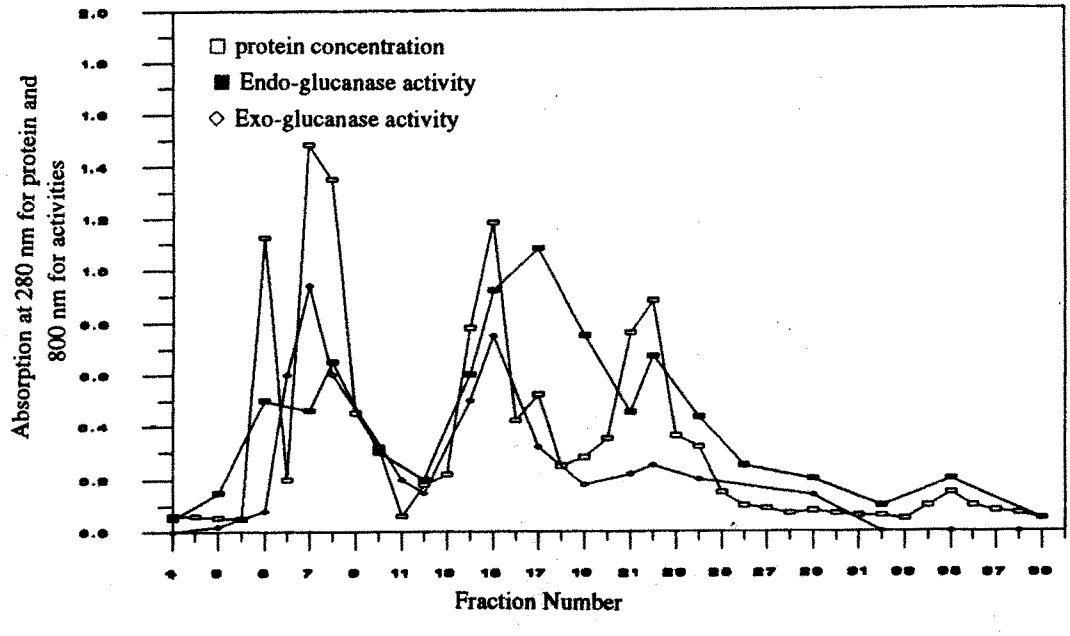

Figure 2. DEAE-Sepharose column chromatography of HMW cellulolytic enzymes of *Cellulomonas* sp. strain O

Picture 1. SDS-PAGE of fractions resulted from HMW cellulolytic enzymes after ion-exchange chromatography on DEAE-Sepharose

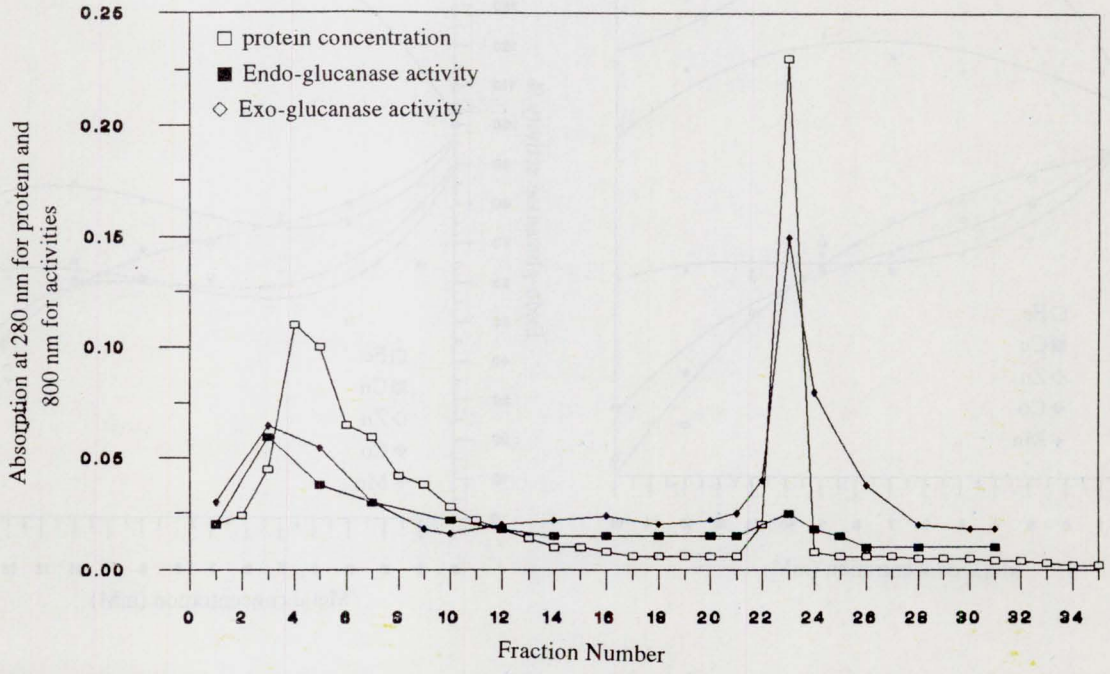

Figure 3. DEAE-Sepharose column chromatography of S1D2 fraction

9 mM, but at higher concentrations activities slowly decreased as the activity of endo- and exo-glucanase were 20 to 30% more than the medium without Mn^{+2}, respectively, at 15 mM.

Discussion

Celullases are often very difficult enzymes for purification because of their tendency to aggregate with other proteins [5]. Our resutls show that the cellulolytic system of *Cellulomonas* sp. strain O does not take the form of a protein complex. This system is composed of at least two endo-glucanases and one exo-glucanase which were purified by Sephacryl S-200 and DEAE-Sepharose, and their homogentiy was shown by SDS-PAGE. They had a molecular weight of 39000 and 70000 daltons for endo-glucanases and 90000 for exo-glucanase. The molecular weight of purified endo-glucanase of other *Cellulomonas* sp. has been shown to be very variable, ranging as high as 130000 in *C. fimi* [4], 57000 and 40000 daltons in *C. fermentans* [3], and as low as 20400 in *C. flavigena* [5]. Two purified endo-glucanases of *Cellulomonas* sp. strain O correspond to two different proteins. SDS-PAGE of proteins demonstrated that they are both monomeric and can not correspond to two different aggregated forms of a single cellulase component as is the case with *C. fermentans* [3] or *Ruminococus albus* [17] cellulases.Nevertheless, it can not be ascertained whether the two endo-glucanases components resulted from post translational modification by proteolysis or chemical substitution of the same protein (e.g. glycosylation) as is the case with endo-glucanases of *C. fimi* [18] or from the production of discrete, structurally unrelated genes. The two purified enzymes should be further characterized with particular attention to their chemical, physico-chemical and enzymological (especially exo-glucanase) properties with a view to elucidating their specific function in enzymatic cellulase degradation.

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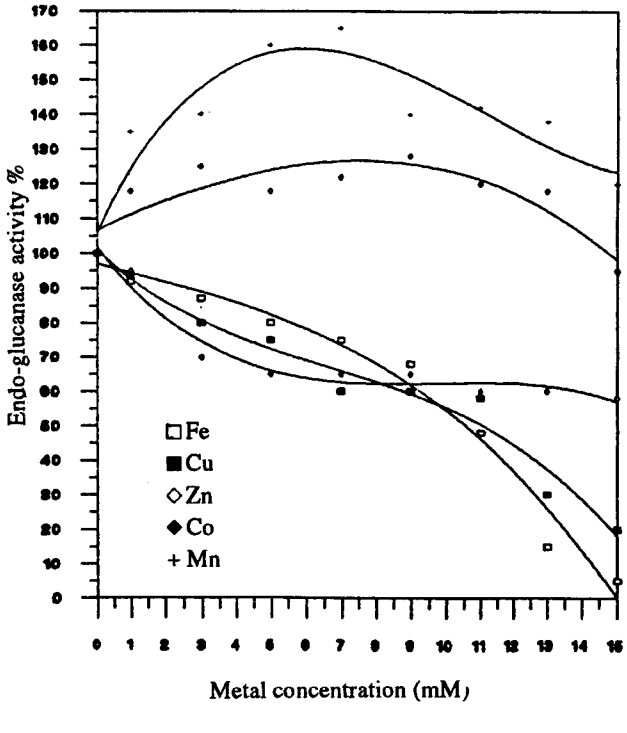

Figure 4. Effect of metals on endo-glucanase activity of *Cellulomonas* sp. strain O

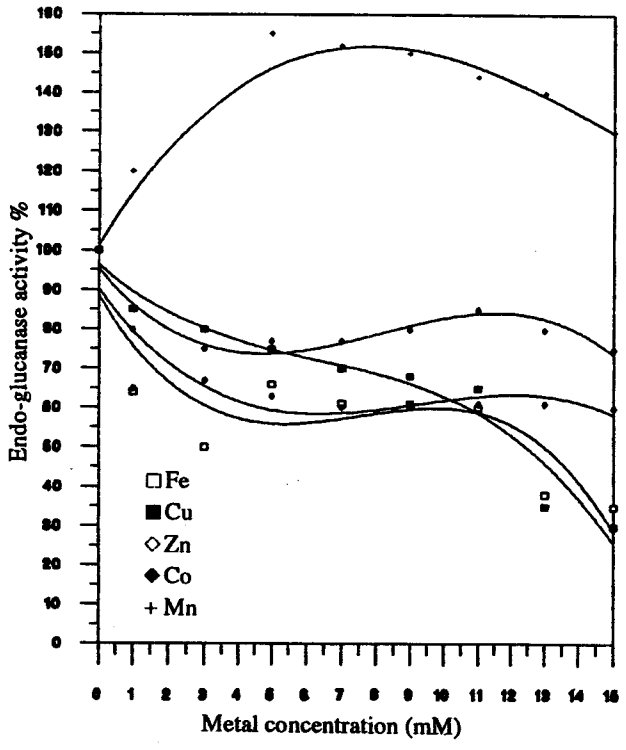

Figure 5. Effect of metals on exo-glucanase activity of *Cellulomonas* sp. strain O

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