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Purification of Endoglucanase From *Trichoderma harzianum*

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Abstract

Cellulases provide a key opportunity for achieving the tremendous benefits of biomass utilization in the long term because of high glucose yields possible, and the opportunity to apply the modern tools of biotechnology to reduce costs. Endoglucanase (endo-1,4-D-glucanase, EC (3.2.1.4), an enzyme of cellulase complex was produced by the fungus *Trichoderma harzianum,* E-58 strain. It was grown on Vogel's medium with different carbon sources like xylan, CMC, corncobs and glucose for 5- days at 180 rpm at 28°C in orbital shaker. Glucose repressed the synthesis of endoglucanase whereas xylan and CMC produced the enzymes in appreciable amount. Optimum pH and temperature of incubation for enzyme activity were 5.5 and 60ºC respectively. The effect of temperature on the endoglucanase activity with Carboyxmethyl cellulose (CMC) as substrate was studied at pH 5.5 and displayed optimum activity temperature 60°C. Maximum enzyme activity of endoglucanase was found to be 0.798 IU /mL by using xylan as a carbon source. The enzyme was purified by ammonium sulfate precipitation and gel filtration (sephadex G -50 and G -200). Purity was analyzed by SDS/PAGE. After gel filtration specific activity of the enzyme was 0.626 IU/mg. SDS/PAGE revealed that EG has only one subunit.

Introduction

Cellulose degrading enzymes (cellobiohydrolases endoglucanases and β-glucosidase) are widely spread in nature, predominantly being produced by microorganisms such as molds, fungi and bacteria. These are the most extensively investigated multicomponent enzyme systems because of their ability to decompose cellulose biomass into glucose, which in turn, can be converted into other valuable chemicals and energy (Mukataka et al; 1998). Cellulose is the main structural constituent of plant cell wall. It is a homopolysaccharide of D-glucose residues linked through β(1, 4) glucoside bonds. Due to its complex structure, the biodegradation of cellulose requires the synergistic action of several hydrolytic enzymes for efficient and complete breakdown. Complete hydrolysis of cellulose requires the action of cellulase system containing Endoglucanase (EC 3.2.1.4), Exoglucanase (EC.3.2.1.91) and β-glucosidase (EC3.2.1.21) (Kubicek *et al*., 1990).

Endoglucanase (EG) functions to increase the effective concentration of accessible end groups for the end wise action of cellobiohydrolase. Hence endoglucanase is assigned the role of initiating the attack on native cellulose (Vidya et al; 1984). The ability of filamentous fungi to secrete large amounts of proteins has motivated their extensive use for the production of industrial enzymes (Cui et al; 1998). Cellulases produced by filamentous fungi have a high industrial utilization. They are widely used in food and feed industries, textile industry and pulp and paper industry (Nakari and Penttila; 1995). Cellulases are also used as solvent in industries (Moo-Young; 1985) in the preparation of medicines, resins and perfumes (Routh et al; 1969). Endoglucanases are required for a good stone washing effect, and EG-II is most effective in removing color from denim.

Here we describe production and purification of EG from a filamentous fungus *T. harzianum*. The data obtained from this and future studies will be used for economic production of cellulases.

MATERIALS AND METHODS

Production of Endoglucanase:

Trichoderma harzianum E 58 strain was grown in slants containing Vogel's medium for the sporulation of *T. harzianum* (Ahmed et al; 2003). Slants were incubated at 28ºC for 24 hours for sporulation and stored at 4ºC. Growth medium containing Vogel's medium was inoculated by a sample of spore suspension. pH of the medium was adjusted to 5.5. Composition of production medium for endoglucanase was same as that of corresponding growth medium except that it was supplemented with CMC, xylan, glucose or corncobs as a carbon source. *A* 10% (v/v) inoculum was used to initiate the EG production. Fermentation of EG was carried out in 250 mL Erlenmeyer flasks under the following conditions: temperature 28 ºC, pH 5.5 and cultivation time 5 days at 180 rpm in orbital shaker. The mycelia were separated by centrifugation and the culture filtrate was used for the enzyme purification.

Activity Assay

Endoglucanase activity was assayed with 1% (w/v) carboyxmethyl cellulose (CMC) as substrate (Vidya et al; 1984) according to Miller method (1959). One milliliter of enzyme solution was incubated for 30 minutes with 1 mL of 1.0% CMC and 1 mL of 50 m*M* sodium acetate buffer (pH 5.0) at 60ºC. The reaction

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was terminated by adding 3 mL DNS reagent, boiled for 10 minutes, cooled and absorbance was noted spectrophotometrically at 575 nm. The international units (IU) of endoglucanase produced per mL (IU/mL) were calculated from standard curve of glucose.

Protein Estimation:

The amount of protein in each sample was determined according to Biuret method (Bardawill and David; 1949) using Bovine serum albumin (BSA) as standard.

Enzyme Purification and Fractionation:

The crude enzyme extract (54 mL) of 5 days old culture of *T. harzianum* grown under optimal fermentation conditions was precipitated using ammonium sulfate (20 % saturation). The mixture was stirred and kept at 4 °C overnight and centrifuged at 10,000 rpm for 15 min at 4 °C. The precipitate was dissolved in 10 ml of 50 m*M* sodium acetate (pH 5.0) and dialyzed overnight against the same buffer. The dialyzed samples were applied to column (30 cm) of sephadex G-50, pre- equilibrated with 50 mM sodium acetate buffer (pH 5.0) Protein was eluted with 50 m*M* sodium acetate buffer, pH 5.0 at a linear flow rate of 30 cm/hr. The fractions with highest absorbance at 280 nm were pooled and subjected to enzyme assay. The most active fractions showing endoglucanase activity were concentrated and loaded on pre- equilibrated column (30 cm) of sephadex G –200. The fractions with highest activity were pooled and enzyme activity was noted.

Polyacrylamide gel electrophoresis

SDS/PAGE on dialyzed samples was performed. Samples were loaded on 10% SDS / PAGE with 4% stacking gel in vertical gel electrophoresis system. Gel was run at 150 V for 10 h. Proteins were stained with coomassie brilliant blue with a solution of 50% methanol, 0.25% coomassie blue and 10% acetic acid in deionized water for 1 hour. Destaining was done out by solution of 7.0 % acetic acid and 5.0% methanol in deionized water for 4- hours. The gel was analyzed on Syngene gel documentation system (Gene Genius Bio Imaging System).

Temperature optima

The optimum temperature was estimated using the endoglucanase-activity assay at various temperature ranges between 30 and 85 °C. Enzyme extract was incubated with CMC, in 50 m*M* sodium acetate buffer, pH 5.0, for 30 minutes at different temperatures. The reducing ends formed were determined using the dinitrosalicylic acid reagent immediately after incubation and the activities were assayed as described above.

Results and Discussion

Enzyme Production on various substrates

Vogel's medium containing glucose as carbon source gave very low endoglucanase activity. Somewhat similar information is also given by Ximenes et al (1996) who found that high glucose repressed the enzyme production. The effect of carbon sources was also studied by Malik et al (1986). They found that with glucose, maximum growth was obtained, but negligible cellulases were produced. These substrates were analyzed at different temperatures but at all temperatures corncobs yielded minimum while CMC and xylan showed better results. CMC was the preferred substrate for the production of endoglucanase purified from cultured broth by Lucas et al (2001). But in the present project we used xylan because maximum production of the enzyme was achieved with xylan as carbon source. Table 1 shows the results of different carbon sources at different temperature ranges.

Table 1. Effect of substrate concentration on endoglucanase activity purified from *T. harzianum*

S. No	*Substrate*	*Endoglucanase activity (IU/ml) Mean* ±***S.D***
1.	CMC	0.0607 ± 0.026
2.	Corncobs	0.0375 ±0.012
3.	Glucose	0.0285 ±0.009
4.	Xylan	0.798 ±0.105

Each value corresponds to mean of at least three independent observations.

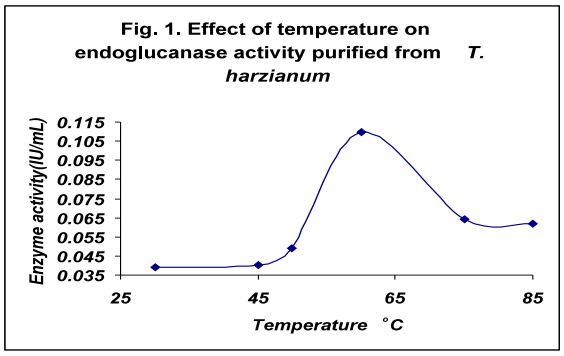
Optimum Temperature

The optimum temperature of the endoglucanase activity was studied in the range of 30-85 °C for different carbon sources. All the carbon sources yielded maximum activity at 60 °C incubation temperatures. Earlier it has been found that the crude and purified enzymes of the cellulase complex had maximum activity at pH 5.0 and temperature ranging between 45 to 65°C (Yazdi et al; 1990). Fig.1 shows that enzyme activity increased with increase in temperature up to 60 °C, but above this temperature the activity decreased. Therefore, enzyme assays were conducted at this temperature. Similarly *Morchella conica was* found to give optimum activity of CMCase at pH 5.5 and 60°C (Cavazzoni and Manzoni; 1994).

Purification of endoglucanase

A summary of purification procedures is presented in Table 2. The crude extract had enzyme activity as 0.798 IU/mL. The total protein concentration was 9.097 mg/mL and the specific activity of the enzyme was 0.087 IU/mg. Similar feature was reported for another cellulase called EG 5 from *Trichoderma reesei*, which also showed a rather low activity towards the common substrates used to assay cellulase activity. Endoglucanase with different enzyme activities (IU/mL) have been produced by several workers (Vidya et al;

1984, Gama et al; 1993, Gadgil et al; 1995 and Lucas et al; 2001).



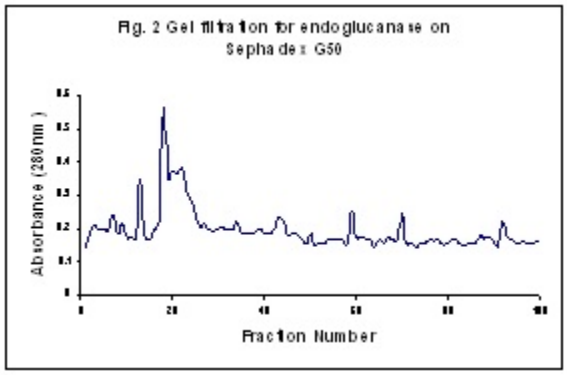
$(NH_4)_2SO_4$ Precipitation

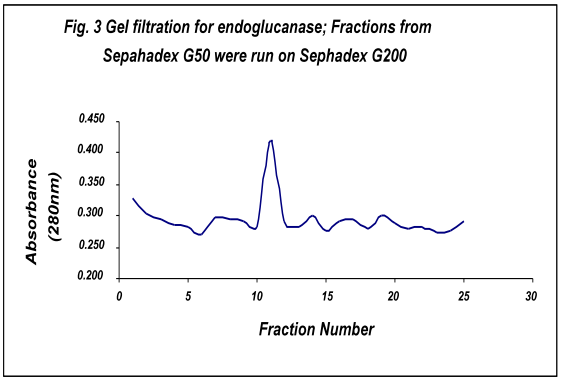
Ammonium sulfate precipitation brings about reduction of total protein content of the original crude extract before the chromatographic purification steps are introduced. EG was precipitated from the crude extract by using fractional ammonium sulfate (0-80%). Good percentage of enzyme was precipitated at 20% ammonium sulfate saturation. Therefore, endoglucanase was partially purified from culture extract by salting out at 20 % ammonium sulfate saturation. The supernatant resulted in 0.365 fold purification, and total protein was reduced from 9.097 to 4.11 mg/ml with 16.5 % recovery of endoglucanase activity The specific activity was 0.032 IU/mg. The residues obtained after 20% $(NH_4)_2SO_4$ saturation showed maximum enzyme activity with specific activity of 0.37 IU/mg and 4.219 fold purification. Protein content was 2.87 mg/ml.

Ammonium sulfate precipitation has been used as a purification technique in experiments by several workers (Vidya et al; 1984, Roy et al; 1991, Lin et al; 1995, Rajoka et al; 1986).

Table 2 A summary of the purification of endoglucanase enzyme from *Trichoderma harzianum*

Sr. No.	Samples	Enzyme activity (1U/mL)	Protein concentration (mg/mL)	Specific activity (1U/mg)	percentage yield	Fold purification
1	**Crude extract**	0.798	9.097	0.087	100	1
2	**$(NH_4)_2SO_4$ precipitation 20% supernatant**	0.132	4.11	0.032	16.5	0.365
3	**20% residue**	1.062	2.87	0.37	13.3	4.219
4	**Gel filtration: Sephadex G-50 (16-19)**	0.447	1.96	0.228	56	2.603
5	**Gel filtration: Sephadex G50 run on G-200 sample (10-12)**	0.72	1.149	0.626	90	7.146





Dialysis

The residue after 20% $(NH_4)_2SO_4$ precipitation was dissolved in buffer and dialyzed against distilled water in dialysis membrane with cut off values of 12,000. EG activity, protein concentration and specific activity were found to be 0.066 IU/ml, 10.81 mg/ml and 0.006 IU/mg, respectively.

Gel Filtration

Further purification was made by size exclusion chromatography, which involves the separation of the molecules on the basis of their molecular weight.

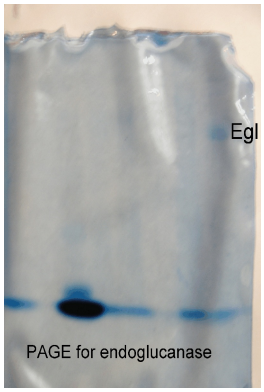
The EG activity was enriched after gel filtration steps. Dialyzed sample was run on Sephadex G-50 column. Fig. 2 shows that the activity is eluted (Fractions: 16-19) as a sharp peak, not well separated from other protein peaks. The purification factor for the size exclusion

chromatography step was 2.603. The specific activity yielded 0.228 IU/mg protein (Table 2) and the total protein was reduced from 10.81 mg/mL to 1.96 mg/ml. The highest active EG fractions obtained from gel filtration Sephadex G-50 column was loaded in the Sephadex G- 200 column. Elutions were performed using 50 m*M* sodium acetate buffer, pH 5.0. Fig 3 shows the results of pooled active peak fractions from Sephadex G-50 run and the activity is confined (Fractions: 10-12) to a well separated, distinctive peak surrounded by a few inactive proteins. Hence, the final purification resulted in 90% yield with a purification factor of 7.146. There is a gain of endoglucanase activity between these steps. Earlier, endoglucanase was characterized and purified from *Aspergillus niger* by Rajoka (2004). There was 13.2 fold improvement in specific activity of enzyme after purification. In the present project we have low EG activity with 2.603 fold improvement in specific activity of the enzyme. This may be due to the difference in fungal species and the growth medium supplied.

SDS Polyacrylamide gel electrophoresis

Purity of endoglucanase was analyzed by SDS/PAGE electrophoresis after gel filtration. (Fig.4) The single band representing pure endoglucanase indicates a single polypeptide chain. Similarly, purification of EG was carried out by Xu et al. (2000) on SDS/PAGE that revealed single band indicating a single polypeptide chain. Several workers have purified an extracellular endoglucanase and cellulases to electrophoretic homogeneity after ammonium sulfate precipitation, gel filtration and Polyacrylamide gel electrophoresis. Endoglucanase-I has been purified by Ding et al. (2001) from the edible straw mushroom, *Volvariella volvacea*. They isolated EG-I from culture fluid by ion exchange, gel filtration chromatography and preparative PAGE. Similarly various isozymes of endoglucanase and xylanase on SDS/PAGE from *Piromyces sp*. have been identified (Samanta et al; 1999).

Fig. 4. Gel showing endoglucanase purified from *Trichoderma harzianum*. The lane with endoglucanase is designated with Egl.



Conclusions

In this work endoglucanase was produced and purified from *Trichoderma harzianum* E 58 strain, grown in Vogel's medium using xylan as carbon source. The optimum temperature for endoglucanase assay was 60°C. The enzyme was purified to 2.603 fold with the help of ammonium sulfate precipitation and gel filtration SDS/PAGE, analysis revealed a single band of the enzyme.

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