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PURIFICATION AND CHARACTERISTICS OF THE ENDO-1,4-β-GLUCANASE FORMED BY THE HYBRID FUNGUS *Trichoderma harzianum*-G-1

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An endo-1,4-β-glucanase consisting of a low-molecular-mass enzyme with a molecular mass of 19,862 Da has been isolated for the culture liquid of the hybrid fungus Trichoderma harzianum-*G-1*.

The interest of researchers in the cellulases of microscopic fungi is due to the fact that they are not only of theoretical but also of great practical value. Purified cellulase enzymes are finding wide use in the national economy for the treatment of various types of cellulose-containing substrates [1].

Our task was to obtain a purified preparation of the endo-1,4-β-glucanase from the culture liquid of the hybrid fungus *Trichoderma harzianum*-G-1 [2], since the endo-1,4-β-glucanase is the main enzyme in the cellulase complex, playing an important role in the breakdown of native cellulose [3-5].

The isolation of an endo-1,4-β-glucanase in a highly purified state is complicated by the presence in cellulase preparations of a large number of components with similar biochemical characteristics and also by the multiplicity of the forms of cellulolytic enzymes.

As a result of the purification of an endo-1,4-β-glucanase preparation obtained from the culture liquid of the hybrid fungus *T. harzianum*-G-1 by precipitation with acetone and purification by passage through a column of Sephadex DEAE A-50 we obtained 100 ml of enzyme liquid, and this was concentrated to 15 ml at 40°C. The optical density at 280 nm of the concentrated enzyme solution and its cellulase activity and protein content were determined.

In the following stage, by purification on a column (2.5 × 50 cm) of TSK-Gel-HW-55 (Fig. 1) we obtained a purified preparation of the endo-1,4-β-glucanase from the culture liquid of *T. harzianum*-G-1. Then, by means of electrophoresis in polyacrylamide gel, we established the homogeneity of the endo-1,4-β-glucanase enzyme preparation.

Sedimentation analysis was conducted in a Spinco E ultracentrifuge at 20°C and 36,000 rpm. The concentration of the enzyme was 2 mg/ml of 0.1 M acetate buffer, pH 4.7. The molecular mass of the endo-1,4-β-glucanase, determined by the sedimentation equilibrium method, was 19,862 Da.

The complete elimination of pigment was achieved and a purified electrophoretically homogeneous preparation was obtained by gel filtration through TSK-Gel-HW-55.

Under the conditions of the experiment, the bulk of the endo-1,4-β-glucanase issued with the free volume, but a certain amount of protein containing a minimum of endo-1,4-β-glucanase activity was retained on the column under the condition of the starting buffer and was eluted with a linear increase in NaCl concentration to 1 M.

The highest degree of purification of the endo-1,4-β-glucanase from the culture liquid of the hybrid fungus *T. harzianum*-G-1 (Table 1) was achieved as the result of the gel filtration of the preparation on TSK-Gel-HW-55, giving a yield of 4.2 mg of protein.

An electrophoregram of the purified preparation in the pH range of 4.8-5.2 showed a single protein band, corresponding to a protein with a molecular mass of ~20,000 in the presence of marker proteins.

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TABLE 1. Purification of the Endo-1,4-β-glucanase from a *T. harzianum*-G-1 Preparation

Stage of purification	Protein, mg	Total number of activity units	Specific activity, units/mg of protein	Activity yield, %	Degree of purification
Initial preparation	7500	5400	0.72	100.0	1.0
Fractionation by ethanol [sic]	1300	3900	3.0	72.2	4.2
Ion-exchange chromatography on Sephadex DEAE-50	76.0	1854	24.4	34.3	33.9
Gel filtration on TSK-Gel-HW-55	4.2	595.4	141.7	11.02	196.9

*Text refers to acetone — Translator.

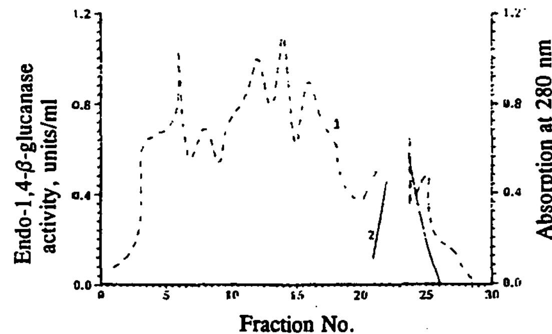

Fig. 1. Purification of endo-1,4-β-glucanase on a column of TSK-Gel-HW-55 (2.5 × 50 cm) equilibrated with 0.05 M acetate buffer; rate of elution 60 ml/h; fraction volume 4 ml: *1*) protein; *2*) endo-1,4-β-glucanase.

Thus, according to the results of electrophoresis and sedimentation analysis, the preparation of endo-1,4-β-glucanase obtained from the culture liquid of the hybrid fungus *T. harzianum*-G-1 was a highly purified low-molecular-mass enzyme with a molecular mass of 19,862 Da, a specific activity of 141.7 units/mg of protein, and a sedimentation coefficient of 3.24 S.

EXPERIMENTAL

T. harzianum-G-1 was grown under deep fermentation conditions at 30°C in Mandels' medium [6], modified by ourselves, in which 2% of ground wheat bran was used as the sole source of carbon.

The enzyme preparation was isolated from a four-day culture liquid of the hybrid by precipitation with acetone (1:4) at 4°C, and its cellulase activity [7] and protein content [8] were determined.

The enzyme preparation was purified by the method of Rodionova et al. [9].

To purify the endo-1,4-β-glucanase, 10 g of the enzyme preparation obtained was dissolved in 100 ml of 0.05 M acetate buffer and the solution was stirred periodically and centrifuged at 11,000 rpm for 20 min. Then 50 ml of the supernatant liquid was transferred to a column (1.67 × 18 cm) of Sephadex DEAE A-50 equilibrated with 0.05 M acetate buffer, pH 5.6. Elution was conducted with the same buffer at a rate of 60 ml/h. Fractions with a volume of 4 ml were collected, and in each test tube we determined the protein content and the optical density at 280 nm.

The following stage of purification was conducted on a column (2.5 × 50 cm) of TSK Gel-HW-55 equilibrated with 0.5 M acetate buffer, pH 5.5. Concentrated enzyme solution (10 ml) was added to the column and elution was performed with the same buffer. Fractions with a volume of 4 ml were collected at the rate of 60 ml/h. The protein content, the cellulase activity, and the optical density at 280 nm were determined in each tube.

To separate the endo-1,4-β-glucanases from β-glucosidases, 10 g of the enzyme preparation was dissolved in 100 ml of 0.05 M acetate buffer with periodic stirring, and the solution was centrifuged at 12,000 rpm for 20 min to eliminate the

insoluble residue. The supernatant liquid, cooled to 4°C, was treated with 1 and 2 volumes of acetone cooled to −20°C. Each time the resulting precipitate was eliminated by centrifugation at 12,000 rpm and was dissolved in the minimum volume of 0.05 M acetate buffer, pH 5.5.

The homogeneity of the enzyme preparation obtained was determined by electrophoresis in 6% polyacrylamide gel. The time of electrophoresis was 2 h 30 min at a current strength of 10 mA per layer of gel and a voltage of 150 V. After the end of electrophoresis the electrophoregrams were fixed in a 0.15% solution of Coomassie Bright Blue (CBB, G-250).

Sedimentation analysis was conducted in a Spinco E ultracentrifuge (Beckman, USA).

The sedimentation coefficient was found from the equation

$$S=\frac{(\lg X_m - \lg X_n)2.303}{W^2 t}.$$

where t is the time, sec; W is the angular speed of rotation; X is the distance from the peak to the axis of rotation; and 2.303 is a conversion factor.

The molecular mass was determined with the aid of the given ultracentrifuge method of sedimentation equilibrium and was calculated from the equation

$$M=\frac{2RT}{(1-V_p)W^2}\bullet\frac{d\ln c}{d X^2},$$

where R is the gas constant; T is the absolute temperature; V is the partial specific volume; $d \ln c$ is the difference in concentration at definite points; dX^2 is the difference in the distances from the center of rotation, cm; p is the density of the solution; $V = 0.75$.

An electrophoretically homogeneous preparation was obtained by gel filtration through TSK-Gel-HW-55.

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