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Biochemistry

Homogenous Preparation and Kinetic and Molecular Indices of Endo-1,4-β-glucanase from the Extremophilic Micromycete *Aspergillus versicolor-83*

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ABSTRACT. From the collection of micromycetes, consisting of microscopic fungi isolated from different ecological niches of the Caucasus and belonging to S. Durmishidze Institute of Biochemistry and Biotechnology, the active producer of cellulase, thermophilic and acidophilic strain of *Aspergillus versicolor* 83 has been selected. The homogenous preparation of endo-1,4-β-glucanase, one of the enzymes of the cellulatic complex of the strain, was obtained and its kinetic characteristics, absorption capacity on an insoluble substrate, the type and extent of inhibition with products, amino acid composition and isoelectrical point were investigated. *© 2007 Bull. Georg. Natl. Acad. Sci.*

Key words*: cellulase producer, cellulase complex, endoglucanase, extremophilic micromycete, cellulose bioconversion.*

The problem of cellulose bioconversion is associated with selection of cellulase producing microorganisms that are capable of instantly and effectively converting different cellulose-containing substrates. From this point of view extremophilic micromycetes and their enzymes, resistant to critical values of temperature and pH, less denaturizing, and retaining enzymatic activity for a long period are of great industrial interest [1].

From the collection of micromycetes, consisting of microscopic fungi isolated from different ecological niches of the Caucasus and belonging to the S. Durmishidze Institute of Biochemistry and Biotechnology, an active producer of cellulases – a thermophilic and acidophilic strain of *Aspergillus versicolor* 83 has been selected. The optimal temperature of its total cellulase activity (following filter paper) is 60°C, and the optimal pH constitutes 3.5.

The protein stability is determined by the summarized effect of different factors, like additional disulfide bonds, non-protein prosthetic groups, metal ions, the peculiarities of the primary structure, etc. [2].

Study of the molecular characteristics of cellulase from *A. versicolor*, as a stable enzyme was interesting. For this purpose it was important to obtain a homogenous preparation of the enzyme.

The cellulase complex consists of three types of enzymes: endo-1,4-в-glucanase, exo-1,4-в-glucanase, and в-glucanase. Endo-1,4-в-glucanase is important to hydrolyze в-1,4 links. The endoglucanase activity is determined with respect to the decrease of polymer substrate viscosity [3]. 1.5% solution of carboxymethylcellulose served as a substrate [3].

The purification scheme of endo-1,4-в-glucanase from *A. versicolor 83* has been worked out. It consists of two stages, involving ion-exchange chromatography on DEAE and SP disks, DEAE 650 Toyperal and gel filtration on HW-55 Toypearl. Anion-exchange chromatography on DEADE disks results in the removal of the essential part of acid proteins and partially of a pigment from the enzyme.

As a result of cation-exchange chromatography, out of the three fractions two endoglucanases, differing in

Table 1

Purification of endoglucanase from deep culture of *A. versicolor* 83.

Stages of purification		Total activity units	Total protein mg	Specific activity unit/mg	Degree of purification	Retained activity %
Initial enzyme preparation		2500	550	4.5	1	100
DEAE (Zeta Prep) disk		2150	90	23.8	5.3	86
SP (Zeta Prep) disk		1950	70	28	6.2	78
Gel filtration HW-55	E_1	350	8	43.7	9.7	I-14
	E_2	1090	10	109	24.2	II-43.6
DEAE- 650 Toypearl	E_1	325	5.5	59	13.1	I-13
	E_2	450	1.8	250	56	II-18

molecular weights and specific activities, have been obtained. For further purification the endoglucanase fraction with higher specific activity was selected. Finally, by means of ion-exchange chromatography on DEAE 650 Toypearl the homogenous fraction of endoglucanase was obtained, according to isofocusing and electrophoresis. The results are demonstrated in Table 1.

In the further stage some physical, chemical and kinetic properties of homogenous endoglucanase were studied. To determine catalytic constant the Michaelis-Menten equation, modified by Laineiver-Berk, was used:

$$\frac{1}{V} = \frac{[K_m]}{[S]} \cdot \frac{1}{V_{max}} + \frac{1}{V_{max}}$$

According to it 1/[S], $1/V$ and a graph drawn in the system of co-ordinates is a line, the angle target of which is K_m/V_{max}; The ordinate intersection point is $1/V_{max}$ and that of the abscissae-$1/K_m$. The graph drawn in the system of those co-ordinates allows determination of V_{max} and K_m. The obtained results are shown in Fig.1 As is seen from the graph, V_{max} of 1,4-β-glucanase from *Aspergillus versicolor* is 74μm·min/mg, K_m-2,5 g/1. The catalytic constant – indicator of the enzyme molecular activity – was determined from CMC (1.5g/1) with respect to the rate of restoring sugar formation.

$$V_{max} = K_{kat} \cdot [E_o] \; K_{kat} = 45 sec^{-1}$$

The specificity constant - $K_{specificity} = K_{kat}/K_m = 18 g/1$

In the course of enzymatic hydrolysis of an insoluble substrate the process of enzyme adsorption on a substrate is quite important, for it is the first stage of substrate degradation. Acting on the postulate: "Better links - better catalysis", the high degree of enzyme adsorption on homogenous endoglucanase insoluble substrate-microcrystalline cellulase was studied by the modified method of Rabinovich [4].

Henry's constant – *Kp*, the coefficient of enzyme equal distribution on a solution and sorbent surfaces – was used as the indicator of absorption degradation.

80% of homogenous endoglucanase is stable and irreversibly absorbs on an insoluble substrate, being the basis for deep and effective hydrolysis of the latter (*Kp* 1.6).

The efficiency of enzyme inhibition by the reaction products is a significant criterion for the evaluation of enzyme technologibility.

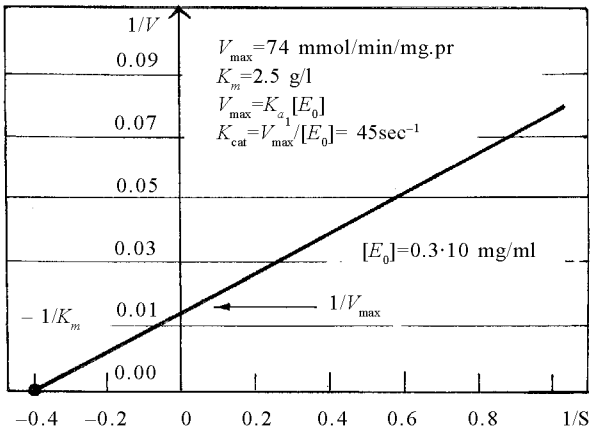

Fig. 1. Relation between the rate of MCC substrate hydrolysis (V-mmol/min per 1mg protein) by the endo-1,4-β glucanase of *Aspergillus versicolor* and substrate concentration (S-mg/ml). Assay conditions: acetate buffer 0.05M, pH4.5, t-55°C.

Therefore the mechanism of endoglucanase inhibition by the reaction product cellulase has been studied. Dyed cellobiose "DC-31" was used as a substrate which in various quantities (2.5-12.5g/l) was added to 4ml 0.05M acetate buffer pH4.5 containing 0.1M cellobiose. The samples were centrifuged at intervals and the optical viscosity of the supernatant (450nμM) was measured. According to the experimental data, following the graph drawn up in Laineiver-Berk coordinates (Fig. 2) it may be suggested that inhibition has a weak, uncompetitive character. In case of dyed substrate the value of Michaelis' constant was 2.2g/l, and that of inhibition totalled 105g/l.

We suppose that weak and uncompetitive inhibition may be determined by a stable and strong adsorption of endoglucanase on a substrate. The endoglucanase binds to the substrate with its outlying parts so firmly that its removal from the substrate surface becomes impossible even via addition of large quantities of inhibitors. Accordingly, competitive inhibition is excluded.

The isoelectric point of the homogenous endoglucanate was determined through analytical isoelectrofocusing by means of a fast system on 45.50. 0.45mm gels. A protein group with well-known isoelectric points (Pi-2.8-6.56) was used as a standard. P_i of the endo-1,4-β glucanase was 4.7.

The composition of endoglucanase amino acids was determined on an amino acid analyzer, triphtophane content was determined spectrophotometrically on 575μm

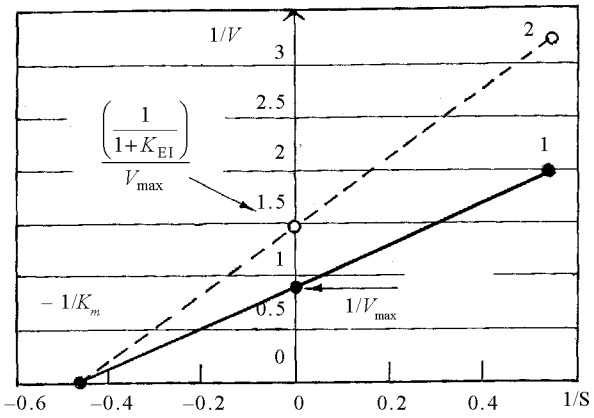

Fig. 2. Influence of cellobiose on the hydrolisis of colored cellulose by the endo-1,4-β glucanase of *Aaspergillus versicolor* 83.

1. Inhibitor's concentration 0.
2. Inhibitor's concentration 34.2g/l.Temperature of incubation 40°C.
3. The linear curve's transaction with *X*-axis gives the meaning of $1/K_m$, and transaction with *Y*-axis – the maximal rate of reaction V_{max}.

with a fluorometer, integrator on the analytical column, by the method of Khorlin [5]. The physical and chemical characteristics of homogenous endo-1,4-β glucanase are given in Table 2.

The results of analysis have shown that endoglucanase contains 326 amino acid residues, among which

Table 2

Physico-chemical characteristics of homogenous endoglucanases

Producer	Molecular weight, Dal.	K_m, g/l	K, kat/sec	V_{max}, mkmol/min	K_{s10}, g/l×min	K_p, l/g	K_i, g/l	P_i	Carbohydrate part
Aspergillus versicolor, thermophile	51000	2.2	45	74	18	2.2	105	4.7	22

wave length. The analysis of SH groups was carried out by titration with chlormercurybenzoate in 0.33 M acetate buffer pH4.6 at 255nμ.

In order to determine the endoglucanase carbohydrate part acidic hydrolysis of a sample was carried out at 100°C for 4 hours. Carbohydrate quantitative analysis was accomplished on HPLC DuPoint sp 800, supplemented

35% are hydrophilic amino acids, 26% - with uncharged polar groups; 2.7% acid amino acids, 11.6% are alkali amino acids; 0.2% cysteine, 22% carbohydrates. According to the literature data, high content of hydrophobic amino acids and low amount of cysteine are characteristic of heat-stable proteins. The carbohydrate part of protein is also an important stabilizing factor.

ბიოქიმია

ექსტრემოფილური მიკრომიცეტის Aspergillus versicolor-83-ის ენდო-1,4-β-გლუკანაზას ჰომოგენური პრეპარატი და მისი კინეტიკური და მოლეკულური მახასიათებლები

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ს. დურმიშიძის ბიოქიმიის და ბიოტექნოლოგიის ინსტიტუტში არსებული კავკასიის სხვადასხვა ეკოლოგიური ნიშნებიდან გამოყოფილი მიკრომიცეტების კოლექციიდან შეირჩა ცელულაზების აქტიური პროდუცენტი, თერმოფილური და აციდოფილური შტამი Aspergillus versicolor-83. მიღებულ იქნა ამ შტამის ცელულაზური კომპლექსის ერთ-ერთი ფერმენტის, ენდო-1,4-β-გლუკანაზას ჰომოგენური პრეპარატი, და შესწავლილ იქნა მისი კინეტიკური მახასიათებლები, უხსნად სუბსტრატზე ადსორბცია, რეაქციის პროდუქტებით ინჰიბირების ტიპი და ხარისი, დადგინდა ამინომჟავური შედგენილობა და იზოელექტრული წერტილი.

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