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Separation of Two Forms of Extracellular β-glucosidase From a Thermotolerant *Aspergillus niveus*

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فصل شكلين من الانزيم الخارج خلوي بيتا ـ كلوكوسيديز من الفطر المتحمل للحرارة *Aspergillus niveus*

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تـم فصـل شـكلين من أنزيم بيتا – كلوكوسيديز (EC 3.2.1.21) من الوسط الزرعي للفطر المتحمل للحرارة *Aspergillus niveus* وذلك بالتنقية الجزئية للأنزيم باستخدام عمود من هلام الكروماتوكرافي نوع *Sephadex G-100* يختلف هذين الشكلين من الأنزيم بالوزن الجزيئي حيث كان *149 kDa* بالنسبة للانزيم الاول GI هـو 97.7 kDa وقد تمت مناقشة وجود أشكال متعددة من الأنزيم كما قد تم مقارنة النتائج مع أنزيم بيت– أكلوكوسيديز للفطريات الأخرى.

Keywords: *Aspergillu niveus, Cellulase, β-glucosidase, molecular weight.*

ABSTRACT

Two forms of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from the culture filtrate of *Aspergillus niveus* were separated and partially purified by gel chromatography on Sephadex G-100 column. The two forms differ from each other with respect to molecular weight, being 194 kDa for βGI and 97.7 kDa for βGII. The results are compared to other fungal β-glucosidases and the multiplicity of the enzyme is discussed.

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Introduction:

β-glucosidase (β-D-glucosidase glucohydrolase; EC 3.2.1.21) is frequently a rate- limiting enzyme in microbial degradation of cellulose to glucose. The enzyme is common among plants, fungi and bacteria. Cellulose is converted to cellobiose by extracellular endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC3.2.1.91). The β-glucosidases, which are usually cell associated in bacteria [1] or mainly extracellular in fungi [2-3], convert cellobiose to glucose. β-glucosidase has aroused a considerable interest primarily because of its involvement in the biological transformations of cellulosic material. The enzyme catalyzes the hydrolysis of various compounds with β-D-glucosidic linkages [4] and plays a crucial role in large-scale saccharification of cellulose by removing cellobiose; an inhibitor of endoglucanase and cellobiohydrolase [2,5,6]. Several fungi such as *Sporotrichum thermophile* [7], *Trichoderma koningii* [8], *Talaromyces emersonii* [9] and *Trichoderma reesei* [2] contain multiple forms of β-glucosidase.

The thermotolerant fungus, *A.. niveus* was isolated from desert soil and found to possess a highly active cellulolytic system. This organism is a hyperproducer of β-glucosidase in comparison with *T.reesei* Rut C30 [10]. The optimum conditions for cellulase, β-glucosidase production and saccharification of biomass were determined for this organism [3].

It is already established that 80.9% of β-glucosidase is extracellular [11] . To determine the multiple forms of β- glucosidase from *A. niveus*, the enzyme was isolated and partially purified using sephadex G-100 gel filtration.

Materials and Methods

Organism. *Aspergillus niveus* Blochwits RMF 7883 was isolated from desert soil [10]. The identification of this species was confirmed by Prof. M. Christensen (Department of Botany, University of Wyoming, USA).

Enzyme production and culture technique. Cultures were grown in 1L shake flasks containing 400 ml of Czapek medium of the following composition (L^{-1}): 3g $NaNo_3$, 1g $K_2 HPO_4$, 0.5g $MgSO_4.7H_2O$, 0.5g KCL, 0.01g $FeSO_4.7H_2O$ and 10 g Whatman filter paper no.1. The medium was buffered with 40 mM KH_2PO_4 and 3g L^{-1} potassium phthalate [3]. Fungal spores from 7 days old slants were used to inoculate flask culture media (approx. $6x10^8$ total spores per flask). Cultures were incubated in an orbital incubator at 120 strokes min^{-1}at 35^0C for 12 days. Mycelia were harvested by centrifugation at room temperature at 2,200xg for 10 min. The supernatant was used for the determination of extracellular enzyme activity. Culture fluid was further clarified using Whatman 0.45μm WCN filters. The enzyme was then concentrated by freeze drying to 1:50 of the original volume.Sodium azide (final concentration 0.02% w/v) was added to all assay buffers to reduce contamination.

Enzyme assays. β-glucosidase activity was assayed as described by Kubicek [12]. Using P-Nitrophenyl β-D-glucopyranoside (PNPG).

Sephadex G-100 gel filtration. A column of Sephadex G-100 (70 x 2.5 cm, Pharmacia, Sweden) equilibrated with 50 mM citrate buffer pH 5 was loaded with enzyme (2.5 mg protein) and eluted with the same buffer. One hundred fractions, each containing 5 ml, were collected.

Molecular weight determination. Molecular weights were estimated by gel filtration chromatography [11]. A column (70 X 2.6 cm) containing Ultrogel AcA 34 equilibrated with citrate buffer pH 5 was used. The column was calibrated using standard markers of known molecular weight, namely blue dextran (2000 kDa), chymotrypsin (25 kDa), yeast hexokinase (100 kDa), aldolase (161 kDa) and pyruvate kinase (237 Kda). The crude enzyme and marker proteins (2.5 mg each) were applied to the column in 1 ml Na-citrate buffer pH 5. The elution of the marker proteins and the enzymes was determined by A_{280} and by activity, respectively.Elution data were plotted as Ve/Vo against log molecular weight [13].

Chemicals. Blue dextran (2000 kDa), Yeast hexokinase (100 kDa) and chymotrypsin (25 kDa) were obtained from Fuka AG, Switzerland. Aldolase (161 kDa) and pyruvate kinase (237 Kda) were from Koch-Light Labs,England. Ultrogel AcA 34 was from LKB, Sweden. All other chemicals were of analytical grade from Koch-Light, Fluka or E. Merck (Germany).

Results and Discussion

When the crude enzyme was subjected to gel chromatography on a column of Sephadex G-100, β-glucosidase activity appeared as two peaks, designated as βGI (large peak) and βGII (small peak). Which were identified in the eluted fractions using PNPG substrate .A typical elution profile is shown in fig.1. Generally, two types of β-glucosidases (aryl-β-glucosidase and β- glucosidase), are produced by microorganisms. The former hydrolyzes aryl β -glucosidases is unable to cleave cellobiose while the latter termed cellobiase is active against both types of substrates. The enzymes from many fungal and bacterial sources fall into this category [1] . The presence of several β-glucosidases commonly appears among cellulolytic fungi. Two extracellular β-glucosidases were purified and characterized from *Macrophomina phaseolina* [14] , *Achlya ambisexualis* [15] and *T. reesei* [2]. In *T. emersonii,* four forms of β-glucosidase, three extracellular (βGI, βGII & βGIII) and one intracellular form (βGIV) have been purified and characterized by McHale & Coughlan [16]. Chirico & Brown [17] reported three forms of extracellular β-glucosidases for *T. reesei* QM 9414. In the rumen fungus, *Orpinomyces* sp. strain PC-2, a single exracellular β -glucosidase was purified from culture filtrate [18].

The molecular weights of βGI and βGII were determined as 194 kDa and 97.7 kDa, respectively by Ultrogel AcA 34 and Sephadex G-100. For comparison, table 1 represents the molecular weights of β-glucosidases for some fungi

Multiple forms of cellulolytic enzymes can be due to multiple genes in bacteria [19]. Different β-glucosidases are coded by separate genes , but multiple forms of the enzyme having different pI values may also be due to differences in charge or in contents of carbohydrates [5]. Separation of multiple forms by gel electrophoresis and characterization of the enzymes are reported in a forthcoming paper.

Table 1: Molecular weights of β -glucosidases for various cellulolytic fungi

Fungi	Enzyme	Molecular weight (kDa)	References
Macrophomina phaseolina	BGI	323.6	Saha *etal.*(14)
	BGII	213.8	
Talaromyces emersonii	BGI	135	Mchale & Coughlan (16)
	BGII	100	
	BGIII	57	
	BGIV	45.7	
Aspergillus fumigatus	BG	380	Kitpreechavanich, Hayashi & Nagai (20)
Orpinomyces sp. strain PC –2	BG	84.4	Chen *etal.* (18)
Aspergillus niveus	BGI	194	The present Study
	BGII	97.7	

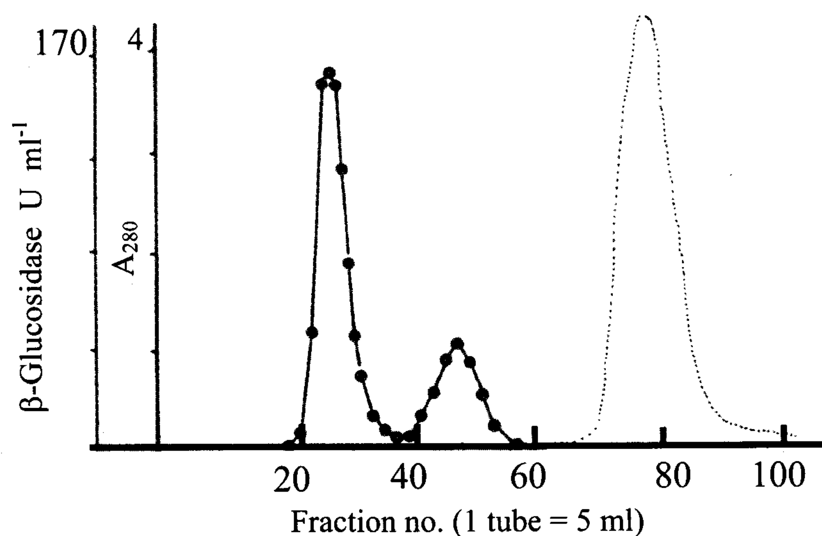

Fig. 1 : Sephadex G-100 chromatography of β -glucosidase from *A.. niveus.* β-glucosidase (•) activity was determined with PNPG. A_{280} (….). Column of 70 x 2.6 cm equilibrated with citrate buffer pH5. Fractions were collected at a flow rate of 1 ml min^{-1}.The large peak is BGI and the small peak is BGII.

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