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Study on heterogeneity of β-glucosidase from *Aspergillus* species by using counter-current distribution

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Abstract

β-Glucosidase plays a key role in cellulose degradation by cleaving its cellobiose units to glucose. The heterogeneity of the enzyme has been studied earlier by isoelectric focusing and several isoforms were found in the culture filtrate of *Trichoderma* and *Aspergillus* species. An aqueous two-phase system is an alternative fractionation method in which proteins are separated according to their net charge and surface properties. A dextran 500–poly(ethylene glycol) 8000 system was used to study the distribution of β-glucosidase of different origins. To investigate the heterogeneity of enzymes 56- and 145-step thin-layer counter-current distributions were carried out. β-Glucosidase produced by *Aspergillus niger* and *Aspergillus carbonarius* has been shown to consist of at least three isoenzymes by applying this method. The three forms of *A. niger* show the corresponding partition ratios with *G* values of 0.13, 0.22 and 0.31; and *A. carbonarius* 0.14, 0.22 and 0.36, respectively. However, the relative amount of these isoforms differs strongly between the two microorganisms.

Keywords: Counter-current distribution; Aqueous two-phase systems; Partitioning; *Aspergillus*; Enzymes; β-Glucosidase

1. Introduction

The enzyme β-glucosidase, present in many microorganisms, is responsible for the conversion of cellobiose to glucose in the cellulose degradation pathway. An aqueous two-phase system can be applied for various purposes in cellulose hydrolysis [1,2], β-glucosidase fermentation [3] and enzyme downstream processing [4,5]. By this method proteins are separated according to their net charge and surface properties. The partitioning of proteins within the two-phase system can be influenced by variation of a number of parameters such as polymer type and concentration, kind of salt present, pH and temperature. Two-phase partitioning used in multiple-step procedures (e.g., counter-current distribution) is a tool to investigate the presence of the different isoforms of an enzyme on an analytical scale. β-Glucosidase has been found in earlier studies by isoelectric focusing to be inhomogenous [6–8]. In a poly(ethylene glycol) (PEG)–dextran two-phase system, β-glucosidase shows much higher affinity to the dextran-rich lower phase than for the PEG-rich upper phase [1–4]. This fact has been used for purification and concentration of the enzyme [3,4].

In the present work we have analysed the heterogeneity of β-glucosidase from two sources, *Aspergil-*

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lus niger and *Aspergillus carbonarius*, by using counter-current distribution [9] of fermentation filtrate with an aqueous two-phase system. Indication of heterogeneity already seen at the normally used number of transfers, 50–60, could be verified by using a greater number of transfers.

2. Materials and methods

2.1. Chemicals

Dextran 500 (M_r=500 000) was purchased from Pharmacia (Uppsala, Sweden). PEG 8000 (M_r= 8000) was obtained from Union Carbide (New York, NY, USA) as Carbowax 8000. All other chemicals were of analytical grade.

2.2. Microorganisms

The fungi *A. niger* BKM F-1305 and *A. phoenicis* QM 329 were cultivated on agar slant containing 5% malt extract and 18 g/l bacto agar, at 30°C then maintained at 4°C. These strains were obtained from the Collection of the Department of Agricultural Chemical Technology, Technical University of Budapest (Budapest, Hungary). *A. carbonarius* KLU-93 was cultivated on Sabouraud's agar slant containing 10 g/l mycological peptone, 16 g/l bacto agar and 40 g/l glucose at 26°C. The strain was maintained at 4°C. *A. carbonarius* was obtained from the Department of Microbiology and Biotechnology, Lajos Kossuth University (Debrecen, Hungary). After 14 days of cultivation the spores were used for inoculation of the below mentioned media.

2.3. Fermentation

Both strains were cultivated in a medium based on Mandels' salt [10] and glucose was used as carbon source. The culture medium was inoculated with a spore suspension of *A. carbonarius* containing 10^6–10^7 conidia/ml and was grown in shake flask on 10 g/l glucose with an initial pH of 5.8.

For *A. niger* fermentation an inoculum was used which was prepared on 5% malt extract for 2 days. The cultivation of *A. niger* was carried out in a 1-l fermenter (Biostat Q, B. Braun, Germany) on a modified Mandels' medium [10] at 28°C and 500 rpm. The medium composition of the stirred-tank fermenter cultivation was: urea 1.41 g/l, $(NH_4)_2SO_4$ 6.58 g/l, KH_2PO_4 3.6 g/l, $CaCl_2$ 0.3 g/l, $MgSO_4 \cdot 7H_2O$ 0.3 g/l, proteose peptone 3.53 g/l, yeast extract 1.18 g/l and trace elements were added: 0.5 ml/l $FeSO_4 \cdot 7H_2O$, 0.16 ml/l $MnSO_4$, 0.14 ml/l $ZnSO_4$ and 0.2 ml/l $CoCl_2$ from 1% solutions together with 20.7 g/l glucose as carbon source. The initial pH of the medium was 5.8–6.0. The working volume was 600 ml medium containing 60 ml of inoculum. The pH was maintained at pH 5.8 with 10% NaOH and 10% H_2SO_4. After 4 days cultivation the fermentation broths were filtered and the supernatant were stored at 3°C.

2.4. Enzyme assay

The β-glucosidase activity was determined according to Norkrans [11] but using 5 m*M* substrate instead of the recommended 1 m*M* concentration. A 0.1-ml volume of sample was mixed with 1 ml of 5 m*M* 4-nitrophenyl-β-D-glucopyranoside in 50 m*M* sodium citrate buffer, pH 4.8 and kept at 50°C for 10 min. The reaction was stopped by addition of 2 ml of 1 *M* Na_2CO_3 followed by 10 ml of water and the absorbance was measured at 400 nm. In the blank, 0.1 ml of water was used instead of the enzyme sample. The activity was defined as μmol of *p*-nitrophenol produced per minute under the assay conditions (IU). Concentration of enzyme was calculated as IU/ml. One absorbance unit corresponds to 1.32 enzyme units per ml. The assays were done in duplicate or triplicate on each sample and the average values were used.

2.5. Two-phase partitioning

Calculated amounts of 40% (w/w) PEG 8000, 20% (w/w) dextran 500, and salt solution (normally lithium phosphate buffer) were dissolved in culture filtrate. After equilibration by careful mixing at room temperature, 23°C, the systems were centrifuged at low speed (1400 *g* for 10 min) using a swing-out rotor. β-Glucosidase content was measured in the top and the bottom phases as well after corresponding dilution.

2.6. Calculations

The activity of partitioned enzyme (IU), m, in the two phases (upper, u and lower, l) is used to calculate the partition ratio, G defined as $G=m_u/m_l$ [12]. The partition coefficient, $K=C_u/C_l$, where C is the volumetric concentration of enzyme (IU/ml) in upper and lower phases, respectively, is calculated from the determined concentrations. However, it may also be obtained from the volumes of the two phases, V_u and V_l and the G value via $K = GV_l/V_u$. In case of the multiple-step counter-current distribution (CCD), theoretical distribution curves were calculated for the possible β-glucosidase components by the equation:

$$T_i = \frac{n!}{i!(n-i)!}\,\frac{G^i}{(1+G)^n}$$

where T_i is the fraction of the substance recovered in tube i and n is the number of transfers [12].

2.7. One-step extraction

The one-step extraction was carried out in a system containing 7% PEG, 7% dextran, 50 m*M* sodium phosphate buffer (pH 5.8) and 10% of culture filtrate. The extraction was performed in graduated and calibrated 10-ml centrifuge tubes in order to determine the phase volumes. After the separation of phases β-glucosidase activity was measured both in upper and lower phases.

2.8. Counter-current distribution

The counter-current procedure has taken place in a partition cellblock, which is made up of two cylindrical plates made of plexiglass (Fig. 1) [9]. The lower plate, a, has a shallow annular groove, 1, which is concentric with the vertical cylindrical axis. A number of shallow cavities, 2, in this groove form the bottom parts of the partition cells and contain the lower phase. The depth of these cavities is 2 mm. The upper plate, b, rests in the groove of the lower plate. The upper plate can rotate about it axis and is guided by the inner and outer edges of the groove. The lower surface of the upper plate, which is in contact with the groove, also has cavities, 3, of the same number and horizontal cross section as the bottom plate cavities. The upper plate cavities contain the top phase.

By turning the upper plate relative to the lower plate each cavity of the rotor can be successively brought into coincidence with each cavity of the lower plate. A circular sequence of partition cells is thus formed following by shaking the new systems. Each upper cavity is proved by a hole, 4, in the top for filling and emptying. A circular cover, c, is used to close the holes. The partition block rests on a horizontal-shaking table, d, with a driving motor and

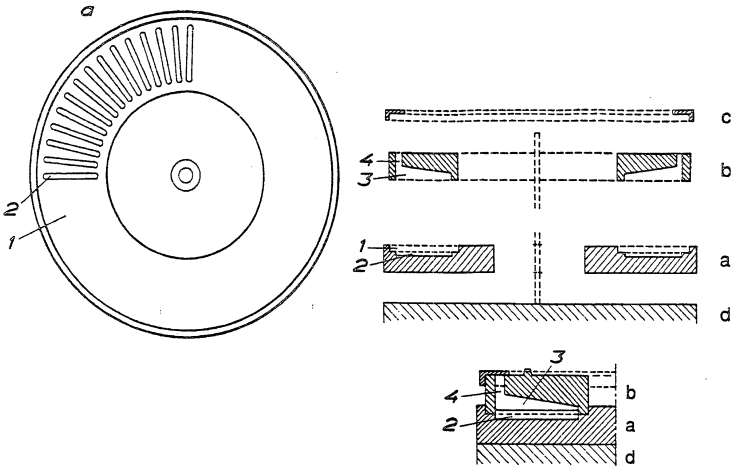

Fig. 1. Drawing of thin-layer counter-current distribution apparatus. For explanation see text. (From Ref. [9], p. 126, with permission).

a device for variation of frequency of shaking. In the center of the table there is also a unit which drives the rotation of the upper plate. All procedures including shaking, settling and phase transfer are guided automatically by a control unit.

The multiple partition processes were performed in the above described thin-layer counter-current distribution machine equipped with 60-chamber plate with bottom phase cavities of 1.05 ml volume was applied (Hahn-Magnet UF, GLG 72 E2, Lund, Sweden). The number of transfers was 56 or 145. In each chamber, 2.75 g of phase system was pipetted containing 7% (w/w) PEG, 7% (w/w) dextran and 50 m*M* Na phosphate buffer, pH 5.8, except for the systems 0, 1 and 2 where 44.3–47.5% of culture filtrate was included. The settling time was 10–12 min and the shaking time 1 min. The experiments were carried out at room temperature, 23°C. After the counter-current distribution, 50 m*M* citrate buffer, pH 4.8, was added to each cavity in an amount of 0.7 ml to give homogeneous solution, which were collected and analysed for β-glucosidase content. CCDs with 56 and 145 steps, respectively, were performed in the same CCD plate. In case of 145-transfer, the plate was totally rotated 2.4 times.

3. Results

3.1. One-step extraction

One-step extraction was carried out in order to determine the partition ratio, *G*, of β-glucosidase obtained by the cultivation of *A. niger* and *A. carbonarius*. Enzymes of both culture filtrates behaved similarly (Table 1). The partition coefficient, *K*, was found to be 0.12 and 0.13 for *A. niger* and *A. carbonarius*, respectively with 96 and 94% recovery based on the added amount of enzyme. The *G*-value of *A. niger* β-glucosidase was calculated to be 0.20 with 83.2% enzyme content in the bottom phase. The most enzyme activity of *A. carbonarius* partitioned to the bottom phase (82.4%) as well, giving a *G*-value of 0.21.

3.2. Counter-current distribution

Each culture filtrate was used in both 56- and 145-step CCDs to examine the heterogeneity of β-glucosidase. The 56-transfer extraction with *A. niger* fermentation broth resulted in one distinct peak. However, the theoretical curve with a *G*-value of 0.2, obtained from the one-step extraction, was unable to cover the experimental curve (Fig. 2A). This indicates the presence of at least two components of β-glucosidase in the *A. niger* culture filtrate (Fig. 2B). The same phenomenon was observed in the case of *A. carbonarius*. Analysing the data, at least two theoretical curves can also be fitted to the experimental curve (Fig. 3A and B). The recovery of β-glucosidase was high, 108 and 91% for *A. niger* and *A. carbonarius*, respectively.

To more clearly observe the heterogeneity of β-glucosidase 145-step CCDs were performed. These experiments confirmed the indication above by giving a more distinct separation of the β-glucosidase components obtained from both *Aspergilli* with 81–94% recovery (Fig. 4A and B).

To compare the two CCDs the *G*-values of theoretical curves have been summarised in Table 2. Concerning the *A. niger* culture filtrate in the 145-step CCD, a third form of β-glucosidase with a 0.14 *G*-value was indicated by analysis of the experimental curve. However, the distribution of fractions was

Table 1
β-Glucosidase partitioned in single-step extraction

	A. niger			*A. carbonarius*		
	Culture filtrate	Top phase	Bottom phase	Culture filtrate	Top phase	Bottom phase
Volume (ml)	0.50	2.90	1.70	0.50	3.00	1.80
Concentration (IU/ml)	3.26	0.09	0.76	0.72	0.02	0.16
Activity (IU)	1.62	0.26	1.29	0.36	0.06	0.28
Recovery (%)	100	16.0	80.0	100	16.7	77.8

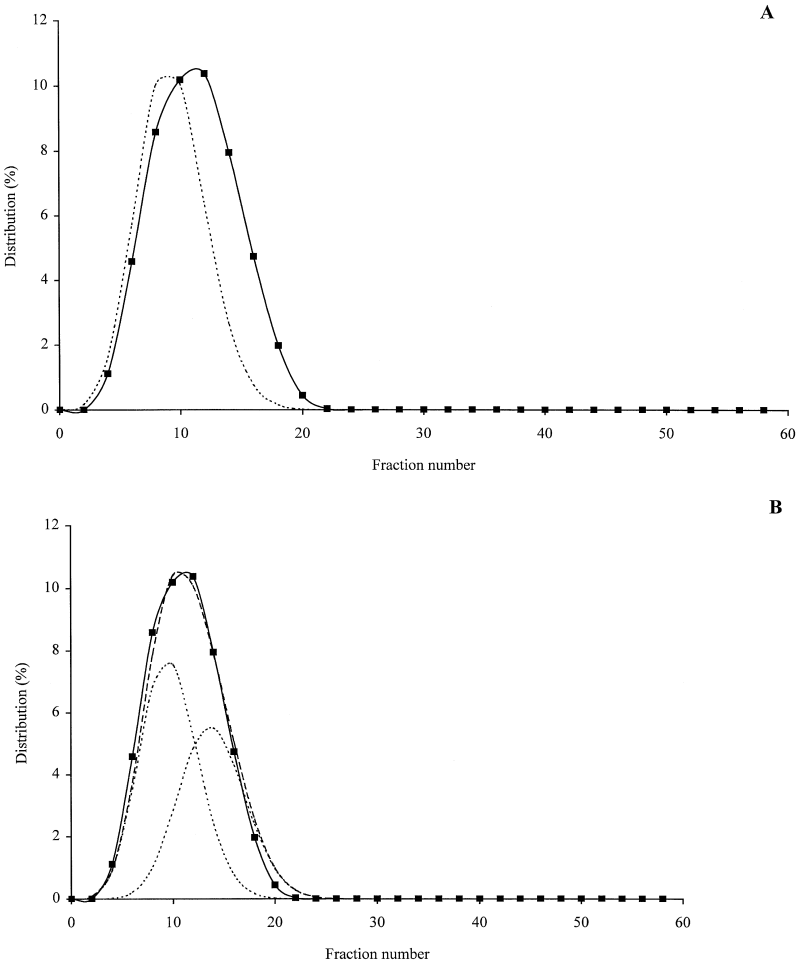

Fig. 2. Counter-current distribution of β-glucosidase from *A. niger* by 56-step extraction. – ■ –, Experimental curve, - - -, theoretical curves, — – —, sum of the theoretical curves. (A) Theoretical curve from the one-step extraction. (B) Fitted theoretical curves.

not found to correlate exactly with that obtained in the 56-step CCD. In the extraction of *A. carbonarius* a new component was also detected but with a higher *G*-value, 0.31, than was found in the 56-step CCD.

Finally, both strains could produce the same β-glucosidase fractions with corresponding *G*-values 0.13–0.14 (form I), 0.22 (form II) and 0.31–0.36 (form III). The relative amount of these hypothetical components differed between the two strains. The culture filtrate of *A. niger* contained 69.8% enzyme fraction with a high *G*-value, 0.36, while *A. carbonarius* had a 47.4% enzyme content with a low *G*-value, 0.13.

4. Discussion

β-Glucosidase from *Aspergillus* strains showed similar partitioning in the aqueous two-phase system with high affinity to the lower dextran-rich phase.

The broadness of the counter-current distribution curve with 56-step extraction indicated the presence of at least two isoforms. However, a longer CCD with a 145-step extraction, separated three enzyme components. The main difference between the enzymes obtained from the two *Aspergillus* strains was the ratio of the amount of isoenzymes. *A. niger* produced 4.7-times more enzyme III than form I and

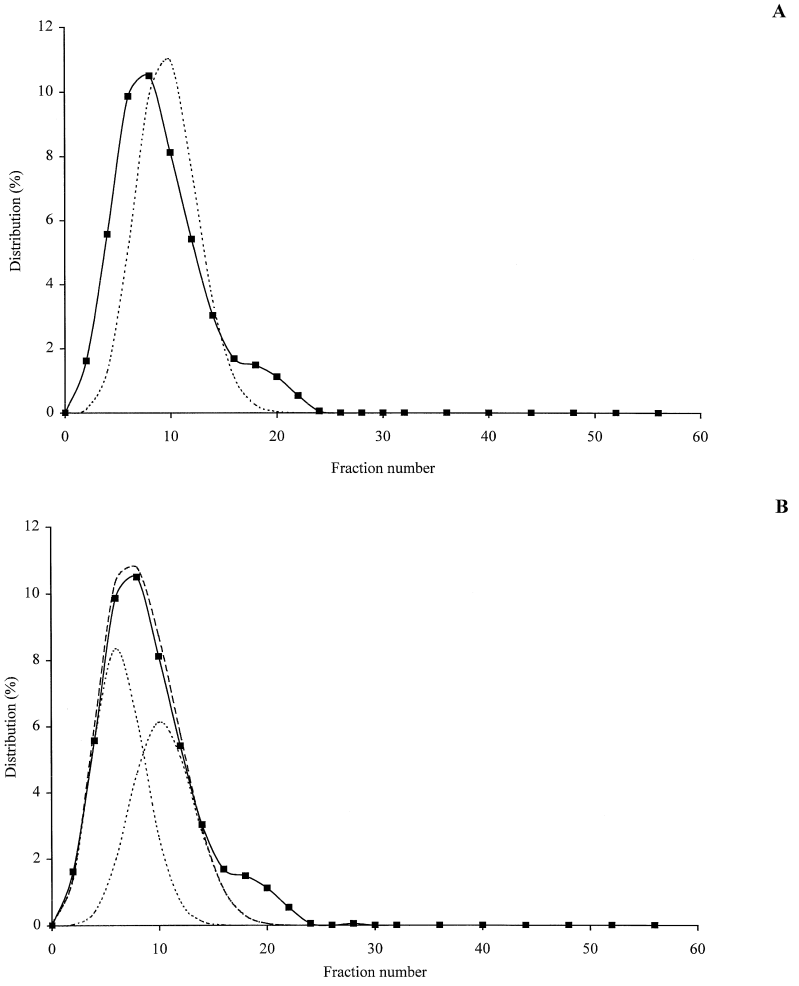

Fig. 3. Counter-current distribution of β-glucosidase from *A. carbonarius* by 56-step extraction. – ■ –, Experimental curve, - - -, theoretical curves, — – —, sum of the theoretical curves. (A) Theoretical curve from the one-step extraction. (B) Fitted theoretical curves.

form II, respectively. In contrast, *A. carbonarius* synthesised 2.9-times of isoform I compared with form II and 1.3-times more than form III (Table 2).

These results confirm the appearance of different isoenzymes in the culture filtrate obtained by the fermentation of *Aspergillus* species. However, by chromatofocusing only one form was found in both culture filtrates with isoelectric point (p*I*) values 3.69 and 4.19 for *A. niger* and *A. carbonarius*, respectively [13].

Even if the proteins have similar isoelectric points, they could differ in net charge at other pH values such as the one used in the aqueous two-phase experiments, pH 5.8. In this method, separation is based on the net charge and the surface properties of protein [9]. The partitioning might be influenced by the carbohydrate content of enzyme. β-Glucosidase from various sources contains different amounts of carbohydrate up to 33% of molar mass [14]. The reason for the various isoforms might be the different hydrolytic functions, which make the fungi able to utilise cellulose and other substrates as well [7,15].

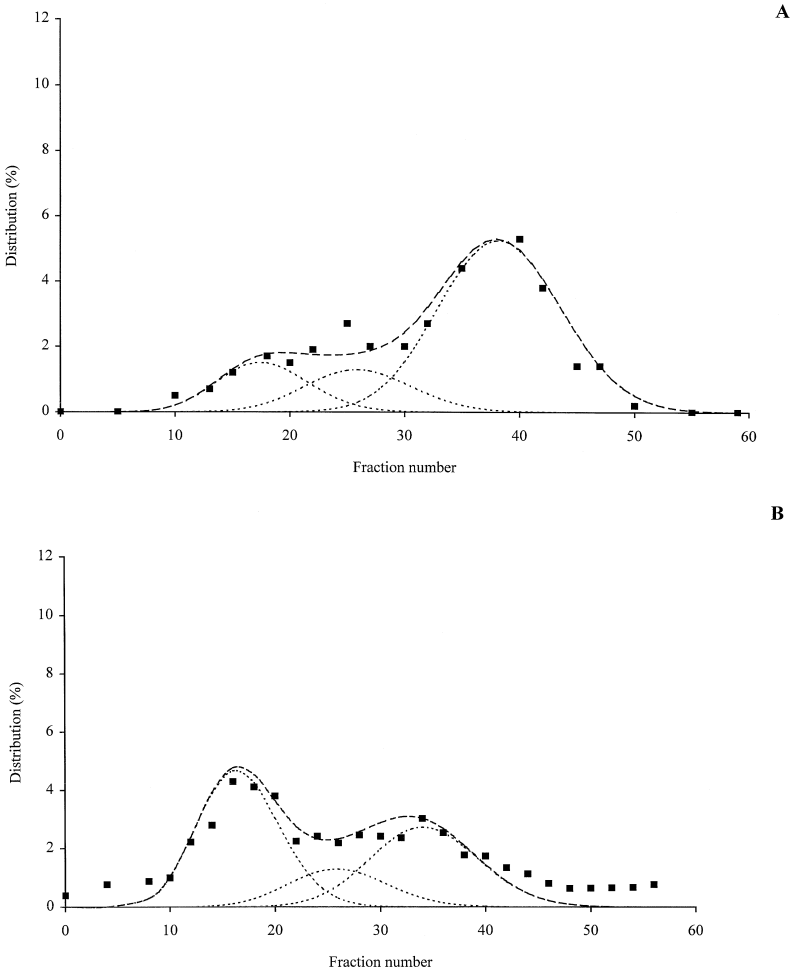

Fig. 4. Fractionation of β-glucosidase by 145-step counter-current distribution. Symbols as in Fig. 3. (A) Separation of *A. niger* culture filtrate. (B) Separation of *A. carbonarius* culture filtrate.

Table 2
Heterogeneity of β-glucosidase showed by counter-current distribution[a]

	Component	*A. niger*		*A. carbonarius*	
		G value	Distribution (%)	*G* value	Distribution (%)
56-step	I	0.21	54.2	0.13	53.3
	II	0.33	45.8	0.23	46.7
145-step	I	0.14	15.0	0.13	47.4
	II	0.22	15.2	0.22	16.3
	III	0.36	69.8	0.31	36.3

[a] *G*-values of the theoretical component and the percentual distributions are given.

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