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Isolation and characterization of α-glucosidase from *Aspergillus niger*

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ABSTRACT

α-Glucosidase is an enzyme widely used in biochemical analytical methods. *Aspergillus niger* was selected as a potential source for its production. Conditions for glucosidase production were optimized and the enzyme was isolated from the culture supernatant by dialysis and anion-exchange chromatography. The activity of the enzyme was determined by maltose hydrolysis to glucose, which was determined using a glucose-specific electrode or by high-performance liquid chromatography. The isolated enzyme was further characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, substrate specificity and fast protein liquid chromatography. The Michaelis constant, optimal temperature and stability of the enzyme preparation were determined.

INTRODUCTION

α-Glucosidase (α-D-glucoside glucohydrolase, E.C. 3.2.1.20) [1] is a hydrolytic enzyme which is used mainly in clinical biochemistry for determination of α-amylase activity [2–4] in blood and serum. This determination is important for the diagnosis of diseases of the gall bladder and pancreas. Fleet *et al.* [5] have suggested that there is a relation between α-glucosidase and human immunodeficiency virus (HIV) activity.

It is found in many microorganisms, however, often together with glucoamylase (α-1,4-glucan-glucohydrolase, E.C. 3.2.1.3). As long as both those enzymes hydrolyse maltose, they are not always properly differentiated. Glucoamylase hydrolyses α-1,4-polysaccharide (glucan) bonds with the release of glucose. It is also possible to break α-1,6 bonds. α-Glucosidase hydrolyses α-1,4 or α-1,6 bonds in short oligosaccharides. Enzymes isolated from different sources sometimes possess different specificity towards maltose and isomaltose, and to *p*-nitrophenyl-α-D-glucopyranoside, sometimes distinguished as maltase and α-glucosidase.

Both extracellular and intracellular α-glucosidase producers and the substrate specificity and other characteristics of isolated enzymes are reviewed by Kelly *et al.* [6]. The production of α-glucosidase by selected microorganisms can be enhanced by selection of a suitable medium, usually containing a substrate [7,8] of the enzyme reaction as an inducer.

The isolation and purification of enzyme from fermentation broth is performed after the separation of cells by centrifugation or filtration. For extracellular enzyme the dialysis of supernatant is recommended [9–11]. Precipitation by ammonium sulphate [10,11] or isopropanol [12] has been described as the next step. Ethanol was not recommended by Martin-Rendon *et al.* [13] because it inhibited the activity of α-glucosidase from *Saccharomyces* and *Candida*. However, precipitation does not separate the amylase activity, and this is a main goal of the purification. The enzyme can be further purified by chromatographic techniques. Gel permeation chro-

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matography [10,14] on Sephadex G-150 has been applied to the separation of α-glucosidase and maltase activity in *Bacillus licheniformis*. Ion-exchange chromatography has been applied to the separation of α-glucosidase and amylase activity on DEAE-Bio-Gel A [12].

Measurement of α-glucosidase activity is usually based on determination of the glucose released by the enzyme. Glucoamylase can be distinguished by its ability to hydrolyse starch [15].

The different authors cited above used different nomenclature for the enzymes which catalyse the hydrolysis of α-glucosidic bonds. Thus the aim of our paper was not only to find a quick and simple method of separating glucosidases with different specificity, but also the proper characterization of these enzymes, produced by *Aspergillus niger*.

EXPERIMENTAL

Materials

Tested microorganisms were obtained from the collection of the Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague.

Chemicals

Yeast autolysate, peptone and casein hydrolysate were from Imuna Šarišské Michalany, Czechoslovakia. *p*-Nitrophenyl-α-D-glucopyranoside, Coomasie Blue R-250, acrylamide, N,N-methylene bisacrylamide and sodium persulphate were from Serva, Heidelberg, Germany. Tris(hydroxymethyl)aminomethane (Tris) was from Fluka, Buchs, Switzerland, N,N,N′,N′-tetramethylenethylenediamine (TEMED) and sodium dodecyl sulphate (SDS) were from Sigma, St. Louis, MO, USA, and starch (Zulkovski) was from Merck, Darmstadt, Germany. Other chemicals used were of reagent grade from Lachema, Brno, Czechoslovakia. Urasol (3.7 m*M* uranyl acetate in 0.15 *M* sodium chloride) was from the Oxochrom glucose diagnostic kit (Lachema).

Instruments

Cultivation was performed in an RT-50 rotary shaker (Developing Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The cooled centrifuge was a Model K-24 from Janetzky, Germany. A 195 D UV spectrometer (Spectromom, Budapest, Hungary) was usd to measure protein content. Ultrafiltration membranes were from Milipore, Milford, MA, USA, Dialysis membranes were from Serva, Heidelberg, Germany. The fast protein liquid chromatography (FPLC) system with a Mono-Q HR 5/5 column was from Pharmacia LKB, Bromma, Sweden. The high-performance liquid chromatography (HPLC) system used for activity measurement consisted of a 64 HPLC pump (Knauer, Bad Homburg, Germany), a 7125 injection valve (Rheodyne, Palo Alto, CA, USA), and an RIDK 102 refractometric detector (Laboratory Instruments, Prague, Czechoslovakia). A 250 × 4 mm I.D. (Tessek Separon SGX RPS 5 μm) with coupled 3 × 30 mm compact glass cartridge guard columns (Tessek Separon HEMA-BIO 1000 SB 10 μm in hydrogen form) and HEMA-BIO 1000 Q 10 μm in hydroxide form, as well as HEMA-cart DEAE cartridges were from Tessek, Prague, Czechoslovakia. The oxygen-selective electrode and MMP 003 low current measurement instrument were from Chemoprojekt, Satalice, Czechoslovakia. Vertical electrophoresis unit EV1 was from Developing Workshops. The Multitemp 2219 cooling unit was from LKB, Bromma, Sweden and the OK-104 conductometer and pH meter were from Radelkis, Budapest, Hungary.

Cultivation media

(a) Complete medium contained 5 g of yeast autolysate, 3 g of peptone, 3 g of casein hydrolysate, 10 g of glucose and distilled water to 1000 ml.

(b) Czapek-Dox medium contained 2 g of $NaNO_3$, 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 10 g of glucose and distilled water to 1000 ml.

(c) Modified Czapek-Dox medium contained the same as above except 10 g of glucose were substituted by 1 g of glucose and 9 g of maltose.

(d) Modified Czapek-Dox medium contained the same as above except 10 g of glucose were substituted by 1 g of glucose and 9 g of soluble starch.

Cultivation of microorganisms

A 150-ml aliquot of culture medium was inoculated with 7 ml of inoculum (medium A inoculated with cells from agar plate for 24 h at 28°C). The cultivation proceeded for 48 h at 28°C on a rotary shaker. The cells were then separated by centrifugation at 1500 *g* for 20 min at 4°C.

For the *Aspergillus niger*, which produced a pellet-form biomass, another inoculation method was used. The spores were washed from the agar plate with sterile distilled water, and the suspension was filtered and used for medium inoculation. Cultivation proceeded for 72 h at 28°C on a rotary shaker. The biomass was then separated by filtration through a Büchner funnel.

Dialysis

Dialysis was performed against 0.01 *M* phosphate buffer (pH 6.5) at 4°C for 16 h with several changes of buffer. The process was controlled by conductivity measurement.

Ultrafiltration

The medium after cultivation and dialysis was ultrafiltered at 4°C through Pellicone membrane with an exclusion limit of 10^4 at 0.5 MPa. After concentration to one-quarter of the original volume the ultrafiltrate was diluted twice and again concentrated to one-quarter of the volume.

Precipitation

The medium remaining after cell separation was precipitated with solid ammonium sulphate for 20 h at 4°C. The precipitate was separated by centrifugation and resolved in 0.1 *M* phosphate buffer pH 5.8.

Anion-exchange chromatography on HEMA-cart DEAE

A 5-ml aliquot of dialysed medium was applied on an equilibrated cartridge (5 ml of distilled water and 5 ml of 0.01 *M* phosphate buffer pH 6.5) from a hypodermic syringe and eluted with 3-ml portions of increasing sodium chloride concentration in phosphate buffer.

Anion-exchange chromatography on Mono-Q HR 5/5 column

A 2-ml aliquot of dialysed medium was applied on a column equilibrated with 0.01 *M* phosphate buffer pH 6.5 and eluted with a sodium chloride gradient at 1 ml/min. Fractions of 1 ml were collected.

Protein content measurement

The protein content was determined [16] from the adsorbance at 260 and 280 nm using the formula $1.45 \times A_{280} - 0.74 \times A_{260}$ (in mg/ml). The cell mass was determined gravimetrically after washing with distilled water and drying to constant weight.

SDS–polyacrylamide gel electrophoresis was performed according to Laemmli [17].

Activity measurement

Enzyme activity was measured by determination of glucose released from maltose. A 1-ml aliquot of 2% maltose in 0.1 *M* phosphate buffer pH 5.8 was incubated with 1 ml of sample for 30 min at 40°C. The reaction was terminated with 0.5 ml of the deproteinization reagent Urasol. After 10 min, the precipitate was separated by centrifugation and the glucose in the supernatant was determined. Two blank controls containing no maltose and no enzyme were measured simultaneously. Substrate specificity was measured for *p*-nitrophenyl-α-D-glucopyranoside and soluble starch using the same procedure.

Glucose determination [18]

The oxygen-selective electrode was covered with a nylon mesh containing immobilized glucose oxidase and catalase. The determination was performed at 30°C in a mixture of 1.4 ml of phosphate buffer and 0.1 ml of sample. Oxygen depletion was measured and evaluated from maxima height. The system was calibrated before each measurement with standard glucose solutions. Alternatively, HPLC determination on a 250 × 4 mm I.D. steel column (Tessek Separon SGX RPS 5 μm octadecyl-modified silica column) was peformed with distilled water as the

TABLE I

EXTRACELLULAR ACTIVITY OF α-GLUCOSIDASE IN THE MEDIUM AFTER CULTIVATION

Microorganism	Activity (nkat/ml)
Endomyces magnusii	0
Hansenula anomala	51.0
Saccharomyces cerevisiae	71.0
Saccharomyces cerevisiae type Malaga	99.6
Saccharomyces cerevisiae type Palestina	99.6
Schizosaccharomyces pombe	63.9
Aspergillus niger	105.0
Penicillium citrinum	112.4
Penicillium brevicompactum	51.3

eluent at a flow-rate of 0.5 ml/min with refractometric detection. When coupled with HEMA-BIO 1000 SB and HEMA-BIO 1000 Q guard columns the system enabled direct injection of buffered samples without disturbance normally caused by the salts. The system was calibrated with glucose and maltose.

RESULTS AND DISCUSSION

Cultivation conditions

From nine tested producers (Table I) *Aspergillus niger* was selected as a promising source owing to its high production rate.

Surprisingly, inoculation with a suspension of spores led to higher activity production than the classical inoculation with a culture inoculum. Many smaller uniform pellets were formed, rather than only a few non-uniform pellets. The activity produced is compared in Table II.

In agreement with the literature, the highest activity production was achieved with maltose as a carbon source (Table III). The maximum activity was produced after 4 days of cultivation later the activity decreased (Fig. 1).

Isolation

As the first step in enzyme isolation after filtration dialysis, ultrafiltration and ammonium sulphate precipitation were tested. The results are given in Table IV. Ultrafiltration caused a high loss of activity, while, probably because of a low protein concentration, precipitation was unsuccessful, no filterable precipitate being formed even under 100% saturation. The filtrate was thus dialysed and further purified by ion-exchange chromatography on anion exchangers. The results from HEMA-cart DEAE separation are given in Fig. 2, while the FPLC separation on Mono-Q column is shown in Fig. 3. The results are summarized in Tables V and VI.

In addition to better recovery in the first case, *i.e.*, with DEAE HEMA cartridges, the method is very quick, simple and does not require special equipment. Good separation of glucosidases with different substrate specificity was achieved in two isolation steps, dialysis and ion-exchange chromatography, which is a significant simplification in comparison with the procedures stated in literature. The degree of purification achieved in the chromatographic step, especially for maltase fraction, was very good (10.5).

Characterization

Two active fractions were isolated, the first exhibiting maltase activity, the second glucoamylase activity. Substrate specificity is compared in

TABLE II

PRODUCTION OF α-GLUCOSIDASE BY *A. NIGER* AFTER DIFFERENT INOCULATION METHODS

Method	Activity (nkat/ml)	Dry mass (g/ml)	Specific activity (nkat per g of dry mass)
Standard	9.0	0.1	90
Suspension of spores	26.7	0.09	297

TABLE III

DEPENDENCE OF EXTRACELLULAR α-GLUCOSIDASE PRODUCTION ON THE CARBON SOURCE

Carbon source	Activity (nkat/ml)
Glucose	7.0
Glucose + starch	11.2
Glucose + maltose	26.7

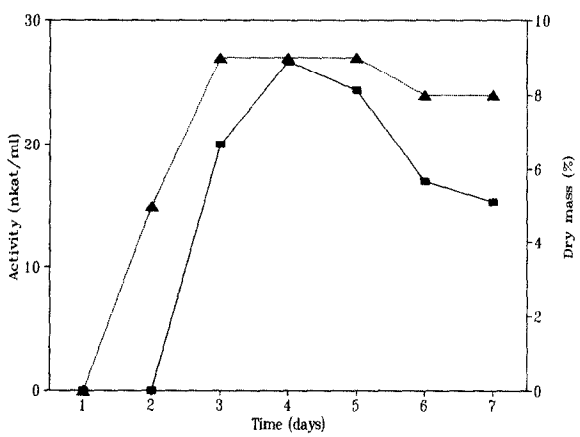

Fig. 1. Time dependence of the α-glucosidase production in *Aspergillus niger*. ■—■ = Activity; ▲···▲ = dry mass.

TABLE IV

EFFECTIVENESS OF FIRST ISOLATION STEPS

Method	Activity (nkat/ml)	
	Before	After
Dialysis	24.0	21.5
Ultrafiltration	24.2	13.0
Precipitation	24.0	No precipitate formed

Table VII. The characteristic parameters for both isolated enzyme fractions are summarized in Table VIII. The dependence of activity on temperature is given in Fig. 4, while the pH dependence of the crude enzyme preparation is shown in Fig. 5. According to SDS-PAGE, two and three protein impurities are present in the enzyme fractions,

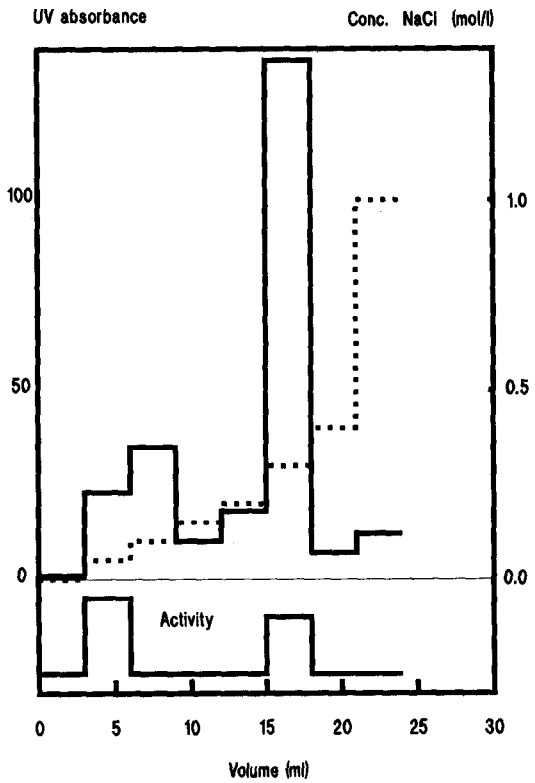

Fig. 2. Anion-exchange chromatography on HEMA-cart DEAE. 1 = α-Glucosidase; 2 = glucoamylase. For conditions see Experimental section.

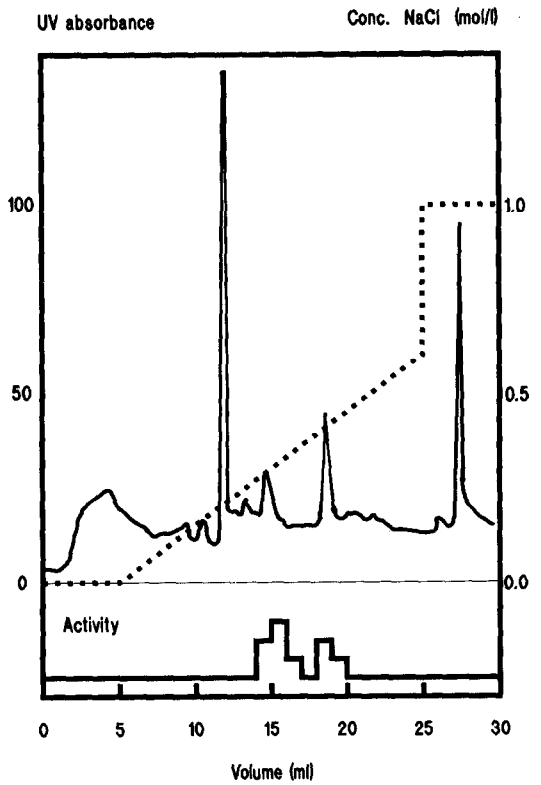

Fig. 3. Anion-exchange chromatography on Mono-Q HR 5/5 column. For conditions see Experimental section.

respectively, none of which possessed α-amylase activity. To obtain a homogeneous preparation, further purification steps would thus be necessary.

Activity determination by HPLC

As a routine method for activity measurement the glucose-selective enzymic electrode was used throughout the work. For comparison, the HPLC

TABLE V

ANION-EXCHANGE CHROMATOGRAPHY ON HEMA-CART DEAE

Sample	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification factor	Recovery (%)
Applied	71.56	1.25	57.32		
Fraction 1	42.00	0.07	600	10.47	92.1
Fraction 2	24.00	0.41	58.54	1.02	

TABLE VI

ANION-EXCHANGE CHROMATOGRAPHY ON MONO-Q HR 5/5

Sample	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification factor	Recovery (%)
Applied	28.00	0.52	53.85		
Fraction 1	13.34	0.05	266.80	4.95	69.07
Fraction 2	6.00	0.11	54.54	1.01	

TABLE VII

SUBSTRATE SPECIFICITY OF ISOLATED FRACTIONS

Glucoside = *p*-nitrophenyl-α-D-glucopyranoside.

Substrate	Activity (nkat/ml)	
	Fraction 1	Fraction 2
Maltose	12.5	7.3
Starch	0	25.0
Glucoside	0	0

TABLE VIII

CHARACTERISTIC PARAMETERS OF ISOLATED ENZYME FRACTIONS

Parameter	Fraction 1	Fraction 2
Substrate specificity	Maltase	Glucoamylase
Weight-average mol. wt. (SDS-PAGE)	131 000	89 000
Optimal temperature	40°C	60°C
Michaelis constant	$7.87 \cdot 10^{-3}$ mol/l	$3.13 \cdot 10^{-2}$ g/ml
(Substrate)	Maltose	Starch
Limiting velocity (V_{lim})	12.93 nkat/ml	82.7 nkat/ml
Remaining activity		
15 days at 4°C	79%	54%
15 days at −20°C	59%	79%
No. of protein impurities	2	3

determination was tested. The HPLC method enables determination of both substrate (maltose) and product (glucose), and has the potential to determine higher oligossaccharides also. A comparison of the results is given in Table IX. The agreement of

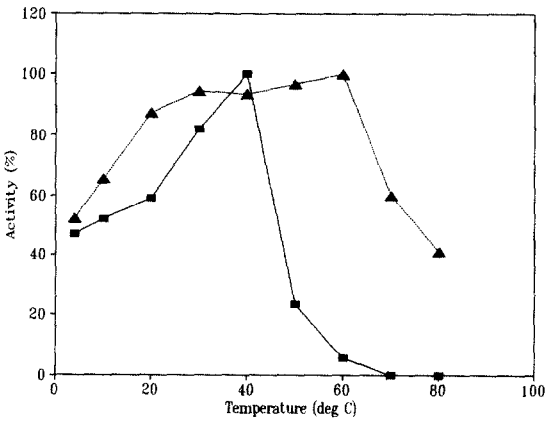

Fig. 4. Temperature dependence of activity of enzyme fractions. Highest activity taken as 100%. ▲···▲ = Glucoamylase; ■—■ = maltase.

both methods is satisfactory; the differences can be probably explained by a wider substrate specificity of the enzyme electrode. The chromatograms are given in Fig. 6. As long as the method takes only a little longer time to perform (analysis time 6 min) and the filtrate after protein precipitation is injected without any pretreatment, it can be an useful alternative method. The advantages will be better specificity and use of lower sample volumes.

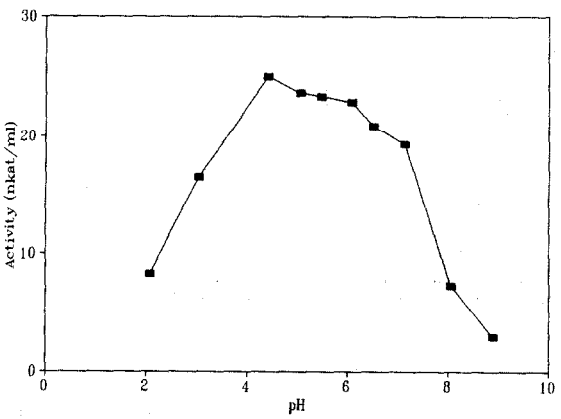

Fig. 5. pH dependence of crude enzyme activity (before separation of fractions).

TABLE IX

COMPARISON OF GLUCOSE DETERMINATION BY ENZYMIC ELECTRODE AND BY HPLC

Sample	Electrode	HPLC
Enzyme sample		
Glucose	6.9 mmol/l	6.14 mmol/l
Maltose		5.88 mmol/l
Blank without enzyme		
Glucose	2.2 mmol/l	0.0 mmol/l
Maltose		10.17 mmol/l
Blank without substrate		
Glucose	0.5 mmol/l	0.45 mmol/l
Maltose		0.22 mmol/l
Activity	14 nkat/ml	19 nkat/ml

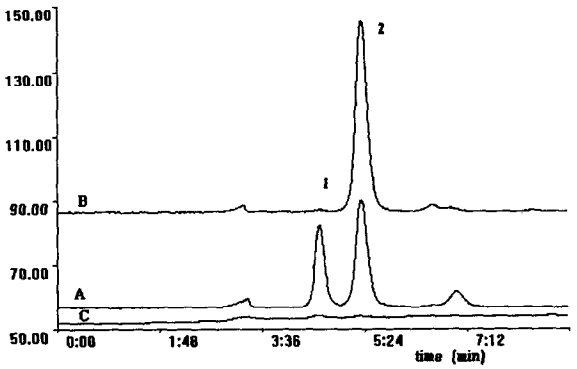

Fig. 6. HPLC determination of glucose and maltose for activity measurement. 1 = Glucose; 2 = maltose. (A) Measured sample; (B) blank (no sample); (C) blank (no maltose).

CONCLUSIONS

Two enzyme fractions isolated from *Aspergillus niger* exhibited maltase and glucoamylase activities as determined by anion-exchange chromatography. Basic characterization of both fractions was performed.

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