

A simple method of affinity chromatography for the purification of glucoamylase obtained from *Aspergillus niger* C

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1. INTRODUCTION

Glucoamylase (α -1,4-glucan-glucohydrolase) (EC 3.2.1.3) is an exosplitting enzyme which removes glucose from the non-reducing ends of starch polymers. This amylase is one of the most important industrial enzymes. It is used on a large scale for the saccharification of starch to dextrins or glucose in food industries [1]. The enzyme has been obtained from *Aspergillus niger* [2] and *A. awamori* [3] and partially purified using conventional chromatographic techniques. There have also been several papers reporting the existence of glucoamylase in *M. rouxianus* [4], *Rhizopus* sp. [5], *Penicillium oxalicum* [6], yeasts [7] and bacteria [8].

The characterization of this enzyme has indicated that glucoamylase of *A. niger* consists of two major components of estimated M_r 63 500 $\pm$ 3000 and 57 000 $\pm$ 2500 [2,9]. The purpose of this study is to describe a very simple affinity chromatography technique of glucoamylase (EC 3.2.1.3) purification from a filtrate of *A. niger* C culture. The results reported also suggest an oligomeric structure of this enzyme, shown only as a glycoprotein of M_r 50 000–100 000 [2,3,9–11].

2. MATERIALS AND METHODS

2.1. Microorganism and culture

Aspergillus niger C was kindly supplied by Professor Z. Ilczuk (Laboratory of Industrial Micro-

biology, University of M. Curie-Skłodowska, Lublin). The culture of *A. niger* C was grown on a shaker (220 rotations/min) at 30°C in 500 ml flat-bottomed flasks. Each flask contained 100 ml of a medium which consisted of: molasses, 0.85%; wheat meal, 3.75%; (NH₄)₂HPO₄, 0.114%; (NH₄)₂SO₄, 0.56%; MgSO₄·7 H₂O, 0.014%; and CaCO₃, 0.014%; prepared as in [12]. The pH of the medium was adjusted to 4.8. The flask containing sterilized medium was inoculated with the *A. niger* C spores dispersed in sterile water. The culture of *A. niger* C was incubated under these conditions for 120 h. The mycelium was removed from the medium by centrifugation at 1000 $\times$ g. The supernatant was desalted on the Sephadex G-25 column (26 $\times$ 9 cm) using as an eluent 5 mM Na-acetate buffer (pH 5.5). The desalted medium was lyophilized and used as a material in affinity chromatography experiments.

2.2. Amylopectin

Amylopectin powder (Koch-Light Labs.) was purified as in [13].

Amylopectin was activated using CNBr and coupled to AH-Sepharose (Pharmacia, Uppsala) as in [14].

Amylopectin was activated with CNBr as in [14] and linked to the amipropyl-controlled pore glass support (pore diam. 500 Å, particle size 125–177 μ m, pierce Chemicals, Rockford IL) using the following steps: 22.5 ml 2% amylopectin CNBr solu-

tion were adjusted with solid NaHCO_3 to 0.1 M and NaCl to 0.5 M and mixed with 15 g alkylamine support. The mixture was shaken for 2 h at 20°C and allowed to cool in a refrigerator for 12 h with periodical shaking. Glass support was filtered off on a glass funnel and washed with 150 ml 0.1 M NaHCO_3 containing 0.5 M NaCl. The glass support was then washed exhaustively on a glass funnel with H_2O and used in affinity chromatography experiments.

Amylopectin-CNBr was coupled to the AH-cellulose (lot. no 10779 Merck) under the same conditions as those for the activation of AH-Sephrose [14].

2.3. Glucoamylase assay

Soluble starch (2%) (BDH-Analar Poole) was dissolved in 0.15 M Na-acetate buffer (pH 4.5) and used as a substrate. Glucoamylase solution (0.5 ml) was incubated for 10 min with 3 ml soluble starch at 60°C. Glucose liberated from starch was determined as in [15]. One unit of enzymic activity was defined as the amount of enzyme which liberates 1 μmol glucose from soluble starch in 1 min.

2.4. Disc gel electrophoresis

Protein electrophoresis was performed in a discontinuous electrolyte system as in [16]. For these purposes 3% and 10% polyacrylamide gels were used. After electrophoresis the proteins bands were stained for 0.5 h with 0.15% Coomassie brilliant blue R-250 dissolved in methanol:acetic acid:glycerol:water (16:2:1:23, by vol.) and destained in methanol:acetic acid:glycerol:water (8:2:1:29, by vol.). The 10% polyacrylamide gels containing 0.5% of soluble starch were used for identification of glucoamylase activity as in [17]. After electrophoresis the gels were incubated for 12 h in 0.15 M Na-acetate buffer (pH 4.5) containing 1% NaCl at room temperature. After this, the gels were fixed for a 1 h in 6% trichloroacetic acid. The enzymatically active bands were revealed by immersing the gels in aqueous solution a I_2 0.005%—KI 0.01%. The gels were photographed using Fotopan FF (Poland) film with a yellow-green filter to obtain better contrast.

2.5. Protein M_r determination

For M_r determination, the enzyme and markers

were separately subjected to electrophoresis as in [16] on polyacrylamide gel—0.1% sodium dodecyl sulfate. The markers used were ($M_r \times 10^{-3}$): catalase (60); bovine serum albumin (67); ferritin (220); thyroglobulin (330); (SDS—PAGE, HMW Kit Proteins, Pharmacia, Uppsala 1).

Protein was determined by the modified method of Lowry [18].

3. RESULTS AND DISCUSSION

The lyophilized filtrate of *A. niger* C culture was chosen as a convenient material for glucoamylase (EC 3.2.1.3) purification since it contained 63% of protein and only 7 protein bands (fig. 1). Among the latter 4 bands showed amyolytic activity.

To exclude the presence of α -amylase (EC 3.2.1.1) activity, the preparation used was incubated with 1 mM (hydroxymercuribenzoic acid Na-salt), PCMB (4-chloromercuribenzoic acid), EDTA (ethylene diamine tetraacetic acid), DTNB (*S,S'*-dithiobis-2-nitrobenzoic acid) and also

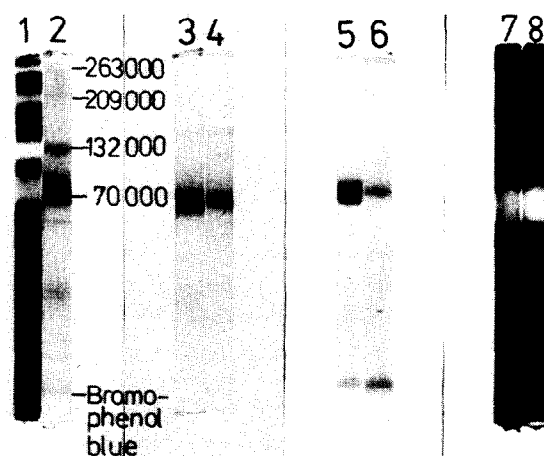


Fig. 1. Polyacrylamide gel electrophoresis of crude and purified by affinity chromatography amyloglucosidase from filtrate after *A. niger* C culture: (1) amylase activity bands of the crude preparation; (2) protein bands of the crude preparation; (3) protein bands of the preparation purified on amylopectin—AH-Sephrose; (4) protein bands of the preparation purified on amylopectin—AA-glass; (5) as in (3) but in the presence of SDS; (6) as in (4) but in the presence of SDS; (7) amyloglucosidase activity bands preparation purified on amylopectin—AH-Sephrose; (8) amyloglucosidase activity bands preparation purified on amylopectin—AA-glass.

heated at 50°C for 1 h. Each of the agents used should inactivate α -amylase [19]. The employed procedures did not modify the number of amylolytic bands or decrease the enzymic activity. In the next experiment the amylolytic active bands were cut out from the polyacrylamide gel after electrophoresis and incubated with starch. All 4 amylolytic active bands catalyze the removal of glucose only from starch. The latter was established using paper chromatography method [3]. The effects of our experiment were similar to those in [3]. According to [3] it was suggested that there were only 4 forms of glucoamylase (EC 3.2.1.3) in the filtrate after *A. niger* C culture.

The main glucoamylase isozyme which corresponded to the greater protein fraction on the electrophorogram had ~64% of enzymatic activity in comparison to the activity of other 3 isozymes (fig. 1). For glucoamylase purification by affinity chromatography technique, CNBr amylopectin was used as a ligand which was bound to three kinds of carriers: AH-cellulose, AH-Sepharose or alkylamine porous glass.

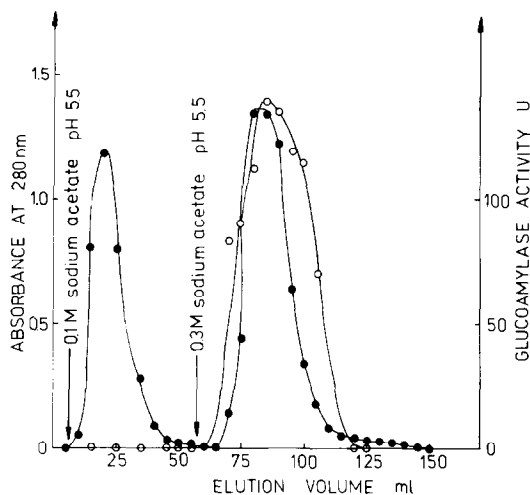


Fig. 2. The elution profile of amyloglucosidase from a column (1.6 × 8.0 cm) of amylopectin-AH-Sepharose 4B: 180 mg lyophilate of filtrate after *A. niger* C culture was dissolved in 5 ml 5 mM Na-acetate buffer (pH 5.5) and applied to the column equilibrated with the above buffer. Chromatography was done at ~5°C; flow rate was ~80 ml/h; 4 ml fractions were collected: amyloglucosidase activity (—○—); absorption at 280 nm (—●—). The column was regenerated with 30 ml 8 M urea.

Glucoamylase from *A. niger* C did not link to AH-cellulose due to affinity forces, as opposed to amylopectin-AH-Sepharose or amylopectin-porous glass. When these carriers were used, glucoamylase was linked and the impurity proteins were eluted from the columns in flowthrough fractions (fig. 2 and 3). Using amylopectin-porous glass as the chromatographic support, more protein impurities were separated from proteins with glucoamylase activity, which increased enzyme purification (fig. 2 and 3). Glucoamylase bound to the amylopectin-AH-Sepharose column was eluted with 0.3 M Na-acetate buffer (pH 5.5) (fig. 2), while the affinity binding strength of glucoamylase to amylopectin-porous glass bed was much stronger. Glucoamylase was eluted from this last affinity column using 0.6 M Na-acetate buffer (pH 5.5) (fig. 3). The glucoamylase activity after amylopectin-AH-Sepharose column was purified from 45–150 U, while specific activity of glucoamylase activity after amylopectin-porous glass column increased from 45–200 U. Polyacrylamide gel electrophoresis of glucoamylase purified by each of the two affinity columns revealed one enzymatic activity band which corresponded to one protein fraction (fig. 1). The latter was observed

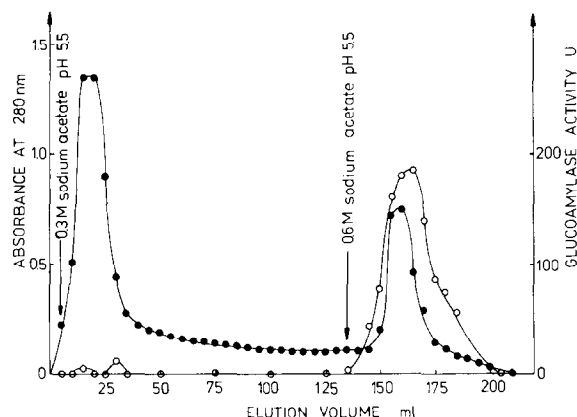


Fig. 3. The elution profile of amyloglucosidase from a column (1.6 × 9.0 cm) of amylopectin-alkylamine porous glass: 150 mg lyophilisate of filtrate after *A. niger* C culture was dissolved in 5 ml 5 mM Na-acetate buffer (pH 5.5) and applied to the column equilibrated with the above buffer. Chromatography was done at ~5°C; flow rate was ~80 ml/h; 4 ml fractions were collected: amyloglucosidase activity (—○—); absorption at 280 nm (—●—).

when electrophoresis was performed in the presence of SDS (fig. 1). When SDS was omitted from the electrophoretic experiments beside the main protein fraction two faint Coomassie blue-positive bands were visibly situated parallel to glucoamylase fractions (fig. 1).

Using the same affinity chromatography columns either with amylopectin-AH-Sepharose or with amylopectin-porous glass glucoamylase purification can be repeated in a lot of cycles. After each cycle of purification the columns were regenerated with 6 M urea. The stability of the columns was checked because the hydrolases could degrade the affinity ligand. In this case, one purification glucoamylase cycle was performed in 2 h at $\sim 5^{\circ}\text{C}$ and buffer pH 5.5. Under such conditions the hydrolysis of amylopectine, used as a ligand was greatly reduced.

The capacity of affinity beds was, respectively: for amylopectin-AH-Sepharose 656 glucoamylase units/ cm^3 of the beds and for amylopectin-porous glass 312.5 glucoamylase units/ cm^3 beds. However, the efficiency of glucoamylase elution from the porous glass column was 32% being greater than from the AH-Sepharose column which was 26% of total enzymatic activity poured onto column.

The purified main fraction of glucoamylase was established to be M_r 70000 by SDS-electrophoretic techniques. Three other glucoamylases had an M_r -value very close to a multiplicity of the above. They were, respectively ($\times 10^{-3}$): 136 ± 5 ; 209 ± 7 ; 263 ± 10 . It was also shown that in the purified glucoamylase (M_r 70000), in denaturing conditions (9 M urea) a new protein fraction was observed which corresponded to M_r 136000.

After these experiments it may be probable that glucoamylase from *A. niger* C has an oligomeric structure. Up to date, this problem has not been solved. In the literature, the M_r estimates of glucoamylase (EC 3.2.1.3) varies from 48000–112000 [2].

The affinity chromatography method described for glucoamylase purification from *A. niger* C is very fast and ensures an output of highly active enzyme preparation. This latter was easy to achieve

since the crude material used for purification of glucoamylase was poor in protein impurities. It is also supposed that these experimental conditions for glucoamylase purification could be used on a greater scale in biotechnology.

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