PRODUCTION AND PURIFICATION OF α-AMYLASE FROM *Aspergillus niger* 33-19 CNMN FD 02A MUTANT FORM

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Abstract. From mutant micelial strain *Aspergillus niger* 33-19 CNMN FD 02A, through alcohol ethylic precipitation of cultural liquid, amylolytic preparation Amilonigrin AS was isolated with 10x degree of purity and a specific activity of 138.3U/mg proteins. α-Amylase from 20mM Tris-HCl extract of Amilonigrin AS was purified to homogeneity by PD-10 column gel filtration and HiTrap™ Q column ion exchange chromatography. A trial for the purification of α-amylase resulted in an enzyme specific activity of 199.68U/mg protein with purification fold 8.9. The analyses of purified α-amylase for molecular weight was carried out by SDS-PAGE electrophoresis, with revealed two polypeptide bands estimated to be 66 and 40.5kDa, probably being two α-amylase izoforms.

Keywords*: Aspergillus niger*, α-amylase, enzyme purification, ion exchange chromatography, SDS-PAGE

INTRODUCTION

Amylases (EC 3.2.1) belong to family 13 of glycoside hydrolase group (GH-13) and catalyze the hydrolysis of amylose and amylopectin from starch, as well as starch derivatives such as dextrins and oligosaccharides. According to enzyme nomenclature system, based on the type of bonds that it hydrolyzes, α-amylase (1,4-D-glucan glucanohydrolase, EC 3.2.1.1) is an enzyme that catalyzes the random endoamylolytic cleavage of 1,4-glucosidic linkages of starch and related components [10, 16, 37]. α-Amylase is a glycoprotein with a single polypeptide chain of about 475 residues. It has two free SH groups and four disulfide bridges and contains tightly bound Ca^{+2} [3]. Calcium ions determine the activity, their structural integrity and stability [24, 34].

Amylases represent about 25-33% of the world market enzymes. They have many practical applications in various fields, being some of the key enzymes of the food, textile and leather industry, manufacture of paper, pharmaceuticals, feed processing and agriculture [6, 14, 33, 35]. In addition, due to the expansion and improvement of biotechnological methods, α-amylases extended their applications in clinical and analytical chemistry, medicine, in fermentation industries at starch saccharification, thus replacing chemical hydrolysis [3, 7, 20, 23].

A new aspect is the use of α-amylase to detergent formulations in laundry industries. Enzymatic detergents are 100% biodegradable and can achieve effective cleaning in just warm water [11, 19].

Although α-amylase can be obtained from several sources, such as plants, animals and microorganisms, enzymes from microbial sources generally have higher demand in industry [36]. The major advantage of using microorganisms in production is capacity to their manipulation to obtain enzyme with desired character. This aspect is due to a number of factors, including productivity, enzyme thermostability and easy cultivation of microorganisms [15, 35].

Industrial production of amylolytic enzymes started more than 100 years ago with a process of production patent of α-amylase from fungus *Aspergillus oryzae*. Studies on fungal α-amylase, especially in developing countries, focused mainly on *Aspergillus* spp. and *Rhizopus* spp., probably due to their ubiquity and non-pretentious nutritional requirements [22, 32]. α-Amylase is derived from a large number of microbial cultures: fungi, yeasts, bacteria and actinobacteria [20]. Fungal α-amylase is preferred in formulations of human and animal consumption, including its ability to act under acidic conditions. At commercial scale α-amylase of fungal origin was found to be more stable than bacterial α-amylase [32].

Commercial producers of α-amylase are *Aspergillus* strains [6]. They have a number of features that make them interesting organisms for industrial applications, such as good fermentation capabilities and high level of enzymes secretion [7]. Filamentous ascomycete *Aspergillus niger* is well known for its ability to produce and secrete a variety of hydrolytic enzymes that contribute to the degradation of plant polysaccharides such as cellulose, hemicellulose, pectin, starch and inulin [36]. The products of these species have obtained GRAS status (generally regarded as safe), which allows them to be used in food and animal fodder obtaining [7].

In recent decades the use of agro-industrial wastes at fungi cultivation expanded [18, 23]. Different vegetal substrates are rich in starch and represent convenient sources of nutrition for the mycelial fungi that α-amylase secrete [36]. This method has high economic value for countries with abundance of agro-industrial residues, so they can be used as cheap raw materials [32]. Cereals bran and flour, potato waste and other starch-component wastes have gained in importance by production of α-amylase from mycelial fungi [38]. The agricultural wastes consist of carbon and nitrogen sources required for growth and metabolism of organisms [15, 35]. The effect of various physico-chemical parameters, different sources of nitrogen, carbon, heavy metals were studied [1, 25].

Although the classic genetic manipulation or recombination DNA techniques are often used to enhance the synthesis of α-amylase from bacteria and fungi, traditional procedures for screening of microorganisms that synthesize enzymes of interest remain attractive [3]. However, selection of an appropriate strain depends on several factors, in particular the nature of the substrate and the growing conditions that are similar to producer cultures [34].

The properties of enzymes and their potential application depend from both producer cultivation circumstances and conditions for enzyme obtaining [36]. Thus, it is important to develop techniques for pure enzymes obtaining, with maximum specific activity [6].

The major objective of this paper is obtaining and purification of α-amylase from *Aspergillus niger* 33-19 CNMN FD 02A mutant strain.

MATERIALS AND METHODS

Microorganism and culture conditions:

Object of study was a mycelial fungi *Aspergillus niger* 33-19 CNMN FD 02A obtained from 1500Gy γ-ray irradiation of parent strain *Aspergillus niger* 33, stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova [5]. Selected mutant was characterized by 1.7-2.1 higher activity of extracellular amylases, which remained stable after 5 years of maintenance [29].

Cultivation of micromycete *Aspergillus niger* 33-19 CNMN FD 02A was carried out in Erlenmeyer flasks of 1000ml volume, under 180-200rpm agitation at a temperature of 28 to 30°C for 6 days. Each flask has 200ml of nutritive medium with the following composition (g/L): starch – 3.0; bean flour – 9.0; wheat bran – 18.0; KH_2PO_4 – 2.0, KCl – 0.5; $MgSO_4$ – 0.5; pH – 3.0.

As seed material served spore suspension in quantity of 10% v/v with density $3 \cdot 10^6$ spores/ml, obtained by washing with sterile water a 12-14 days culture grown on malt-agar oblique columns [31]. The content of spores was determined microscopically with Goreaev counting camera [4].

Enzyme extraction:

For extracellular α-amylase isolation, at the end of cultivation the medium was separated from biomass by filtration. The filtrate was centrifuged at 4000-6000rpm for 20 minutes. The amylolytic enzyme complex was extracted from supernatant with 96% ethanol cooled to -15°C in ratio 1:4 C_2H_5OH, sedimentation duration - one hour, $CaCl_2$ concentration – 0.2% [29, 30]. Obtained sediment was separated through 20 minutes centrifugation at 6000 rpm and dried at 20-22°C. Dried powder was further investigated and purified. The enzyme activity and protein content were recorded.

Enzyme assay:

The α-amylase activity was determined according SKB modified method [7, 9]. The reaction mixture (15ml) contained 10ml of buffered (0.2M acetate buffer, pH 4.7) 1% soluble starch (Sigma) solution as the substrate and 5ml adequately diluted enzyme sample. Incubation was performed at 30°C for 10 minutes. The reaction was terminated and developed with iodine solution in ratio 0.5ml reaction mixture/50ml iodine solution (5mg iodine and 50mg KI in 100ml 0.1N HCl) for 5min. One unit of activity was defined as the amount of enzyme, which catalyzes hydrolysis of one gram soluble starch up to dextrins with different molecular weight; hydrolysis range 30% under the assay conditions.

Protein determination:

Protein was estimated according to the Lowry method of taking crystalline bovine serum albumin as the standard [13].

Enzyme specific activity determination:

The α-amylase activity was calculated per milligram protein from the enzyme preparation according to the following equation: (U/mg) Specific activity = enzyme activity / protein content.

α-Amylase purification:

Gel filtration. 75mg dried enzyme preparation was washed with 3ml 20mM Tris-HCl buffer (pH 7.0), centrifuged for 10min at 14000rpm. The supernatant was collected. The extract (2.5ml) was applied to PD-10 column (Amersham Pharmacia Biotech) previously equilibrated with 20mM Tris-HCl buffer, pH 7.0. The column was eluted with the same buffer.

Ion exchange chromatography. The collected fraction was loaded onto an anion exchange HiTrap™ Q (5ml) column at a flow rate 1ml/min using FPLC System (Amersham Pharmacia). Column equilibration was performed with 20mM Tris-HCl buffer. Unbound protein was eluted with linear gradient of 0-0.5M NaCl in the same buffer. At each stage of purification amylolytic activity and protein content was determined. Active fractions were pooled.

Gel electrophoresis:

The α-amylase purity was checked by SDS-PAGE (15%), carried out by Laemmli method [12]. Gel was stained with coomasie Brillant Blue R-250. The SDS-PAGE molecular weight markers were β-galactosidase (116kDa), phosphorylase b (97.4kDa), bovine serum albumin (66.2kDa), alcohol dehydrogenase (37.6kDa), carbonic anhydrase (28.5kDa).

Statistical analysis:

All the experiments performed thrice and the results are presented as the mean of three. The level of significance is $P<0.05$ [17].

RESULTS

At sedimentation with 96% ethanol and drying of amylolytic complex from micromycete *A. niger* 33-19 CNMN FD 02A cultural liquid, local amylolytic preparation named **Amilonigrin AS** was obtained**,** with 10x degree of purity [29] and a specific activity of 138.3U/mg proteins (Table 1). Respective degree of purity is acceptable for zootechny, food, laundry and light industry [8].

α-Amylase application in medicine and pharmacy shall require an advanced degree of purity of enzyme preparations. Thus, investigation of α-amylase extraction from amylolytic preparation Amilonigrin AS and purification by gel filtration method and ion exchange chromatography (Table 2) show the increase of α-amylase specific activity to 1232.61U/mg protein, a yield of 25% and a purification coefficient of 8.9 times.

Elution profile of α-amylase and protein content in the separated 25 fractions by ion exchange chromatography (Fig. 1) indicates the presence of small amounts of α-amylase, separated only in fractions 17-20, with peak resulted in fraction 18, presenting an activity of 133.12U/ml and a protein content of 0.108mg/ml. The data presented are calculated for 1.5 dilution that was used for ion-exchange chromatography solution.

Active fractions were collected, pooled and applied to SDS-PAGE electrophoresis to test their purity. At the same time from amylolytic preparation Amilonigrin AS proteins were isolated and applied to electrophoresis to compare the initial purity of preparation and after processed to all stages of purification (Fig. 2).

As presented in Fig. 2A, preparation Amilonigrin AS shows a totality of polypeptide bands with different molecular weights. After ion exchange chromatography two polypeptide bands with apparent molecular masses of 66kDa and 40.5kDa were identified (Fig. 2B), indicating the purification of exocellular α-amylase from *A. niger* 33-19 CNMN FD 02A mutant form. In the polypeptide spectrum of proteins, extracted from the amylolytic preparation, the bands of 66kDa and 40.5kDa does not appear, demonstrating once again the extremely heterogeneous composition of preparation and the present of a very low amount of α-amylase enzyme.

Thus, by gel filtration and ion exchange chromatography, amylolytic preparation Amilonigrin AS, obtained at micromycete *A. niger* 33-19 CNMN FD 02A cultivation, was purified by inorganic and organic residues which were present after sedimentation of cultural liquid with alcohol ethylic. This contributes to increase of α-amylase purity by 8.9 times compared to the initial purity.

Table 1. Amylolytic properties of preparation Amilonigrin AS.

Protein (%)	α-Amylase activity (U/g)	Specific activity (U/mg)
23.5	32554.5	138.3

Table 2. Purification scheme of α-amylase from amylolytic preparation Amilonigrin AS.

Purification Steps	Volume (ml)	α-Amylase activity (U/ml)	Total α-Amylase activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Amylolytic preparation solution (25mg/ml)	3	813.86	2441.58	5.875	17.625	138.30	100	1
Extract	3	422.88	1268.63	1.908	5.724	221.63	52	1.6
PD-10 column gel filtration	3	390.12	1170.36	1.173	3.520	332.45	48	2.4
HiTrap™ Q column ion exchange chromatography	3	199.68	599.05	0.162	0.486	1232.61	25	8.9

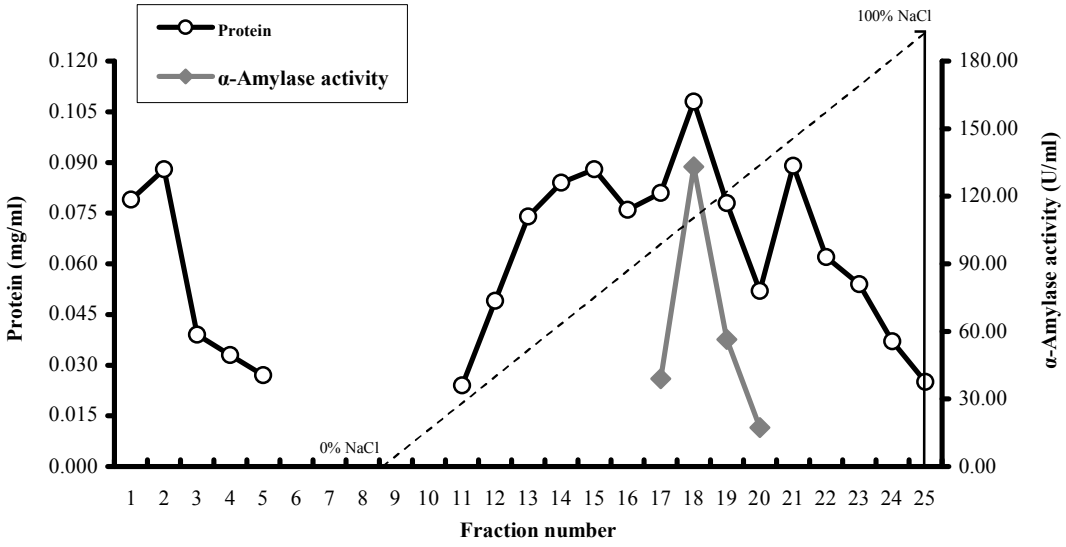

Figure 1. Purification of α-amylase from amylolytic preparation Amilonigrin AS by ion exchange chromatography. Sample: 2ml of α-amylase preparation after gel filtration through PD-10 column. Column: HiTrap™ Q (5ml). Flow rate: 1ml/min. Buffer A: 20mM Tris-HCl, pH 7.0. Buffer B: A+0.5M NaCl. Gradient: 0% B for 27ml, 0%-100% B in 75ml.

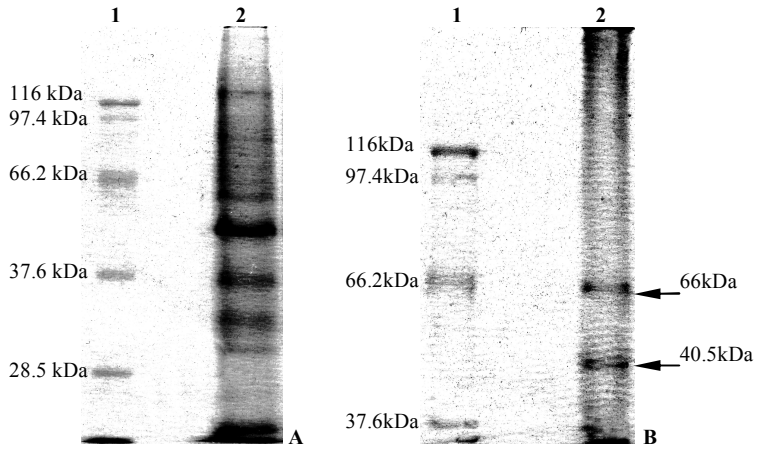

Figure 2. SDS-PAGE electrophoresis of *A. niger* 33-19 CNMN FD 02A α-amylase. A. Lane 1: Standard proteins, Lane 2: Ethylic alcohol precipitation; B. Lane 1: Standard proteins, Lane 2: Ion exchange chromatography sample.

DISCUSSIONS

Identification of new hiperproducers of α-amylase, their directed cultivation, enzyme isolation and purification presents increasing interest because of their expansion in various branches of industry. Scientific literature describes methods for obtaining α-amylase in a crystalline form from a variety of animal sources, plant and microbial. A comparative study of their chemical composition indicates their similarity. These enzymes catalyze the same chemical reaction regardless of the source from which they are extracted [36]. The methods of α-amylase isolation, purification and study are also identical.

The purification of thermostable α-amylase from the newly isolated fungi *Acremonium sporosulcatum* was performed involving ammonium sulfate precipitation, Sephadex G-100 gel filtration and DEAE cellulose-50 ion exchange chromatography. The enzyme was shown to 58kDa by SDS-PAGE and was purified 2.1 fold [35]. α-Amylase from *Trichoderma harzianum* grown on mandarin peel was purified using DEAE-Sepharose and Sephacryl S-200 columns. The purified enzyme had molecular weight of 70kDa at SDS-PAGE [15]. Also, α-amylase from *Aspergillus flavus* strains was purified to homogeneity using NH_4HS0_4 precipitation and Sephadex G-200 gel filtration which conduct to increase of enzyme concentration and specific activity. SDS-PAGE showed a single band equal to molecular weight of about 56kDa [6, 28].

The traditional purification of α-amylase produced by various strains of *Aspergillus ssp*. is identical with related methods [7, 23, 27, 37]. The related by Asad et al. [3] a single step purification by $(NH_4)_2S0_4$ precipitation and dialysis using EDTA of α-amylase from *Aspergillus niger* grown on waste bread medium resulted in almost 7 times increase in enzyme specific activity. α-Amylase from *A. niger* JGI 24, purified by ammonium sulphate fractionation, resulted in 1.49 fold increase in enzyme activity compared to crude extract. Dialyzed and subjected to SDS-PAGE precipitated α-amylase showed a molecular weight of ~43kDa [35].

Also, these methods have been applied at isolation and purification of α-amylase obtained from *A. niger* 33-19 CNMN FD 02A mutant form. Enzyme precipitation from cultural liquid with ethanol, gel filtration and ion exchange chromatography provided obtaining of a high purified α-amylase, that is demonstrated by SDS-polyacrylamide gel electrophoresis. Emphasis of two polypeptide bands after ion exchange chromatography with 66 and 40.5kDa allows to assume existing of two α-amylase izoforms. Same results are obtained at investigation of α-amylase from other biological objects. A new fungal *Aspergillus oryzae* strain isolated from old sweet soy sauce produce two extracellular α-amylases: AmyA and AmyB. The enzymes were purified to homogeneity through fractional acetone precipitation, size exclusion and ion exchange chromatography and the estimated molecular masses by SDS-PAGE were 50 and 42kDa. The monitoring of α-amylase synthesis indicated that AmyB could be formed from the proteolysis of AmyA [26].

Three forms of α-amylases, Amyl I, Amyl II and Amyl III, were purified by several column chromatographys from *Aspergillus awamori,* which was isolated in Indonesia. They were identified with molecular weights of 49, 63 and 97kDa by SDS-PAGE and manifest different catalytic function on starch [2].

By α-amylases purification from microbial strain *Bacillus subtilis* X-23 a complete (Ba-L) and truncated (Ba-S) forms of enzyme was identified. The deduced molecular masses of two purified enzymes were 67 and 47kDa. The investigations show that two izoforms were produced from the same α-amylase gene and that Ba-S arose from Ba-L by the processing of its carboxyl-terminal region during the *B. subtilis* cultivation [21].

Thus, in this research for the first time it was demonstrated the efficient purification of α-amylase from mutant strain *Aspergillus niger* 33-19 CNMN FD 02A that leads to increase of specific enzyme activity by 8.9 times compared to the initial purity of amylolytic preparation, with a yield of 25%. At the same time the synthesis of two type of α-amylase was shown, which according to experimental results of

some researches most probably is the result of cultivation procedure.

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