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Production, Purification and Characterization of Extracellular α-Amylase from Mutant Strain *Aspergillus Oryzae* MSPP

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Abstract

Aspergillus oryzae is well known species and it has an important role in the fermentation of oriental food products and in industrial application as a producer of hydrolytic enzymes. An extracellular α-amylase enzyme from mutant strain *A. oryzae* MSPP was purified to electrophoretic homogeneity by a simple procedure including ammonium sulfate precipitation, anion-exchange chromatography and Sephadex gel filtration. All purification steps were checked by PAGE. The molecular mass of the purified α-amylase was estimated to be 52 kDa. The enzyme had a pH optimum at 4.7 and was stable in the range of pH 4.7 to 10. The optimal temperature for the highest α-amylase activity was measured at 50˚C. Half-life of the enzyme was 30 min at 50˚C. Stability of the purified enzyme mainly depended on the presence or absence of soluble starch in the reaction mixture. Michaelis constant (Km) for soluble starch as a substrate was estimated to be 5 mg/ml.

Keywords: α-amylase, *Aspergillus oryzae*, gel filtration, ion exchange chromatography

Резюме

Aspergillus oryzae е добре известен вид и има важна роля в получаването на азиатски ферментационни хранителни продукти и намира приложение в индустрията като продуцент на хидролитични ензими. Извънклетъчният ензим от мутантен щам *A. oryzae* MSPP е пречистен до електрофоретична хомогенност чрез опростена процедура, включваща преципитация с амониев сулфат, анийона обменна хроматография и Sephadex гел филтрация. Всички пречистващи стъпки са проверявани с полиакриламид гел електрофореза. Молекулната маса на пречистеният ензим е 52 kДа, pH оптимумът е 4.7, а pH стабилността е в обхват от 4.7 до 10. Оптималната температура, при която α-амилазата проявява най-висока активност е 50˚C. Полуживотът на ензима е 30 минути при 50˚C. Стабилността на пречистения ензим главно зависи от присъствието или отсъствието на разтворимо нишесте в реакционната смес. Изчислена е и константата на Михаелис (Km) за разтворимо нишесте като субстрат в концентрация 5 мг/мл.

Introduction

Amylases are among the most important and widely used enzymes which spectrum of application have widened in many sectors such as clinical, medicinal and analytical chemistry (Kumari *et al*., 2012). Besides their used in starch saccharification they also find application in food, baking, brewing, detergent, textile, paper and distilling industry (Pandey *et al*., 2000; Singh *et al*., 2014).

α-Amylases (1,4-α-D-glucan-glucohydrolase; E.C. 3.2.1.1.) are endo-amylases catalyzing the hydrolysis of internal α-1,4-D-glucosidic linkages in polysaccharides (as starch, glycogen, dextrins, etc.). They can be derived from several sources, but the enzymes produced by microorganisms generally meet industrial demands (Fogarty, 1983; El-Fallal *et al*., 2012; Elmansy *et al*., 2018). Studies in enzyme production are pointed to optimize the fermentation processes, to obtain high active producers by mutagenesis, genetic engineering and to receive enzymes with specific properties as cold resistance, thermostability, etc. (Doyle *et al*., 1989; Shaw *et al*., 1995; Kirimura *et al*., 1997; Petrova *et al*., 2000).

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Lots of industrial enzymes are derived from filamentous fungi belonging to the genus *Aspergillus*. The fungi and their products are used in many fields, especially in food and food processing industry for many decades, which resulted in a so-called GRAS status (generally regarded as safe) for some of the strains such as *Aspergillus oryzae*.

In this study α-amylase enzyme from a highly active mutant strain *A. oryzae* MSPP was purified and some properties were determined.

Materials and Methods

Strains, media and culture conditions:

The strain used in this study is *A. oryzae* MSPP obtained by chemical mutagenesis of parental strain *A. oryzae* PP from Microbial collection of the Department of Biotechnology, Faculty of Biology, Sofia University. The parental strain was used in patent industrial technology BG 61573 B1 for production of fungal α-amylase enzyme (Peev *et al*., 1998).

A. oryzae MSPP, a hyper-producer of α-amylase was maintained on Sabouraud agar. The strain was incubated at 28°C for 7 days and stored at 4°C. Inoculum for the fermentation was obtained on medium with the following composition (g/L): Czapek mineral salt medium with glucose – 20, insoluble starch – 10, soybean flour- 30 and maize extract – 10 ml. Initial pH was 6.3. The composition of fermentation medium for production of α-amylase enzyme was optimized by mathematical modeling (Box-Wilson method – data not shown) and contained (g/L): insoluble starch – 60, soybean flour- 30, wheat bran – 10, $NaNO_3$, - 3, KCl – 0.5, $MgSO_4$ $x5H_2O$ – 0.5, K_2HPO_4 – 1, $FeSO_4$ – 0.01 and pH of the medium was 6.3.

The cultivation process was done in a 5 L bioreactor with 3 L medium (work volume) at 28°C for 120 h. The bioreactor was inoculated with 10% (v/v) 24 hours inoculum. The cultivation was carried out at the following conditions: 1.5 L/L/min aeration for 72 hours and until 120 hour at 1 L/L/min, stirring at 600 rpm at 28°C. Mycelial mass was removed by filtration and the resulting cell free filtrate was used as starting material for enzyme purification.

Assay methods

Enzyme activity assay

α-Amylase activity was determined by measuring a decrease in the amount of soluble starch (1%) used as a substrate in the reaction mixture by starch – iodine method (Ruchlyadeva and Goryacheva, 1966). That assay method has a correlation with SKB method. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 mg starch per minute under assay optimum conditions. Protein assay was determined by method of Lowry (Lowry *et al*., 1951).

Electrophoretic methods:

PAGE and SDS-PAGE were performed on 12% polyacrylamide gel according to Laemmli (1950) to estimate the molecular mass of the purified amylase using standard protein kit from Farmacia.

Purification of α-amylase

Fractional precipitation with ammonium sulfate: Culture filtrate from 120 h with highest α-amylase activity was saturated with solid ammonium sulphate at two steps to take the concentration from 40-80%. Two hours after adding first dose of ammonium sulphate at 4°C under constant stirring protein mixture was clarified by centrifugation at 7000 x g for 20 min at 10°C. The supernatant was further saturated to 80% with ammonium sulphate and the same procedure was repeated. The precipitate containing α-amylase was dissolved with 10 mM potassium phosphate buffer, pH 6.6 and subjected to ultrafiltration on Amicon (PM 10 membrane) until residual amounts of ammonium sulphate were washed and used for chromatographic purification. Amylolytic activity and protein content were determined in the saturated fractions.

Ion-exchange chromatography: Anion-exchange chromatography on Fractogel TSK DEAE-650 (Merck) column (26x60mm) was used for further purification. The ultrafiltrate from step 1 was put on the column, equilibrated with 10 mM potassium phosphate buffer, pH 6.6 and was washed with the same buffer until unbound material eluted out. Then three step gradient elution with phosphate buffer pH 6.6 (100 mM, 500 mM and 500 mM plus 1 M NaCl) was performed at a flow rate of 50 ml/h. Fractions of 10 ml were collected from the start of the gradient. All received fractions were checked for α-amylase according to assay and active fractions were collected and again concentrated by ultrafiltrated apparatus (Amicon, PM 10 membrane).

A sample of the concentrated enzyme was applied to a Sephadex G-100 (fine) column (16x750mm) which had previously equilibrated with 0.05 M phosphate buffer (pH 6.6) containing 0.05 M NaCl. The flow rate was 12 mL/h and the volume of each fraction was adjusted to 2 mL. All

active fractions were collected and concentrated by ultrafiltration.

Re-chromatography was carried out at the same conditions. The resulting purified enzyme was stored at 4°C.

Effects of temperature and pH on enzyme activity

To investigate the effect of temperature on enzyme activity, an enzyme analysis was performed at 10, 20, 30, 35, 40, 45, 50, 55 60, 70 and 80°C. The effect of pH was studied at pH 3 - 6 with acetate buffer, at pH 6 - 8 with potassium phosphate buffer and at pH 8 - 10 with glycine-sodium hydroxide. Each of the buffers had final concentration 100mM.

Effects of temperature and pH on stability of the enzyme

To measure the effect of temperature, the enzyme solution was incubated at 30 - 80°C for different time intervals: 10, 20, 30, 60, 90, 120 min and then the remaining activity was determined according the α-amylase assay conditions. The effect of different pH buffers (described above) on enzyme stability was investigated for two hours. After incubation with each buffer the relative activity was assayed.

Effect of substrate concentration on enzyme activity

Soluble starch in different concentrations from 0.4 to 1.5% (in 0.1 step) was used to analysis the effect of substrate concentration on the enzyme activity.

Effect of metal ions.

Metal ions as K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Al^{3+} were added to the substrate solution to a final concentration of 10 mM. The enzyme reaction was performed at 30°C for 10 min and relative activity was assayed.

Results

A. oryzae MSPP produced high levels of an extracellular α-amylase (Fig. 1). Maximum production of enzyme in bioreactor was observed at 120 h – 536 U/mL. After the fifth day of fermentation α-amylase activity was decreased. Then the culture broth was filtrated to remove cells and debris and the supernatant was collected. Only 150 mL of the filtrate was used for further purification of the enzyme.

The results of the supernatant partial precipitation with ammonium sulfate were summarized in Table 1.

The amylolytic activity in the fraction 0-40% was low and insignificant. A major part of amylase enzyme was determined in the supernatant. Second precipitation to 80% showed increased both in protein concentration and amylolytic activity.

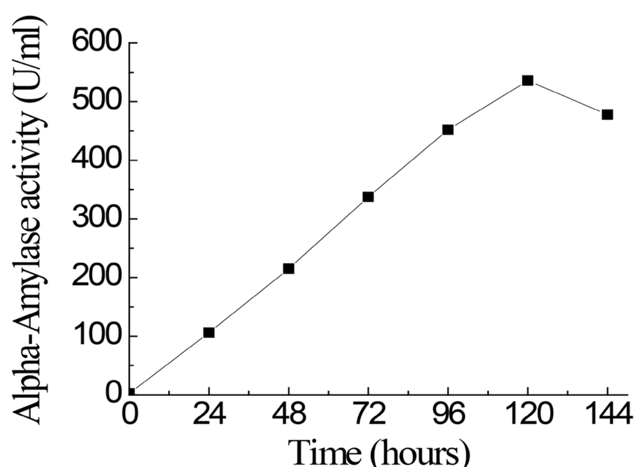

Fig. 1. Dynamic of α-amylase activity during cultivation of *A. oryzae* MSPP in bioreactor at 28°C and aeration from 0-72 h 1.5 L/L/min and 1 L/L/min from 72 h onward.

Table 1. Partial purification of the starch-hydrolyzing enzyme from culture of *Aspergillus oryzae* MSPP by ammonium sulphate precipitation.

Percent of saturation (fractions)	Protein (mg/ml)	Amylolytic activity (U/ml)
0-40	1.9	10.2
40-80	12	900

A sample from this fraction was used for anion-exchange chromatography on DEAE Fractogel column. To remove ammonium salts, the probe was ultrafiltrated and concentrated on Amicon with 10 mM potassium phosphate buffer and then was applied to the column. α-Amylase was eluted as two peaks with 0.1 M and 0.5 M phosphate buffer, pH 6.6. The second peak had lower enzyme activity – 10% of the total amylase activity (Fig. 2). Similar results have been detected by Chung during the purification of α-amylase by *Thermococcus profundus* DT5432 (Chung *et al.*, 1995). According their experimental data the authors considered that these α-amylases were isoenzymes.

DEAE-650 column equilibrated with 10 mM potassium phosphate buffer (pH 6.6) - flow rate of 50 mL/h, fractions volume – 8 mL. The protein fractions were eluted with the same buffer by stepwise gradient (0.1 M, 0.5 M phosphate buffer and 0.5 M phosphate buffer plus 1M NaCl). The fractions between 10 and 18, containing α-amylase activity, eluted with 100 mM buffer, were combined, concentrated and applied to Sephadex G-100 column. It was observed two main fractions separated at the conditions of gel filtration – one, containing

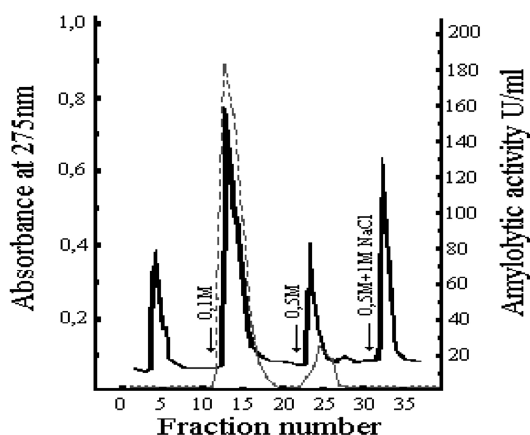

Fig. 2. Elution profiles of α-amylase activity (---) and an absorbance (—) of a Fractogel TSK

α-amylase activity (fractions 35-55) and another with larger molecular mass, a low protein content and a presence of carbohydrates (determined by Molisch test- data not shown).

Re-chromatography was carried out at the same conditions (Fig. 3B).

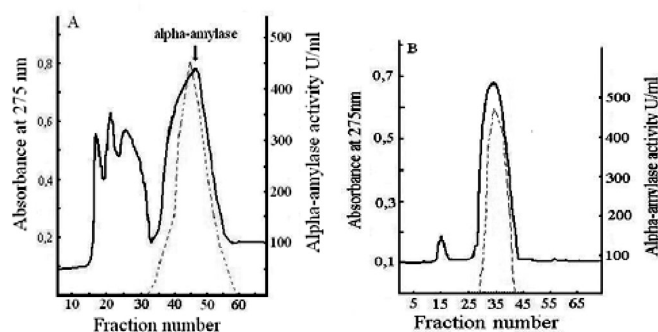

Fig. 3. Gel filtration chromatography of the concentrated active fractions after DEAE –Fractogel on Sephadex G 100; A: — absorbance, --- amylase eluted profile; B: re-chromatography: flow rate of 12 mL/h and the fractions volume – 2 mL.

negligible quantities were observed on the polyacrilamide gel (Fig. 4B).

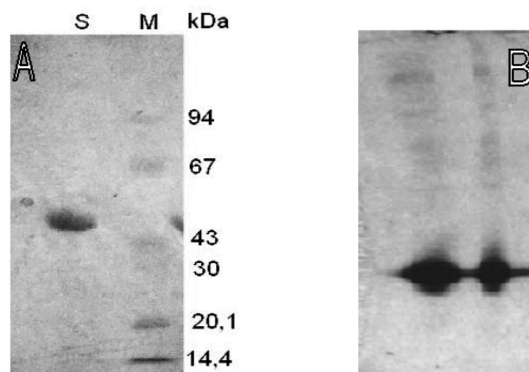

Fig. 4. Polyacrylamide gel electrophoresis: A: SDS-PAGE of the purified α-amylase: M – test kit (14.4 kDa – α-lactalbumin, 20.1 kDa – trypsin inhibitor, 30 kDa – carboxyanhidrase, 43 kDa – ovalbumin, 67 kDa - albumin, 94 kDa – phosphorylase); S – purified enzyme; B: Native PAGE of the culture filtrate.

These results demonstrated that *A. oryzae* MSPP synthesized high levels of the extracellular α-amylase, which is the main protein in the culture filtrate. This explains the low fold of purification which is enough to obtain electrophoretic homogeneity of the enzyme. The high yield allowed easily and widely application of the α-amylase in industry.

Effect of temperature and pH on α-amylase activity

Maximum activity was registrated at 50°C (Fig. 5A). That temperature was an optimum for growing of the mesophylic strain *A. oryzae* MSPP. A temperature above 60°C a rapid decrease of activity was demonstrated.

The optimum pH of α-amylase was found to be at 4.7 (Fig. 5B). The enzyme activity was higher

Table 2. Purification steps of α-amylase from mutant strain *A. oryzae* MSPP

Steps	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold of purification	Yield %
Crude enzyme	150	60674	2160	28.3	1.0	100
$(NH_4)_2SO_4$ precipitation	85	54740	1411	38.8	1.4	90.2
DEAE Fractogel	6	36252	751.2	48.3	1.7	78
Sephadex G-100	2.7	35961	625.6	57.5	2	71

The results of purification procedures were summarized in Table 2. The final preparation had 71% yield and 2.0 fold of purification.

The purified enzyme had a single band on SDS-PAGE with molecular mass approximately 52 kDa (Fig. 4A). Native PAGE was performed with properly diluted culture broth. Several proteins in

than 80% in a range of 3.7 to 5.7 and was stable for 2 hours at 30°C. The incubation of the enzyme at higher pH values (over 7.8) leaded to quick lost of relative amylase activity – 10% at pH 8.6.

Effect of temperature and pH on α-amylase stability

Thermal inactivation of the purified α-amyl-

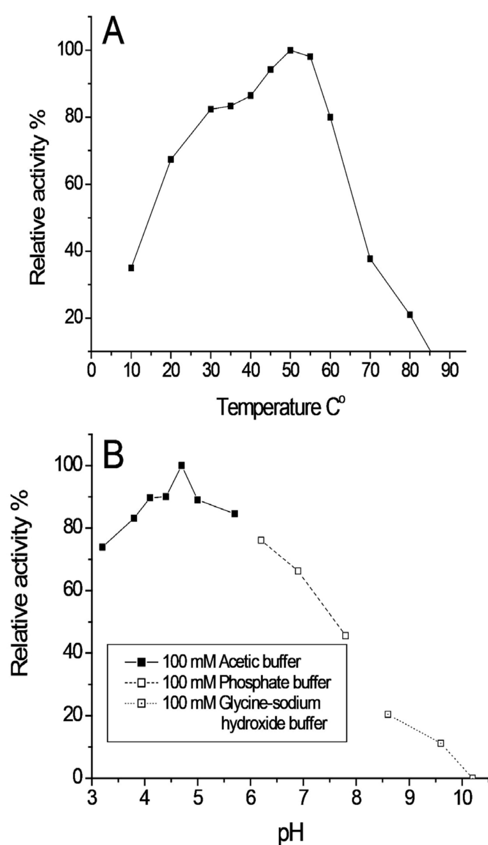

Fig. 5. Effects of temperature (A) and pH (B) on α-amylase activity.

ase was investigated by incubating the enzyme in the absence of substrate at various temperatures (Fig. 6) in 100 mM phosphate buffer pH 6.6. The enzyme activity was preserved 100% for two hours at temperature 30°C.

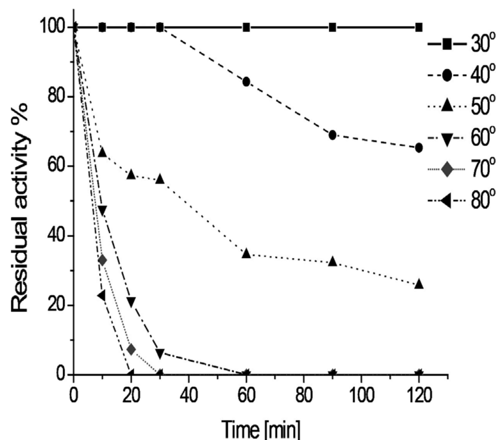

Fig. 6. Influence of temperature on α-amylase stability

The α-amylase activity was 100% for 30 min at 40°C, but at the end of the temperature treatment enzyme showed 30% decrease of the activity. Half-life of the enzyme at 50°C was 30 min. Temperatures higher than 50°C caused a quick decline of the amylase activity.

The effect of pH was also investigated. The enzyme was more stable at pH 4.7-10.1 – residual activity was more than 50% for two hours and lower at pH 3.2 (residual activity 32 %) (Table 3).

Table 3. Influence of pH on α-amylase stability

pH	Residual activity %
3.2	32.36
4.1	38.49
4.7	68.67
5.6	66.12
7.8	67.84
8.6	64.21
10.1	60.14

Effect of substrate concentration on enzyme activity.

The substrate concentration in the range from 0.4 % to 0.6 % caused exponential increase on amylase activity (Fig. 7).

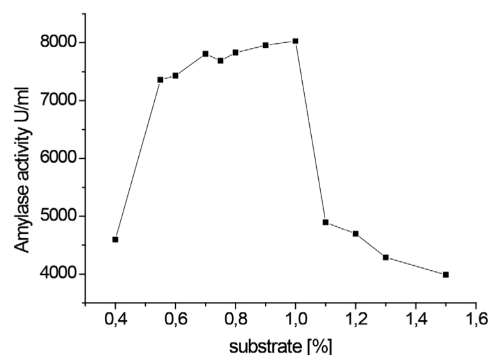

Fig. 7. Influence of the substrate concentration on the purified enzyme

Further increasing of starch concentration showed a slight grow of the amylase activity and after 1% substrate concentration the activity sharply decreased. The linear rate of the absorbance change was measured to give the initial velocity (v). The K_m was calculated by Lineweaver-Burk plotting, with at least six initial substrate concentrations. The Km value for soluble starch as a substrate was estimated to be 5 mg/mL (date not shown).

Effect of metal ions on α-amylase activity

Cations, each at a level of 10 mM, had various effects on the purified amylase (Table 4). The metal ions, except Ca^{2+} decreased amylase activity during the incubation at 30°C for 10min. Mg^{2+}, Mn^{2+} and Zn^{2+} caused 80% restoration of amylase activity. Al^{3+} ions had 57.5% inhibition effect on catalytic activity of α-amylase.

Table 4. Effect of metal ions on α-amylase activity

Metal ions (10 mM)	Relative activity %
Control	100
Ca^{2+}	104.3
Mg^{2+}	85.6
K^{+}	71.4
Cd^{2+}	71.5
Zn^{2+}	81.7
Ni^{2+}	69
Al^{3+}	42.5
Cu^{2+}	61.7
Mn^{2+}	81.3

Discussion

Extracellular α-amylase from mutant strains *A. oryzae* MSPP was purified to electrophoretic homogeneity and partially characterized. In comparison with other fungal amylases, the properties of *A. oryzae* MSPP α-amylase were in agreement with the reported data. The purified α-amylase had molecular weight of 52 kDa, optimum temperature at 50°C, optimum pH 4.7, pH stability was observed in the range of pH 4 to pH 8 for 2 hours (over 80% activity). The half-life of the enzyme at 50°C was 40 min (mesophilic enzyme) and Km estimated for soluble starch was 5 mg/ml. The effect of metal ions showed no requirements of cations.

At this stage of investigation, α-amylase appeared to have common characteristic of α-amylases (Kariya *et al*., 2003, Nagamine *et al.*, 2003). The α-amylase from *A. oryzae* MSPP was synthesized in high quantities in the culture broth (Fig. 4B – native PAGE). A pure α-amylase enzyme with electrophoretic homogeneity was obtained only after two-fold of purification with high yield 71%. Further studies and with the help of protein engineering and immobilization techniques can improve the enzyme activity and stability (Kumari *et al*., 2012, Tonova, 2018, Al-Najada *et al*., 2019) in higher range.

The purified enzyme is a potential candidate for application in the industry – food, pharmaceutical and clinical sectors where high purity amylases are required.

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