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PURIFICATION AND CHARACTERAIZATION OF -α-AMYLASE ISOLATED FROM *ASPERGILLUS FALVUS VAR. COLUMNARIS*

E. M. El-Safey* and M. S. Ammar**

***Department of Botany and Microbiology, Faculty of Science, Al-Azhar University. Assiut branch,**
****Fermentation Biotechnology and Applied Microbiology (Ferm-BAM) Center, Al-Azhar University. Madient Nasr Cairo, Egypt.**

ABSTRACT :

The amylase family of enzymes is of great significance due to its wide area of potential application.
α-amylase from *Aspergillus flavus* var. *columinaris* isolate was produced under optimum conditions and subjected for purification and characterization. The enzyme was purified by ammonium sulfate precipitation and sephadex G200 filtration. A trial for the purification of α-amylase resulted in an enzyme with specific activity of 6471.6 (units/mg prot/ml) with purification folds 9.97 times. The α-amylase activity increased as the increase of enzyme concentration. The optimum substrate concentration (starch) was 0.2 % (w/v) while the optimum incubation temperature was 35ºC. The purified α-amylase enzyme had a maximum activity at pH 6.2, after 30 h of incubation.

INTRODUCTION:

α-amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants (Octávio *et al*, 2000). Amylases (α-amylase) are among the most important enzymes in present-day biotechnology. The enzyme has found numerous applications in commercial processes, including thinning and liquefaction of starch in alcohol, brewing and sugar industries (Riche`le, *et al*, 1998).

The amylase family of enzymes is of great significance due to its wide area of potential application such as application in pharmaceutical and a clinical sector requires high purity amylases. Thus, it is significant to develop economic processes for their purification to obtain pure enzymes with maximum specific activity (Pandey *et al*, 2000).

In fungi, detailed studies on α-amylase purification have largely been limited to a few species of fungi (AbouZeid, 1997 and Khoo, *et al*, 1994). Nevertheless, Amirul *et al*, (1996) produced alpha-glucosidase, alpha-amylase and two forms of glucoamylase from. *Aspergillus niger* grown on a liquid medium containing raw tapioca starch as the carbon source.

On the other hand, as bacterial α-amylase have generally been produced from the strains belonging to genus *Bacillus*, several attempts have been made at their purification and characterization, from both mesophilic and thermophilic strains (Pandey *et al.*, 2000). An

extra-cellular α-amylase produced by *Bacillus thermooleovorans* NP54, was partially purified using acetone (80%[v/v] saturation), and 43.7% recovery of enzyme with 6.2-fold purification was recorded (Malhotra *et al*, 2002). Amirul *et al*, (1996) purified α-amylase, alpha-glucosidase, and glucoamylase to homogeneity by ammonium sulfate precipitation, ion-exchange and two cycles of gel filtration chromatography. In addition to that, an extracellular α-amylase produced by *Lactobacillus fermentum* was purified by glycogen precipitation and ion exchange chromatography. The purification was approximately 28-fold with 27% yield (Talamond *et al.*, 2002). This article describes the purification and characterization of the α-amylase enzyme which produced under solid state fermentation (SSF) conditions by *Aspergillus flavus* var. *columinaris*.

MATERIALS AND METHODS:

Enzyme purification:

α-amylase purification steps were previously mentioned by El-Safey, (1994). This included the following steps:

Step 1. Enzyme production and preparation of cell free filtrate :

***Aspergillus flavus* var. *columnaris* was grown under the entire of optimized parameters using solid state fermentation (SSF) conditions as previously mentioned by El-Safey and Ammar (2002). The filtrate broth (crude α-amylase) was collected and centrifuged at 4000 rpm for 15 min at 4ºC and then filtered through a sterile sintered glass G-4 in order to obtain a cell free filtrate (CFF). After performing a test for sterility, 200 mls of the CFF containing α-amylase were collected and their amylolytic activity and protein content were determined.**

Step 2. Ammonium sulfate fractionation:

200 ml of the crude α-amylase enzyme were first brought to 20% saturation with solid ammonium sulfate (enzyme grade) as mentioned by Dixon and webb (1964). The precipitated proteins were regimented by centrifugation for 15 min at 500 rpm. The resulted pellet was dissolved in 5 ml of 0.2 M phosphate buffer at pH 6.2. The left supernatant was applied again with ammonium sulfate to achieve 40, 60, 80, and 100% saturation. Both enzyme activity and protein content were determined for each separate fraction.

Step 3. Dialysis against distilled water and buffer:

The obtained ammonium sulfate precipitate (in solution) was introduced into dialysis bag against distilled water for 3 h, followed by dialysis against phosphate buffer at pH 6.2. The obtained α-amylase enzyme preparation was concentrated against crystals of sucrose and kept in the refrigerator at 5ºC for further purification.

Step 4. Application on column chromatographic technique:

Preparation of the gel column and the fractionation procedures was determined as previously mentioned by Ammar (1975). For this purpose, a Pharmacia column (2.6×7.0 cm) has been used. Sephadex G-200 (Pharmacia, Upsulla, Sweden) "practical size 200 µ" was also used. 0.2 M phosphate buffer of pH 6.2 was used and the slurry was allowed to swell for 3 days at room temperature (≈22±1ºC). Sodium azide (0.02%) was added to prevent any microbial growth. Applying a mixture of blue dextran

2000 and bromophenol blue determined the void volume.

One ml of the enzyme preparation sample was applied carefully to the top of the gel and allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. Fifty fractions (5 ml each) were collected.

Amylotic activity and protein content were carried out for each individual fraction. Sharp peaks of fractions obtained after applying Sephadex G 200 column were collected and investigated for the properties of the partially purified α-amylase enzyme.

Enzyme activity:

The α-amylase enzyme activity were made as previously mentioned by El-Safey and Ammar, (2002).

Protein determination:

The protein content of α-amylase enzyme was determined by the method of Biuret as mentioned in Chykin, (1966).

Determination of the specific activity of α-amylase enzyme

The specific activity of the α-amylase enzyme protein was expressed in terms of units/mg protein/ml according to the following equation:

Specific activity =enzyme activity/ protein content (mg/ml)

Characterization of α-amylase:

Effect of different enzyme concentrations:

This experiment was performed to investigate the effect of different concentrations of α-amylase enzyme on their activities. The purified α-amylase enzyme dilutions were, 0.0075, 0.0150, 0.0300, 0.0600 and 0.120 (mg protein/ml).

Effect of different substrate concentrations:

This experiment was carried out to study the effect of different substrates (starch) concentrations on purified α-amylase. Different soluble concentrations (w/v) were used, viz. 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 respectively.

Effect of incubation temperature:

This experiment was performed by incubating α-amylase at different temperatures viz.: 10, 25, 30, 35, 40, 50 and 60 ºC respectively.

Effect of different pH values:

This experiment was planned to investigate the effect of different pH values on purified α-amylase activities. The purified α-amylase was incubated at different pH values viz.: 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 using phosphate buffer (0.2 M), using pH meter a model Jenway 3020.

Effect of incubation period:

The purified α-amylase was incubated for different incubation time's viz.: 1, 6, 12, 18, 24, 30 and 36 h at 35 ºC, respectively.

RESULTS AND DISCUSSION:

Purification of α-amylase enzyme:

α-amylase enzyme was produced under solid-state fermentation (SSF) conditions from Nile hyacinth by *Aspergillus flavus* var. *columnaris* (El-Safey and Ammar, 2002). α-amylase was purified by ammonium sulfate precipitation and Sephadex G200 filtration. As shown in Table (1) ammonium sulfate precipitation resulted in specific activity of 3670.51 (units/mg prot/ml) and purification

folds 5.66 times (Fig. 1, A). The α-amylase was subjected to Dialysis against sucrose resulted in specific activity 4023.63 (units/mg prot/ml) and purification folds 6.20 times (Table 1).

A trial for the purification of α-amylase enzyme resulted in specific activity of 6471.6 (units/mg prot/ml) with purification folds 9.97 times (Table 1 and Fig. 1, B). Several investigators studied the purification of amylase enzymes. Purified α-amylase enzyme produced from *B. stearothermophilus* S-92 under both mesal (30ºC) and thermal (55ºC) incubation conditions after 24 h by ammonium sulfate precipitation; column chromatography and polyacrylamid gel electrophoresis columns was reported by Abd El-Rahman (1990).

Table (1): Purification profile of α-amylase produced by *Aspergillus flavus* var. *columnaris*.

Purification Step	Enzyme volume (ml^{-1})	Enzyme activity (units/ml^{-1})	Protein content (mg^{-1}/ml^{-1})	Total activity (units/ml^{-1})	Total protein (mg^{-1}/ml^{-1})	Specific activity (units/mg^{-1}, $prot/ml^{-1}$)	Purification folds
Cell free filtrate	200	266.07	0.41	53214	82	648.95	1
Ammonium SO_4 fractionation (60 % saturation)	200	660.69	0.18	132138	36	3670.51	5.66
Dialysis against sucrose crystals	15	2213.09	0.55	33196.35	8.25	4023.63	6.20
Sephadex G-200 chromatography	5	3235.8	0.5	16179	2.5	6471.6	9.97

In addition Khoo, *et al*, (1994) purified α-amylase enzyme produced by *Aspergillus flavus* using ammonium sulfate precipitation and ion-exchange chromatography and found that the enzyme is homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recently Abou-Zeid, (1997) purified extra-cellular α-amylase enzyme from *Aspergillus flavus* by a starch adsorption method. Moreover, Chakraborty *et al.*, (2000) investigated thermostable α-amylase enzyme and purified it to homogeneity by ammonium sulfate fractionation and TECC on DEAE-cellulose column.

Fig. (1): The relation of the applied ammonium sulfate concentration to the corresponding precipitated activity of α-amylase (A), The fractionation pattern of α-amylase produced by Aspergillus flavus var. columnaris on sephadex G-200 column chromatography (B).

Properties of the purified α-amylase:

Results in Fig. (2,A) indicated that as α-amylase concentration increased the α-amylase activity increased. This behavior is in accordance with the observations of West, *et al.* (1967) who stated that within fairly wide limits the speed of enzyme concentration is directly proportional to the enzyme concentration. Abd El-Rahman, (1990) and El-Safey, (1994) previously reported the same observation.

The α-amylase activity reached the maximum with an optimum substrate (starch) concentration of 0.2% with enzyme activity 50.118 units/ml. Any increase or decrease of substrate concentration gave a corresponding decrease in α-amylase activity (Fig. 2, B). Kuiper, *et al*, (1978) reported that the maximum activity of α-amylase enzyme was obtained at 1.67 % of substrate (starch) concentrations. In addition to that, Abd El-Rahman, (1990) concluded that, the optimal concentration of starch for maximum α-amylase activity was between 2–3%. Moreover, El-Safey, (1994) reported that, the optimal substrate (starch) concentration in reaction mixture of the MM-α-amylase enzyme was found to be 0.1 % (w/v) corresponding to 2% (w/v) for RH-α-amylase enzyme. The effect of temperature on the activity of the purified amylase is shown in Fig. (2,C). The optimum incubation temperature for purified α-amylase enzyme was 35ºC. the purified α-amylase activity reached up to 26.915 units/ml. While the temperature below or above 35 ºC exhibited lower activities of α-amylase. Other investigators were reported that, the optimum temperature for maximum purified α-amylase activity was 30ºC (Strumeyer and Fisher, 1982). The same finding was reported by El-Safey, (1994) who indicated that, the purified MM-α-amylase displayed maximal activity at 30ºC corresponding to 50ºC for purified RH-α-amylase. On the other hand, Abd El-Rahman, (1990) and Lin *et al*, (1998), concluded that, the optimum temperature for purified α-amylase ranging from 30 to 85 ºC.

Moreover, Khoo *et al*, (1994) reported that, the optimum temperature for purified α-amylase was 55ºC. Chakraborty *et al*, (2000) found that the maximum activity of a thermostable purified α-amylase was observed at 50ºC. Moreover, Odibo and Ulbrich-Hofmann, (2001) concluded that the optimum temperature for the enzymes were 60 ºC for α-amylase and 70ºC for glucoamylase, respectively.

The enzyme activity of the α-amylase was determined at different pH values. As shown in figure (2, D) the pH for maximal activity is 6.2 with 25.11 units/ml.

α-amylases produced by thermophilic bacteria are active at pH ranging from 5.5 to 8.5 (Uchino and Katano, 1981). Moreover, El-Safey, (1994) concluded that the optimum pH values for MM-α-amylase enzyme was ranged between 5.8 and 6.4 for RH-α-amylase. In addition, Amirul *et al*, (1996) found that the optimal activities of the purified enzymes were found to have pH optimum of 4.2 and 4.5 for GA1 and GA2. Khoo *et al*, (1994) reported that the α-amylase enzyme was found to have maximum activity at pH 6.0. Abou-Zeid (1997) found that the optimal activity of the purified enzyme was achieved at pH 7.0. Moreover, Chessa *et al*, (1999) reported that the optimum pH value for maximum α-amylase activity was at found pH 7.5. Chakraborty *et al*, (2000) concluded that the purified α-amylase showed a wide range of pH tolerance and maximum activity was observed at 7.0. Moreover, Malhotra *et al*, (2002) concluded that the purified α-amylase showed a maximum activity at the optimum pH value of 8.0. In addition, Lin *et al*, (1998) recorded that, the optimal pH for alpha-amylase enzyme activity was 9.0. Nevertheless, Odibo, and

Ulbrich-Hofmann, (2001) reported that the optimum pHs for enzymes were found to be 5.0 for α-amylase and 6.0 for glucoamylase.

The results (Fig. 2, E) indicated that, as time increased the enzyme activity increased. The optimum incubation period for α-amylase activity was obtained at 30h. (25.11units/ml) beyond which the enzyme activity get affected due to the length of incubation period.

Fig (2): The properties of purified α-amylase, Enzyme concentration (A); Substrate (starch) concentration (B); Temperature(C); pH values (D) & Incubation periode (E).

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تنقية ودراسة خواص إنزيم ألفا-أميلاز من أصل ميكروبى المنتج تحت ظروف تخمير منابت صلبة من نبات ورد النيل

الصافى محمد الصافى ، مختار صالح عمار

يهدف هذا البحث إلى تنقية ودراسة خواص إنزيم ألفا–أميلاز الذى سبق إنتاجه تحت ظروف الإنتاج المثالية للمنابت الصلبة من فطرة *Aspergillus flavus* var. *columinaris* على مطحون نبات ورد النيل، وتم تنقية إنزيم ألفا–أميلاز بواسطة Ammonium sulfate precipitation and sephadex G200 filtration .

ودلت النتائج على أن specific activity للأنزيم النقى هى (units/mg prot/ml) 6471.6 مع purification folds 9.97 times . وتم دراسة العوامل المختلفة ومدى تأثيرها على نشاط إنزيم ألفا–أميلاز النقى، كما دلت النتائج على أنه كلما زاد تركيز الإنزيم زاد نشاطه. ووجد أن أنسب تركيز لنشاط الإنزيم من النشا هو 0.2 (w/v) % ، وأنسب درجة حرارة لإنتاج إنزيم ألفا–أميلاز هى ٣٥° ودرجة الأس الهيدروجينى المثلى هى ٦.٢، وأنسب فترة تحصين لنشاط أنزيم الألفا–أميلاز هى 30 h.