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Purification and kinetic properties of a novel β-amylase from Penicillium *citrinum* AS-9

[a]**Abdel-Mohsen S. Ismail***, [b]**Milada S. Abdel-Malik**, [a]**Ahmed I. Al-Diwany** and [b]**Ahmed F. Afifi**

[a]*Chemistry of Natural and Microbial Products Dept., Pharmaceutical and Drug Industries Research Institute., National Research Centre, Dokki, Giza, Egypt P.O. Box: 12622*
[b]*Dept. of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Roxy, Cairo, Egypt*

Abstract

β-amylase-rich preparation produced by the soil fungal strain *Penicillium citrinum* AS-9 was completely purified within the succeeding steps; ultrafiltration, acetone fractionation and gel filtration on Sephadex G-150 column. Acetone fractionation recovered the highest β-amylase activity and the 85% acetone fraction exhibited 3.5-fold activity that of the crude enzyme. The column affected 8.6-fold purification for β-amylase, which produced maltose as the major product of starch hydrolysis. The pure enzyme showed a Km value of 17.5 mM and Vmax of 17.5 Umg-1 protein, applying Woolf plot and exhibited its maximum velocity at pH 7.1 and 50°C. In absence of substrate, thermal treatments of the enzyme solution at pH 5.2 and 5.5 had the most adverse effects on the enzyme activity $CaCl_2$ (1mM) activated the enzyme, while each of Co^{2+}, Fe^{3+}, cysteine, cystine, I2 and p-chloromercuribenzoate (PCMB) had different inhibitory effects. Maltose as the enzyme product at final concentration of 80 mM strongly inhibited the enzyme.

Keywords: Starch, β-amylase-rich preparation, Penicillium citrinum AS-9, Chromatographic purification, kinetic properties.

1. Introduction

Microbial amylases have successfully replaced the chemical hydrolysis of starch [1]. In recent years the spectrum of amylase applications has widened in many fields, such as clinical, pharmaceutical, chemical, textile, food and brewing industries with the coming of new frontiers in biotechnology [2-3]. β-amylase (EC 3.2.1.2, α-1,4-glucanomaltohydrolase) acts in exo-fashion, from the non-reducing chain ends of amylase, amylopectin or glycogen and hydrolyses alternate glucosidic linkages producing the β-anomeric form of maltose. β-amylase is unable to pass α-1,6-glucosidic linkages in amylopectin and glycogen [4]. β-amylases are very important technologically, mainly for the production of pure maltose, which is used as brewing adjuncts, by subjecting a starch consisting predominantly of amylopectin to the action of pure β-amylase and by dialyzing the reaction mixture against water [5]. These enzymes occur widely in higher plants as barley, oats, wheat, soybean and sweet potatoes. Their existence as extracellular enzymes in microorganisms was reported [6-9]. Nevertheless, till now most of the β-amylase published articles belonged to the plant type, but of the microbial and particularly the fungal are uncomprehensive and few.

The present article was thus undertaken to investigate the purification of β-amylase-rich enzyme preparation produced by the soil fungal strain *Penicillium citrinum* AS-9, characterization and kinetic studies of the pure fungal β-amylase.

2. Experimental

2.1. Microorganism

The fungal strain *Penicillium citrinum* AS-9 was isolated from cultivated soil at Alexandria and kindly identified through the help of the staff of the Commonwealth Mycological Institute at Kew, Surrey, Ferry Lane, England. It was maintained on Waksman's medium [10].

2.2. Cultivation

Transfers were made from the subcultures to Czapek's agar slopes, which were then incubated at 30°C for 7 days. Cultivation was in 250-mL Erlenmeyer conical flasks, each containing 50 mL of the following medium (g/L): soluble starch, 30; NaH_2PO_4, 4.7; $CaCl_2$, 0.2; $MgCl_2$, 0.2;

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KCl, 1 and NaCl, 0.02, pH was adjusted before autoclaving to 5.0.

Seven days old culture was employed to prepare the enhanced fungal inoculum. 10% (v/v) enhanced inoculum pellets were used to inoculate each flask. After incubation at 30°C in a thermostatic incubator for 6 days under static conditions, the culture medium in each flask was filtered off to separate mycelium from filtrate, which applied as the enzyme source.

2.3. Media
2.3.1. Czapek-Dox, Czapek's agar medium [10]
This was used for culture maintenance and stock cultures.
2.3.2. Fungal growth enhancement medium [4]
This was employed to enhance the fungal growth and composed of (g/L): D-Glucose, 10; peptone, 5.0; yeast extract, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.05, pH was adjusted to 7.2.

2.3.3. β-amylase optimized production medium
The optimized β-amylase production medium by the fungal strain *P. citrinum* AS-9 consisted of (g/L): Sodium nitrate, 15; soluble starch, 30; NaH_2PO_4, 4.7; $CaCl_2$, 0.2; $MgCl_2$, 0.2; KCl, 1.0 and NaCl, 0.02, pH was adjusted to 5.0 before autoclaving.

2.4. Preparation of the crude β-amylase
The culture filtrates were collected and centrifuged in a refrigerated centrifuge at 2300g, to separate the transparent filtrate; the clear culture filtrate was ultrafiltered with Millipore pelican cassette system (Filter type PTGC, porosity 10,000 MW). The ultra-filtrate was lyophilized and homogenized.

Fractional precipitation of the lyophilized enzyme was achieved by salting out with ammonium sulphate or by precipitation with ethanol, acetone or tannic acid, using solution of the lyophilized enzyme preparation. Each precipitated fraction solution was dialyzed overnight against distilled water in a refrigerator, then assayed for amylase activity to choose the most suitable fraction for purification.

2.5. Enzyme purification
The enzyme fraction precipitated at 85% acetone concentration was used for further purification by column chromatography technique. The last-named fraction was dissolved in distilled water and dialyzed against 0.02M-phosphate buffer, pH 7.1. The dialyzed enzyme sample was applied to Sephadex G-150 column (60 x 1.5 cm), which was equilibrated with 0.02 M-phosphate buffer, pH 7.1. Stepwise elution was carried out in

succession with phosphate buffer (pH 7.1) of different concentrations as follows: 0.02 M (110 mL). 0.05 M (110 mL), 0.1 M (75 mL) and finally 0.1M-KCl in 0.1 M-phosphate buffer, pH 7.1 (65 mL). The 5-mL fractions were collected by fraction collector at a flow rate of 30 mL/h, using peristaltic pump. The protein content and amylase activity of each fraction were then determined.

The fractions, which showed amylase activity, were thereafter pooled and lyophilized. A check of the purity of the enzyme component showing amylase activity was undergone by disc polyacrylamide gel electrophoresis, according to Davis, [12] and Ornstein, [13] methods.

2.6. Paper chromatography
Hydrolysis products of pure amylase were detected by chromatography on Whatman No.1 paper, using descending technique with the solvent system n-butanol: acetic acid: water (2:1:1 by volumes) and detection was effected by spraying with aniline phthalate reagent [14].

2.7. Protein measurements
This was done by the micro-Kjeldahl method, according to A.O.A.C, [15]. Regarding protein contents of the enzyme fractions obtained by column chromatography and of the pure amylase were determined by ultraviolet absorption at 260 nm.

2.8. Measurement of amylase activity
This was undergone according to the method described by Plummer, [16]. The reaction mixture contained 1.5 mL enzyme solution and 1.5 mL of 0.5% (w/v) saline starch in 0.2 M-phosphate buffer, pH 7.1, and was incubated for 5 min at 55°C (optimum conditions for crude β-amylase). Similar reaction mixtures using heated inactive enzyme were prepared as control. The reducing sugars released were determined by the method described by Nelson, [17] and Somogyi, [18]. One enzyme activity unit (U) was defined as the amount of enzyme that produces 1μmole of reducing sugars as maltose/min. Saccharification (%) of soluble starch by amylase was also determined.

3. Results and Discussion

Ultra-filtration of β-amylase-rich culture filtrate from *Penicillium citrinum* AS-9 effected 1.5-fold purification of amylase (Table 1). Fractional precipitation of ultrafiltrate was carried out by different agents and yielded 26 fractions including 6 with ethanol, 5 with acetone, 7 with

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tannic acid and 8 with ammonium sulphate, of these precipitants, tannic acid and ethanol were less efficient in this respect, by which only 11.7 and 10.08% amylase recovered activities were recorded, respectively. However tannic acid recovered 47.67% of the total protein, while

ethanol recovered most of the used protein (95%). On the other hand, ammonium sulphate yielded the lowest protein and amylase activity recovery (39.5 and 4.5%, respectively).

TABLE 1: Summary of the purification steps of the crude β-amylase from *Penicillium citrinum* AS-9.

Purification step	Total protein (mg)	Recovered protein (%)	Specific activity (U/mg protein)	Total amylase activity (U/total protein)	Fold purification	Yield (%)
1. Culture filtrate	527.00	100.00	2.10	1106.70	1.00	100.00
2. Ultrafiltration	368.90	70.00	3.15	1162.04	1.50	105.00
3. 85%acetone fraction	120.00	22.77	7.35	882.00	3.50	79.70
4. SephadexG-150						
a) Fractions 8-21 (component I)	54.42	10.33	13.75	748.28	6.55	67.61
Fraction 13(the peak)	11.25	2.14	24.30	273.38	11.57	24.70
b) Fractions 32-41 (componentII)	27.26	5.17	0.04	1.09	0.02	0.10
c) Fractions 59-70 (component III)	14.94	2.84	-	-	-	-

Acetone yielded the highest recovery of both protein (97.2%) and amylase activity (90%). Of all the fractions obtained by the four precipitants used, that brought about by precipitation at 85% acetone showed the highest amylase activity (79.7 and 89% of the initial activity and the recovered activity by acetone fractionation, respectively) and also, the highest specific activity (7.35 U mg-1 protein), which reached 3.5-fold that of the culture filtrate (Table 1). Whatanabe et al., [19] underwent fractional precipitation of the fungal β-amylase from the culture of *Rhizopus javanicus* with acetone 40-66%. Also, bacterial amylase from Bacillus lentus was partially purified by acetone fractionation [20]. Accordingly, this fraction (85% acetone) was considered as a partially purified enzyme preparation and loaded on Sephadex G-150 column.

Stepwise elution of the column with phosphate buffer yielded 70 fractions (Table 1). The column affected the recovery of 96.52% of the applied protein (120 mg). The first protein component (fractions 8-21) which eluted with 0.02M-phosphate buffer (pH 7.1) was the major and comprised about 45.35% of the recovered protein. Majority of amylase activity (67.61%) was recovered by this component. This enzyme component showed 6.55-fold purification and its peak (fraction 13) exhibited 11.6-fold purification.

The second and the third components recovered 22.72 and 12.45 % of the applied protein, respectively, and had either negligible or no amylase activity (Table 1).

The major protein component was characterized by its sharp peak indicating its purity. This was substantiated by disc polyacrylamide gel electrophoresis, which indicated the homogeneity of the purified enzyme. In this regard, Kurimoto [21] purified β-amylase from β-amylase-rich preparation of vegetable matter by separation on Sephadex gel or by inactivation of the associated α-amylase. Also, Sakano et al., [22] purified β-amylase of *Pseudomonas stutzeri* to electrophoretic homogeneity by adsorption on starch granules and chromatography on Sephadex G-100. In addition, Femi-Ola, [8] and Femi-Ola et al., [9] achieved 6.4-fold purification of *B. cultilis* β-amylase by Sephadex G-150 column chromatography.

Hydrolysis time-course study by paper chromatography of soluble starch products released by the pure enzyme at different time intervals, revealed that maltose was the major product with a very little glucose and oligosaccharides quantities as shown on the related chromatograms.

Extended hydrolysis yielded constantly higher amounts of maltose and this provided an unequivocal evidence that the pure enzyme was β-amylase.

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In this respect, the pure β-amylase from *Streptomicus hygroscopicus* afforded syrup of starch hydrolysate comprising maltose (71.3%), dextrin (12.9%), oligo sugars (11.7%) and glucose (4.1%) after complete saccharification of starch [23].

The general properties of *P. citrinum* AS-9 β-amylase were investigated. Higher soluble starch concentrations than 1% (w/v) led to decrease in enzyme velocity and saccharification potency, indicating that this concentration was a limiting factor for enzyme activity (Table 2). On the other hand, the linear Woolf-plot ([starch]/v versus [starch]) indicated a Km value of 0.35%, w/v (17.5 mM) and Vmax of 17.5 U mg-1 enzyme protein. These values indicated good enzyme capabilities for technological applications. Similar Km value was reported for amylase from the yeast *Schwinniomyces alluvius* [24], while lower value was reported for the pure β-amylase from thermophilic strain *Bacillus sp.* [25]. On the other hand, El-Aassar et al., [19], reported a Km value of 0.283% and Vmax of 9.2 Umg-1 enzyme protein for *Bacillus lentus* pure α-amylase.

The increase of enzyme protein concentration from 50 to 200 μg/mL led to significant increase in both enzyme activity and saccharification potency, above which the two parameters decreased (Table 2) and this pointed out the normal relation of the enzyme concentration and its velocity [26].

The enzyme reached its maximal activity at 50°C and pH 7.1, whereby the enzyme showed 16.5 Umg-1 protein and 42.47% saccharification in the reaction lasting for 5 min, Extension of the reaction for 20 min, led to the maximum starch saccharification by the pure β-amylase, accompanied with little decrease in enzyme activity (Table 2). Similar values for optimum pH and temperature for other β-amylases were also recorded [27-28] and [8]. It is worthy to note that, in most cases, saccharification of starch by pure β-amylase was a criterion of its activity.

In absence of the substrate, the pure fungal β-amylase showed various degrees of stability in phosphate buffer (Table 3). The stability depended on the pH of the buffer, the temperature and the time of exposure. The enzyme was comparatively more stable at neutral and slight acidic (7.1, 5.9) than acidic (5.2, 5.5) pH values. It is worthy to note that the enzyme lost 90 and 85% of its original activity when left at 60°C for 5 min, only at pH 5.2 and 5.5, respectively. At pH 7.1 and 50°C, the enzyme lost about 50% of its activity when preheated for 20 min. Under the same conditions, but in the presence of the

substrate, the enzyme showed considerable activity (80% of the original), also, the enzyme lost more than 70% of its activity when preheated at higher temperature 60°C for 20 min. (Tables 2 and 3).

TABLE 2: Properties of the pure β-amylase from *P. citrinum* AS-9.

Property	β-amylase activity (U/mg protein	Starch Sacchari fication (%)
1. Substrate (soluble starch) concentration (%)[a]	0.25	7.54	79.00
	0.50	7.85	50.00
	1.00	15.00	40.66
	1.50	14.00	27.00
	2.00	13.50	20.00
2. Enzyme protein concentration[a] (ug/mL)	50.00	6.23	16.01
	100.00	14.53	33.70
	150.00	14.62	38.90
	200.00	16.50	42.40
	250.00	15.02	40.66
	500.00	15.00	40.66
3. pH of the reaction[a]	6.20	4.56	11.52
	6.60	6.85	17.60
	6.70	11.62	29.20
	7.10	16.50	42.47
	7.70	3.08	7.92
4. Temperature of the reaction[a] (°C)	40.00	4.57	11.76
	45.00	8.00	20.59
	50.00	16.50	42.47
	55.00	11.00	28.31
	60.00	5.40	13.90
	65.00	2.08	5.35
5. Extension of the reaction[b] (min)	2.00	6.58	7.00
	5.00	16.47	42.40
	7.00	14.92	56.43
	10.00	14.61	77.85
	20.00	13.73	80.00

a) Time of reaction * 5 minutes.
b) Optimum conditions in 1, 2, 3 & 4 were employed.

These results collectively demonstrate the known stabilizing effect of the substrate on the pure enzyme in the enzyme-substrate complex during the enzymatic reaction [26]. Similar findings were reported for pure β-amylase from *Rhizopus javanicus* [19] and for other microbial amylases [29] and [20].

The effect of some agents on the pure fungal β-amylase was also studied. In this case, the enzyme was preheated with the substance before mixing with the substrate (Table 4). Calcium chloride at a final concentration of 1 mM brought about 15.72% activation, indicating its stabilizing action at this concentration on the pure enzyme, however, the higher concentrations resulted in decrease of the enzyme activity. Some others, partially inhibited the enzyme, among those Co^{2+}, Fe^{3+}, and Cystine. Complete inhibition occurred with iodine even at low concentrations. Although, the inhibitory effects of PCMP, cysteine and reduced glutathione suggested the presence of reactive sulfhydryl groups in the active sites of

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the enzyme molecule, no conclusive evidence was however provided in this respect.

TABLE 3: Thermal and pH stability of the pure β-amylase from *P. citrinum* AS-9

pH of the enzyme solution	Temperature of heating (°C)	Time of heating (min.)	Residual β-amylase activity (%)
7.1 (Control)	-	0	100.0
5.2	50	20	13.58
		40	10.39
		60	12.48
	55	5	39.62
		10	32.70
		15	12.56
	60	5	22.46
		10	14.47
		15	10.00
5.5	50	20	25.79
		40	15.06
		60	17.00
	55	5	50.64
		10	49.05
		15	24.53
	60	5	27.17
		10	23.57
		15	15.00
5.9	50	20	30.25
		40	20.00
		60	15.00
	55	5	65.00
		10	57.86
		15	24.53
	60	5	38.05
		10	24.60
		15	20.00
7.1	50	5	80.36
		10	70.28
		20	50.08
	55	5	70.30
		10	58.00
		20	55.00
	60	8	52.40
		10	45.00
		20	29.20

Concerning the β-amylase activation by Ca^{2+} was reported by Ajayi and Fagade, [6], who found that the addition of Ca^{2+} to the reaction system of β-amylase of any of *Bacuillus circulans* (S1), *B. megaterium* or *B. polymyxa*, improved its activity. In addition, Adejuwan,[7] reported the same for *Aspergillus flavus* L β-amylase.

The inhibitory effect of KH_2PO_4 may be due to the drop of pH of the reaction and this is in harmony with the pH stability results of the pure β-amylase. Besides, maltose at high concentrations > 20 mM resulted in strong inhibition and this may be probably due to the product inhibition phenomenon of the enzyme by maltose.

4- Conclusion

Beta-amylase enzymes are of great importance due to their multiple uses in industry, medicine, pharmaceutical and food preparations, and in molecular biology researches. Most researches

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dealt with plant β-amylase enzymes, while microbial, few have touched on it. This research sheds light on the production of an enzymatic preparation that is very rich in beta-amylase enzyme in optimal conditions, and the study of purification of this enzyme preparation in sequential steps, starting with the ultra-filtration of the product solution and ending with separation by column chromatography, in order to study its general, operational and kinetic properties, which have been accurately determined. All these properties indicate the validity of this purified product for various applied uses with a high degree of stability that qualifies for long-term storage.

TABLE 4: Effect of some co-factors and inhibitors on the activity of pure β-amylase from *P. citrinum* AS-9.

Substance added	Final concentration of substances (%)	Relative activity (%)	Starch saccharification (%)
None	-	100.00	42.47
$CaCl_2$	0.1	6.16	2.61
	0.01	92.77	39.33
	0.001	115.72	49.07
$CoCl_2$	0.1	18.00	7.80
	0.01	25.90	10.97
	0.001	41.51	17.70
$FeCl_3$	0.1	20.00	8.88
	0.01	31.88	13.52
	0.001	50.72	21.47
Iodine	0.1	0.00	0.00
	0.01	0.00	0.00
	0.001	0.00	0.00
PCMB	0.1	18.48	7.84
	0.01	31.15	13.33
	0.001	36.16	15.33
Cysteine-HCl	0.1	0.0	0.0
	0.01	10.87	4.67
	0.001	36.16	15.33
Cystine	0.1	50.63	21.47
	0.01	63.40	26.93
	0.001	76.08	32.27
Reduced Glutathione	0.1	0.0	0.0
	0.01	1.81	0.77
	0.001	16.30	6.93
Gibberellin	0.1	0.0	0.0
	0.01	24.45	10.40
	0.001	67.92	28.80
KH_2PO_4	0.1	0.0	0.0
	0.01	23.73	10.08
	0.001	42.77	18.13
Maltose	0.08	0.08	0.10
	0.04	7.38	0.90
	0.02	85.00	36.18

Conflicts of interest

The authors declare no conflicts of interests.

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