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PURIFICATION AND CHARACTERIZATION OF β-AMYLASE FROM CANARY GRASS *Phalaris minor* SEEDS

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By
M. A. Mohamed

Department of Molecular Biology, National Research Centre, Giza

ABSTRACT

The major anionic isoenzyme (A_2) β-amylase from canary grass *Phalaris minor* seeds was purified to apparent homogeneity. A molecular weight of about 53,000 and 51,000 Da was established for both native (Sephacryl S-300) and denatured enzymes (SDS-PAGE), respectively, suggesting that the enzyme is monomeric. The enzyme was determined to be a β-amylase by its inability to hydrolyze β-limit dextrin and to release dye from starch azure. The enzyme exhibited a sharp pH optimum at 5.5 and a *Km* value of 6.7 mg ml^{-1} using soluble potato starch as substrate. Moderately branched glucans (amylopectin) were better substrates for *P. minor* β-amylase A_2 than less branched or non-branched (amyloses) or highly branched (glycogens) glucans. The enzyme was susceptible to inactivation by heavy metal ions (pb^{+2}, Cu^{+2}, Hg^{+2}) and sulfhydryl reagents such as *p*-HMB, indicating the sulfhydryl nature of the enzyme. The enzyme was partially inhibited by Schardinger maltodextrins , with α-cyclohexaamylose being a stronger inhibitor than β cycloheptaamylose. The enzyme was noncompetitively inhibited by its end product, maltose, with a *Ki* of 11.1 mM. This study of the catalytic properties of *P.minor β-amylase* A_2 indicates the importance of the enzyme as a starch degrading enzyme. The results are compared with those reported for other plant β -amylases that are of

industrial importance.

Key words: *β-amylase ,canary grass, Gramineae, Phalaris minor, purification.*

1. INTRODUCTION

β-Amylase (EC 3.2.1.2, aα-1,4-D-glucan maltohydrolase) has gained a considerable attention owing to its widespread utilization in the medical and industrial fields. It is employed in food (Tkachuk and Tipples, 1966), beverage (Sohn *et al.*, 1996; Kihara *et al.*, 1998) and pharmaceutical industries (Ray *et al.*, 1995) for conversion of starch into maltose. Cereal grains have been generally regarded as the most practical source of plant β-amylase (Doyen and Lauriere, 1992; Grime and Briggs, 1995; Igyor *et al.*, 1998).

β-Amylase catalyzes the liberation of β-maltose from the non-reducing end of α-1,4-glucans, leaving a β-limit dextrin when degrading starch and amylopectin (Thoma *et al.*, 1971; Vikso-Nielsen *et al.*, 1997). β-Amylase unlike α-amylase, is synthesized during ripening and not synthesized *de novo* during germination of seeds (Thoma *et al.*, 1971; Yamamoto, 1995). In dry cereal seeds, β-amylase is accumulated in the endosperm in both free (active) and bound (inactive) forms which can be extracted with saline solution and reducing agents, respectively (Lauriere *et al.*, 1992; Yamamoto, 1995). During germination, the enzyme undergoes posttranslational modification, during which bound β-amylase is converted into the free active form (Sopanen and Lauriere, 1989; Doyen and Lauriere, 1992; Grime and Briggs, 1995; Loreti *et al.*, 1998).

The physiological roles of β-amylase in plant cells are not well understood. Traditionally, β-amylase has been associated with starch degradation. The entire pathway of starch degradation has been associated to various combinations of activities of α-amylase, β-amylase, starch debranching enzyme and α-glucosidase (Lizotte *et al.*, 1990; Ravikumar *et al.*, 1997). Beck and Ziegler (1989) also reported that β-amylase may play a role in the mobilization of starch during seed germination or sprouting of tubers.

β-Amylase had been purified and characterized from germinating maize grains (Doyen and Lauriere, 1992; Subbarao *et al.*, 1998), tap roots of alfalfa (Boyce and Volenec, 1992), pea epicotyl (Lizotte *et al.*, 1990) and the phloem of *Arabidopsis thaliana* (Wang *et al.*, 1995; Zeeman *et al.*, 1998). Recently, the molecular properties of the purified enzyme from leaves of potato (Vikso-Nielsen *et al.*, 1997), *Bacillus polymyxa* (Sohn *et al.*, 1996; Niziolek, 1997) and *Clostridium thermosulfurogenes* (Reddy *et al.*, 1998) have been described.

A considerable body of information has accumulated with respect to cereal β-amylases. Although the data encompass the biochemistry, physiology and production of the enzyme, they are largely restricted to cereal genera (*Hordeum*, *Triticum*, *Sorghum* and *Zea*) of family Gramineae which are economically important and have priority as food for human and animals. Therefore, further investigations should be oriented toward other species of the Gramineae for β-amylase production. The present study provides the first detailed description of purification and characterization of β-amylase from canary grass *Phalaris minor* seeds as a member of Aveneae tribe of family Gramineae.

2. MATERIALS AND METHODS

2.1. Plant material

P.minor seeds were purchased from the local market.

2.2.. Chemicals

Soluble potato starch, amylose, maltose, amylopectin, glycogen, iodoacetamide, *N*-ethylmaleimide, carboxymethyl (CM) cellulose, *p*.hydroxymercuribenzoic acid (*p*-HMB),α-cyclohexaamylose, β-cycloheptaamylose, Remazol Brilliant Blue (RBB)-dye, starch azure, 5,5'-dithio-(2-bisnitrobenzoic acid) (DTNB), dithiothreitol (DTT) and diethylaminoethyl (DEAE) cellulose were purchased from Sigma Chemical Co. (St Louis MO). Molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals, Sweden. Trichloroacetic acid (TCA)

was obtained from Riedel-De Haen Co. (Seelze Hannover, Germany). All resins and reagents for electrophoresis were products of BDH Chemical Ltd., and other chemicals were of analytical grade.

2.3. Enzyme assays

2.3.1. β-Amylase assay

β-Amylase activity was routinely assayed by measuring the rate of generation of reducing sugars from starch (Okon and Uwaifo, 1984; Sohn *et al.*, 1996). Assay reaction mixture contained in 1.0 ml: 50 mM citrate-phosphate buffer, pH 6.0 , 0.5 ml 2% (w/v) soluble potato starch and appropriate dilution of enzyme preparation. The reaction mixture was incubated for 15 min at 37°C and terminated by adding 0.5 ml of alkaline dinitrosalicylic acid solution prepared according to Bernfeld (1955) and then placed immediately into a boiling water bath. Colour was fully developed after 5 min, the absorbance was read, after cooling, at 540 nm and maltose was used as a standard. One unit of enzyme activity was defined as the quantity of enzyme that released 1μ mol maltose h^{-1} under standard assay conditions. Specific activity was expressed in units mg^{-1} protein. In all experiments, values were determined in triplicates.

2.3..2. α-Amylase assay

This assay was carried out during the first purification steps to estimate the contamination of β-amylase fractions by α-amylase. α-Amylase activity was determined by measuring the release of the RBB-dye from starch azure (Doehlert and Duke, 1983; Witt and Sauter, 1995). Assay reaction mixture contained in 1.0 ml: 50 mM sodium acetate buffer, pH 5.6, 0.6 ml 0.9% (w/v) freshly boiled substrate, 10 mM $CaCl_2$ and appropriate dilution of enzyme preparation. The reaction mixture was incubated for 15 min at 37°C and terminated by addition of 0.25 ml 50% TCA. After centrifugation to remove nondigested substrate, the absorbance of the supernatant was measured at 595 nm using RBB-dye as a standard. One unit of enzyme activity was defined as 1 μ mol RBB-dye released h^{-1} under standard assay conditions. In all the experiments, values were determined in triplicates.

2. 4. Protein determination

Protein was determined either by measuring the absorbance at 260/280 (Warburg and Christian, 1942) or by the method of Bradford (1976) using bovine serum albumin as a standard.

2. 5. Buffers

Buffers were prepared according to Gomori (1955), and the final pH was checked by an EIL pH meter Type 7020.

2.6. Purification of *P. minor* β-amylase

Unless otherwise stated, all steps of purification extraction of β-amylase were performed at 4-7°C using 50 mM sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl for extraction and ammonium sulfate precipitation (Buffer 1), 50 mM sodium acetate buffer, pH 5.6 (Buffer 2) for CM-cellulose and Sephacryl S-300 chromatography and Tris-HCl buffer, pH 6.5 (Buffer 3) for DEAE-cellulose chromatography.

2.6.1. Preparation of crude extract

Crude extract of β-amylase was prepared by homogenizing 10 g of ground *P. minor* seeds in Buffer 1 using Sorvall Omni Mixer-17106 for 15 min. The homogenate was centrifuged at 16,500 X g for 30 min at 4-7°C and the supernatant was saved. The precipitate was reextracted with the same buffer and recentrifuged. The two supernatants were pooled and designated as crude extract.

2.6.2. Ammonium sulfate fractionation

Solid ammonium sulfate to 20% saturation was added to the crude extract and the mixture was stirred for 30 min with continuous cooling. After centrifugation, the precipitate was saved. The ammonium sulfate concentration was then increased in the supernatant up to 80% saturation and the mixture was stirred for another 30 min, the precipitate was collected by centrifugation for 15 min at 11,400 X g and the two precipitates were designated as I and II, respectively. The precipitates were dissolved in Buffer 1 and dialyzed overnight against Buffer 2. The precipitates formed during dialysis were discarded after centrifugation at 5,000 X g. The activity

of α- and β-amylases were measured in the two supernatants.

2.6.3. CM-cellulose chromatography

The dialyzed ammonium sulfate fraction II was applied directly to a CM-cellulose column (20 X 1.6 cm i.d.) preequilibrated with Buffer 2. The exchanged material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.4 M prepared in Buffer 2 at a flow rate of 60 ml h^{-1} and collected in 5 ml fractions. Fractions exhibiting β-amylase activity were eluted at 0.0 and 0.1 M NaCl, and designated β-amylases A and B, respectively. The fractions that were enzymatically the most active were pooled and dialyzed overnight against Buffer 3.

2.6.4. Chromatography on DEAE-cellulose

The dialyzed pooled CM-cellulose fraction of β-amylase A was applied to a DEAE-cellulose column (20 x1.6 cm i.d.) preequilibrated with Buffer 3. The exchanged material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.4 M prepared in Buffer 3 at a flow rate of 60 ml h^{-1} and collected in 5 ml fractions. Fractions exhibiting β-amylase activity were eluted at 0.1 and 0.3 M NaCl, and designated β-amylases A_1 and A_2, respectively.

2.6.5. Chromatography on Sephacryl S-300

The pooled active fractions of β-amylase A_2 were concentrated by using polyethylene glycol 1000 and applied on the top of Sephacryl S-300 column (95 X 1.6 cm i.d.) preequilibrated with Buffer 2. The active fractions of β-amylase were pooled and stored at -20 C for characterization.

2.6.6. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis under nondenaturing conditions was performed in 7.5% (w/v) acrylamide slab gel according to the method of Davis (1964) using a Tris-glycine buffer, pH 8.3. Protein bands were stained with Coomassie brilliant blue R-250.

2.6.7. Molecular weight determination

Molecular weight was determined by gel filtration using

Sephacryl S-300 (Porath and Ernback, 1967). The column (95 X 1.6 cm i.d.) was calibrated with myoglobin (17,200), bovine serum albumin (67,000), alcohol dehydrogenase (150,000), catalase (240,000) and ferritin (440,000). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). SDS-Molecular weight protein markers, phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), trypsin inhibitor (20,000) and α-lactalbumin (14,400) were applied with unknown samples and used for the calibration curve.

3. RESULTS AND DISCUSSION

3.1. Purification of *P.minor* β-amylase

Free β-amylase was extracted from *P. minor* seeds using saline buffer according to Sopanen and Lauriere (1989). β-Amylase predominates, and occurs abundantly in ungerminated cereal grains where α- amylase content is extremely low (Thoma *et al.*, 1971; Yamamoto, 1995). Elimination of any contaminating α-amylase was achieved by ammonium sulfate precipitation at 20% saturation.

Table (1): * Purification scheme for *P. minor* β-amylase A_2.

Fraction	Total protein (mg)	Total activity (units)**	Specific activity (units mg^{-1}protein)	Fold purification	Recovery%
Crude extract	183.1	3218	17.6	-	100.0
20-80% $(NH_4)_2SO_4$	99.1	2648	26.7	1.5	82.3
CM-cellulose:					
0.0 M NaCl (A)	56.4	2254	40.0	2.3	70.0
0.1 M NaCl (B)	19.8	249	12.6	0.7	7.7
DEAE-cellulose for β-amylase A					
0.1 M NaCl (A_1)	16.8	233	13.9	0.8	7.3
0.3 M NaCl (A_2)	9.2	1781	193.6	11.0	55.3
Sephacryl S-300 for A_2	4.1	1431	349.0	19.8	44.5

*Each value represents the average of two experiments.

**One unit of β-amylase was defined as the amount of enzyme that released 1 µmol maltose h^{-1} under standard assay conditions.

This step resulted in a decrease in total protein (46%) with only a slight increase in fold purification (1.5). Fractions containing β-amylase activity eluted from the CM-cellulose, DEAE-cellulose and Sephacryl S-300 columns were identified by their ability to hydrolyze soluble potato starch and their failure to release RBB-dye from starch azure. The purification of *P. minor* β-amylase is summarized in Table 1.

As shown in Fig. (1), the CM-cellulose column resolved β-amylase into two isoforms A (major) and B (minor), where the enzyme A was 9.1 times higher than β-amylase B. Therefore, β-amylase A was subjected to further purification. By DEAE-cellulose column (Fig. 2) two forms of β-amylase A were resolved (A_1 and A_2). β-Amylase A_2 was further purified on a Sephacryl S-300 column (Fig. 3) to obtain pure enzyme with increased specific activity. β-Amylase A_2 was purified to homogeneity to a final specific activity of 349.0 units mg^{-1} protein which is consistent to the values of 276, 300 and 674.2 units mg^{-1} protein reported for germinating maize grains (Doyen and Lauriere, 1992), leaves of potato *Solanum tuberosum* (Vikso-Nielsen *et al.*, 1997) and tap roots of alfalfa (Boyce and Volenec, 1992) β-amylases, respectively. The purification of *P.minor* β-amylase A_2 reached 19.8 fold over the crude extract which is consistent with that reported for *B. polymyxa* (22.5) (Sohn *et al.*, 1996) and it was 2.1 higher than that reported for tap roots of alfalfa β-amylases (Boyce and Volenec, 1992).

3.2. Molecular weight

The native molecular weight of *P.minor* β-amylase A_2 was calculated from Sephacryl S-300 calibration curve (Fig. 4) to be 53,000 ± 4,700 Da. This value was confirmed by SDS-PAGE where subunit molecular weight of the enzyme was calculated from the calibration curve (Fig. 5) to be 51,000 Da as single subunit. The molecular weight of *P. minor* β-amylase A_2 resembles the most characterized cereal enzymes, of sorghum (Okon and Uwaifo, 1984), maize (Subbarao *et al.*, 1998) and wheat (Kato *et al.*, 1974) β-amylases which have molecular weights of 53,000, 56,000 and 57,500

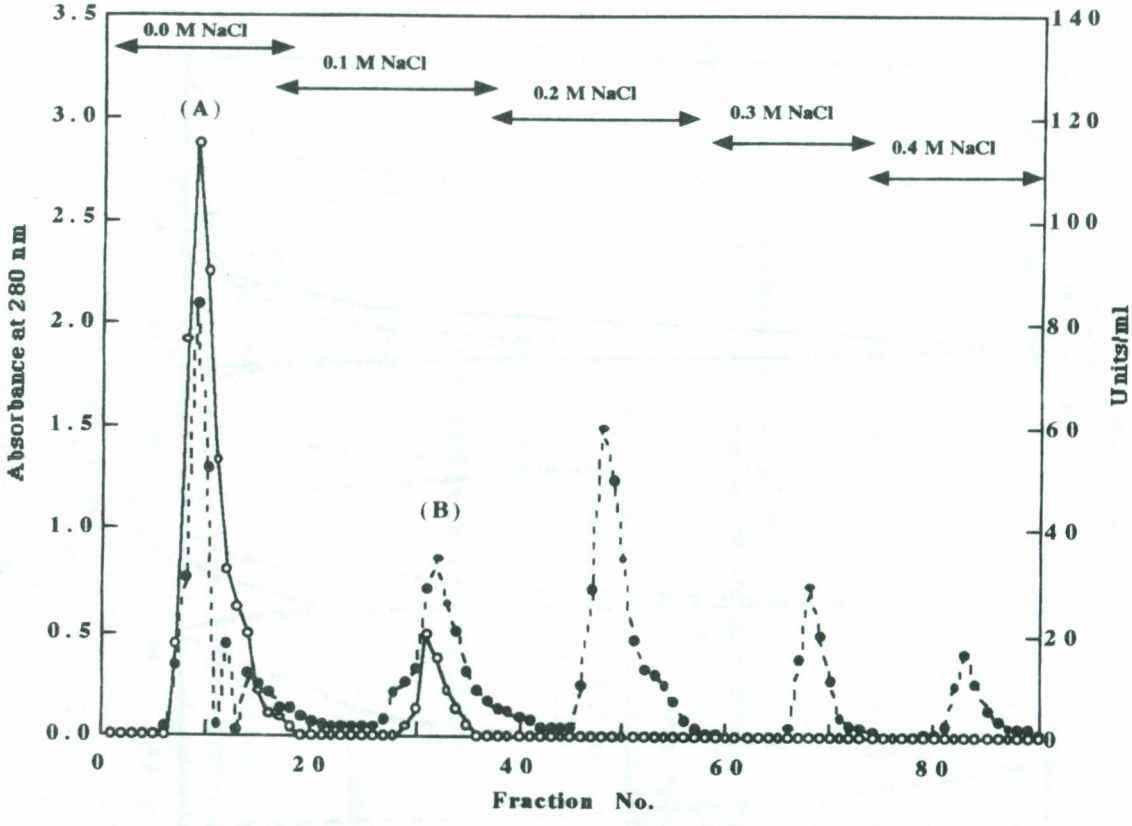

Fig.(1): A typical elution profile for the chromatography of *P. minor* β-amylase ammonium sulfate fraction on CM-cellulose column (20X 1.6 cm i.d.) preequilibrated with Buffer 2 at a flow rate of 60 ml h^{-1} and 5 ml fractions. Absorbance at 280 nm (-------),β-amylase activity (0_____0)

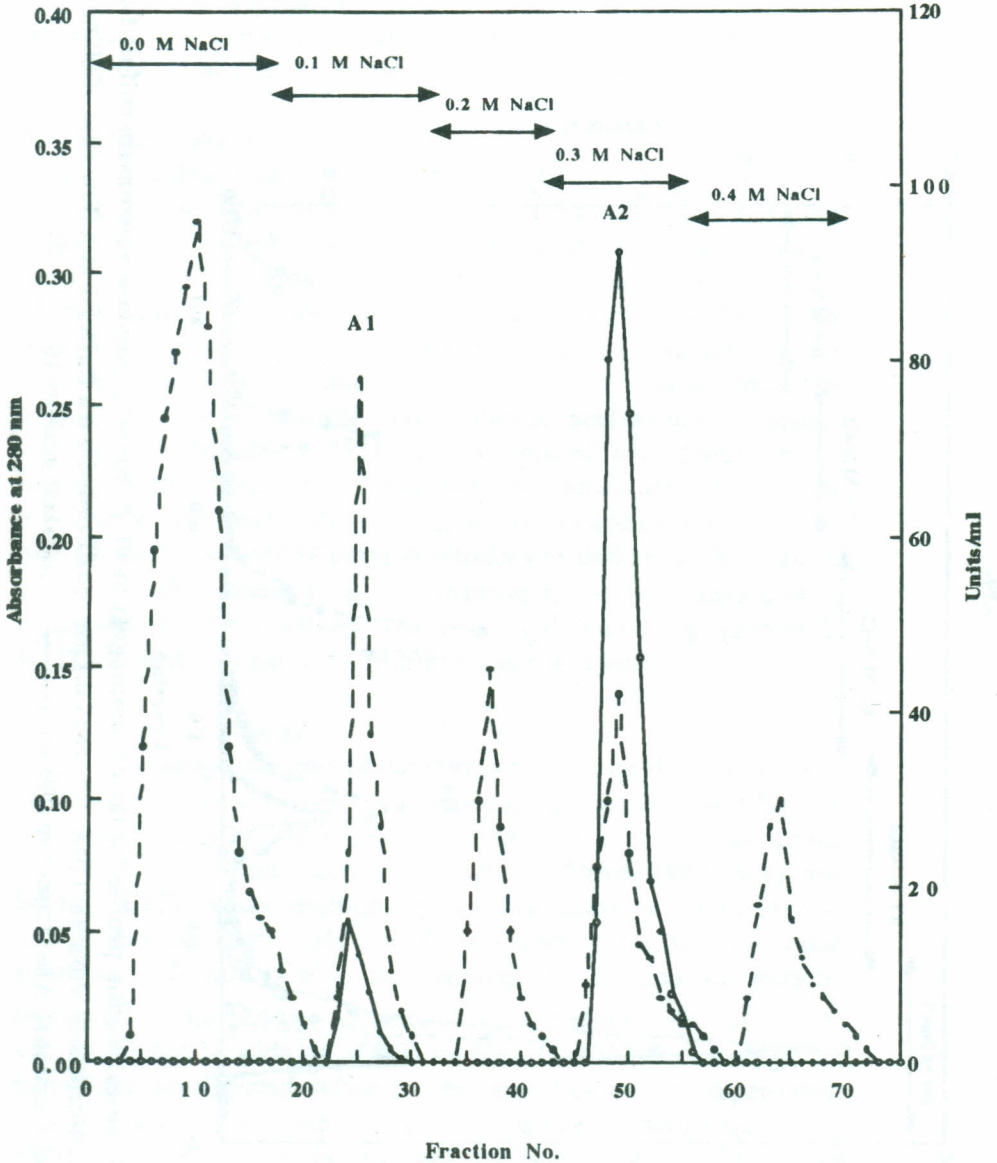

Fig. (2):A typical elution profile for the chromatography of pooled CM-cellulose fractions of *P. minor* β-amylase A on DEFA-cellulose column (20X 1.6 cm i.d.) preequilibrated with Buffer 3 at a flow rate of 60 ml h^{-1} and 5 ml fractions. Absorbance at 280 nm --------),β-amylase activity (0_____0)

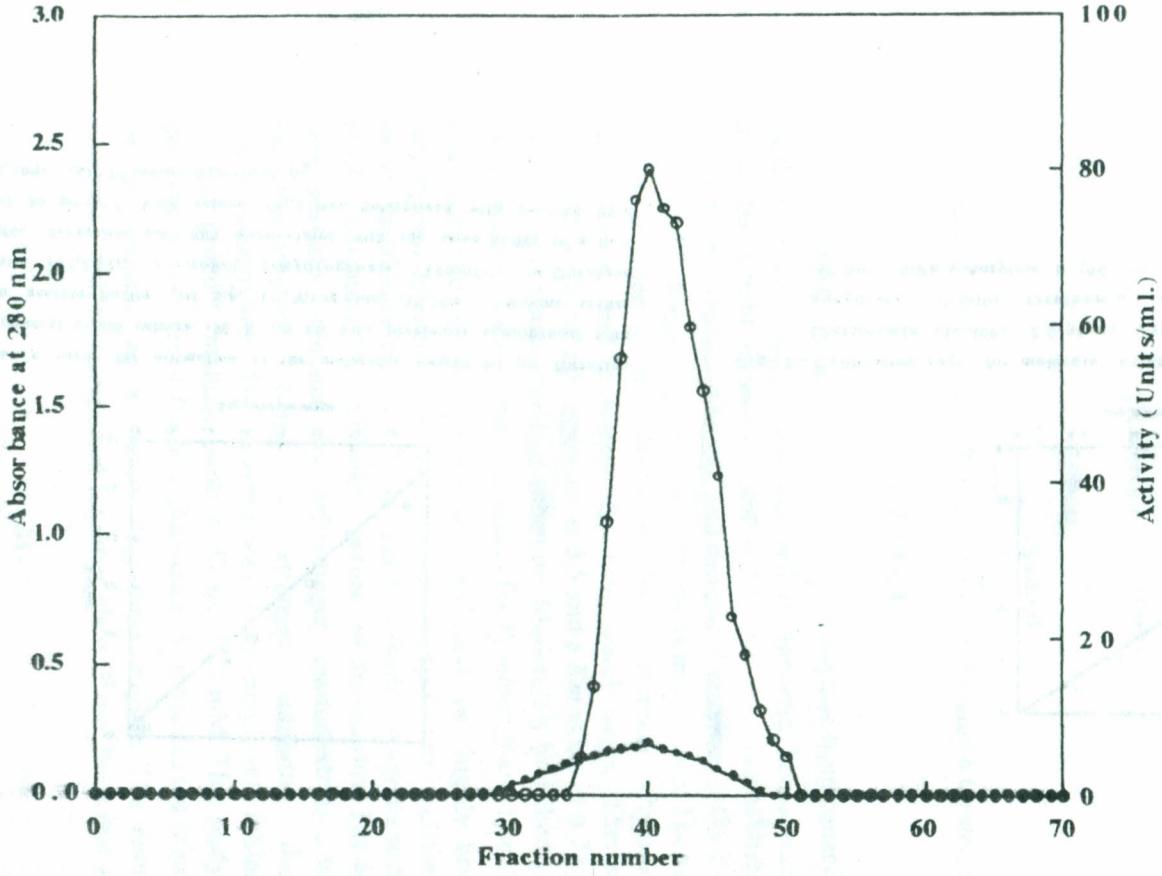

Fig. (3): A typical elution profile for the chromatography of concentrated *P. minor* β-amylase A_2 on Sephacryl S-300 column (90 X 1.6 cm i.d.) preequilibrated with Buffer 3 at a flow rate of 20 ml h^{-1} and 3 ml fractions. Absorbance at 280 nm (.——.),β-amylase activity (0_____0).

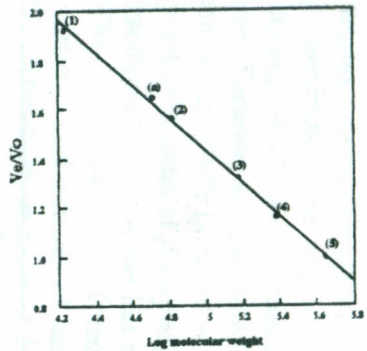

Fig.(4): Calibration curve for estimation of the molecular weight by gel filtration on Sephacryl S-300 column (95 X 1.6 cm i.d.) previously equilibrated with sodium acetate buffer, pH 5.6. 1- Myoglobin (17,200); 2-Bovine serum albumin (67,000); 3-Alcohol dehydrogenase (150,000); 4-Catalase (240,000); 5-Ferritin (440,000) were eluted with the same buffer at a flow rate of 20 ml h^{-1} Void volume (V_0) was determined with Dextran blue (2,000,000) (a) *P. minor* β-amylase A_2.

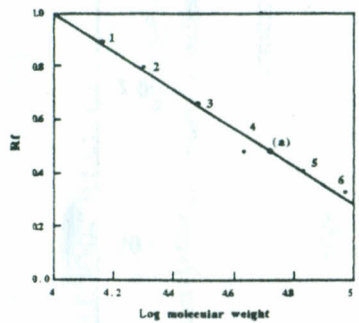

Fig.(5): Calibration curve for molecular weight determination by SDS-PAGE. 1-α-Lactalbumin (14,400); 2-Soybean trypsin inhibitor (20,000); 3-Carbonic anhydrase (29,000); Ovalbumin (43,000); 5-Bovine serum albumin (67,000); 6-Phosphorylase b (94,000). (a) *P. minor* β-amylase A2

Da, respectively. It was also consistent with that purified from pea epicotyl (55,000) (Lizotte *et al.*, 1990), mustard (58,000) (Subbaramaiah and Sharma, 1990) and from bacteria *B. polymyxa* (53,000) (Sohn *et al.*, 1996). On the contrary, it was found to be lower than that reported for leaves of potato *S. tuberosum* (111,000) (Vikso-Nielsen *et al.*, 1997), *C. thermosulfurogenes* (186,000) (Reddy *et al.*, 1998) and sweet potato *Ipomoea batatas* (207,000) (Chang *et al.*, 1996) β-amylases, respectively.

3.3. Characterization of *P. minor* β-amylase A_2

3.3.1. Thermal stability

P.minor β-amylase A_2 was similar to other β-amylases in their requirement for sulfhydryl reagents to stability (Lizotte *et al.*, 1990; Subbaramaiah and Sharma, 1990). The thermal stability of *P.minor* β-amylase A_2 in the presence and absence of DTT is shown in Fig. (6). Both enzymes are unstable at temperatures above 40°C similar to pea epicotyl (Lizotte *et al.*, 1990) and potato leaves (Vikso-Nielsen *et al.*, 1997) β-amylases. While a loss of 52% was recorded in the activity of DTT-free enzyme upon incubation for 15 min at 50°C, the DTT-containing enzyme lost only 22% of its activity. Subbaramaiah and Sharma (1990) reported that inclusion of DTT in storage media of mustard (*Sinapsis alba*) β-amylase had a pronounced effect on stabiliy of mustard β-amylase.

3.3.2. *Km*

P.minor β-amylase A_2 followed Michaelis Menten - Kinetics with soluble potato starch as substrate and had apparent *Km* value of 6.7 mg ml^{-1} with *Vmax* of 111 μ mol maltose ml^{-1} h^{-1} (Fig. 7) which was congruent with that reported by Doehlert *et al.* (1982) for light and heavy alfalfa *Medicago sativa* β-amylases (*Km* values of 5.9 and 6.8 mg ml^{-1} and *Vmax* of 640 and 130 μ mole maltose ml^{-1}, respectively). On the other hand, it was 2.8 and 4 times higher than that reported for mustard, *S.alba* (Subbaramaiah and Sharma, 1990) and pea epicotyl, *Pisum sativum* (Lizotte *et al.*, 1990) β-amylases, respectively.

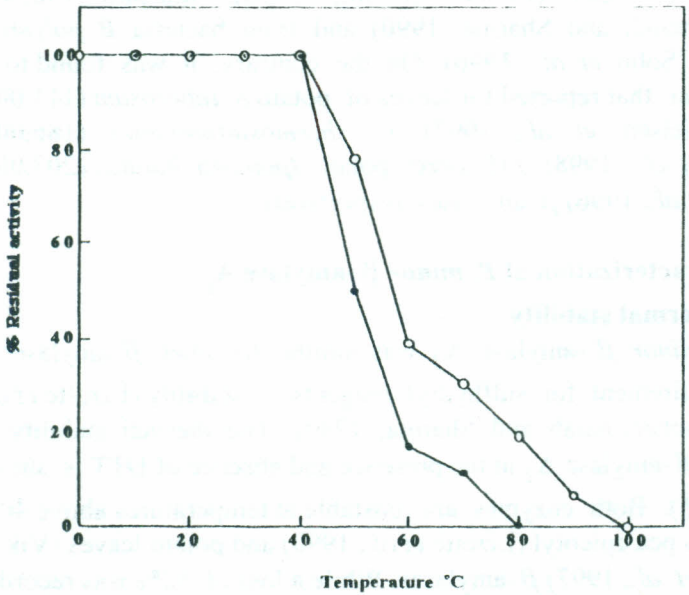

Fig.(6): Effect of temperature on stability of *P. minor* β-amylase A_2 in presence and absence of DTT. The reaction mixture contained in 1 ml: 50 mM citrate-phosphate buffer, pH 5.5, 1.5 units of enzyme, 10 mM DTT (o——o) and in absence of DTT (• ——•). The reaction mixtures were incubated at various temperatures for 30 min prior to substrate addition, followed by cooling in ice bath. The residual activity was measured and the activity at zero time was taken as 100% activity. Each point represents the average of three replicates.

3.3.3. Substrate specificity

The activity of *P. minor* β -amylase A_2 with respect to its ability to hydrolyze several glucans is shown in Table 2. The

Table (2) : Relative activity of *P.minor* β-amylase A_2 toward different glucans as substrates. All assays were incubated for 15 min at 37°C in 50 mM citrate-phosphate buffer, pH 5.5 containing 2.0 units of enzyme and 2% (w/v) glucan. Amylopectin was taken as 100% activity. Each value represents the average of three replicates.

Substrate	Relative activity(%)
Soluble potato starch	86.4
Amylopectin	100
β-Limit dextrin	0.0
Amylose	31.3
Glycogen	25.7
Soluble corn starch	67.0
Xylan	0.00
Pullulan	0.00

enzyme preferred branched substrates, *i.e.*, amylopectin, soluble potato starch and soluble corn starch, over less branched or nonbranched substrates. The rate of amylose (nonbranched glucan) hydrolysis was less than half that of branched starches, probably because amylose is an essentially linear molecule, with fewer non-reducing ends available for enzymatic attack. However glycogen, a highly branched glucan, was not hydrolyzed as rapidly as amylopectin, presumably due to steric hinderance as reported by Lizotte *et al.* (1990). *P. minor* β -amylase A_2 did not hydrolyze pullulan, indicating the absence of debranching enzyme of this preparation, nor β -limit dextrin, suggesting that the enzyme was exoamylase and free of other starch hydrolases. Similar results had been reported for pea epicotyl (Lizotte *et al.*, 1990), cotyledons of mustard (Subbaramaiah and Sharma, 1990), alfalfa tap roots (Boyce and Volenec, 1992) and potato leaves (Vikso-Nielsen *et al.*, 1997) β-amylases.

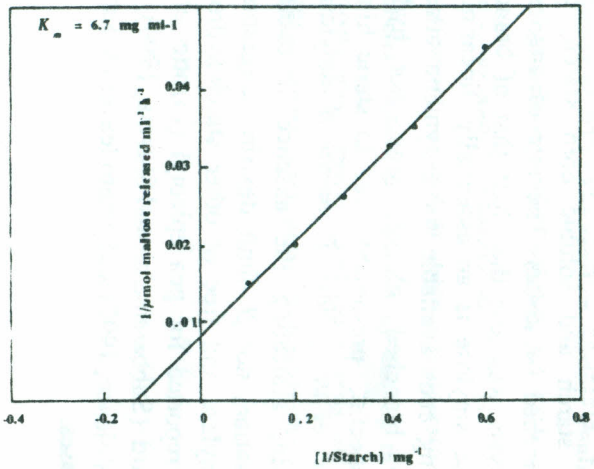

Fig. (7):Lineweaver-Burk plot relating *P. minor* β-amylase A_2 reaction velocity to strach concentration. The reaction mixture contained in 1 ml: 50 mM citrate-phosphate buffer, pH 5.5, 2 units of enzyme and different concentrations of starch. Each point represents the average of three replicates.

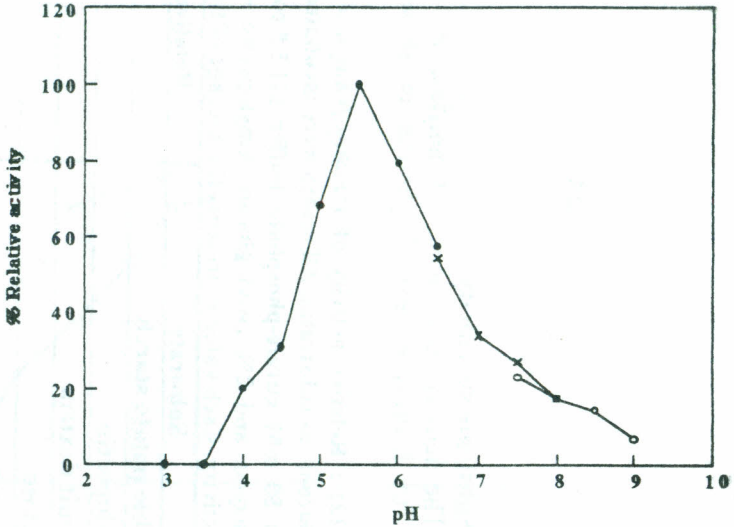

Fig.(8): pH optimum of *P. minor* β-amylase A_2. The reaction mixture contained in 1.0 ml: 15 mg starch, 1.5 units enzyme and 50 mM citrate-phosphate buffer (pH 3.0-6.5) (.__.), phosphate buffer (pH 6.5-8.0) (x—x) and Tris-HCl (pH 7.5-9.0) (0—0). Each point represents the average of three replicates.

3.3.4. pH optimum

Hydrolysis of soluble starch with *P. minor* β-amylase A_2 was maximal at pH 5.5 in citrate-phosphate buffer (Fig. 8). This result is in good agreement with that reported for wheat (Tkachuk and Tipples, 1966), barley (Lundgard and Svensson, 1987) and germinating maize grains (Doyen and Lauriere, 1992) β-amylases. On the contrary, *P. minor* β-amylase A_2 had a pH optimum lower than that reported for tap roots of alfalfa (7.5) (Boyce and Volenec, 1992), potato leaves (6.5) (Vikso-Nielsen *et al.*, 1997) and *C. thermosulfurogenes* (6.0) (Reddy *et al.*, 1998) β-amylases.

3.3.5. Effect of heavy metal ions

Heavy metal ions could be arranged in a descending order, according to their inhibitory effect on the enzyme activity as $Hg^{+2}>Pb^{+2}>Cu^{+2}>Zn^{+2}>Ag+$. Other metal ions had little or no inhibitory effect on the enzyme activity (Table 3). These results are in agreement with that reported for sweet potato (Chang *et al.*, 1996), pea epicotyl (Lizotte *et al.*, 1990) and mustard (Subbaramaiah and Sharma, 1990) β-amylases. The fact that β-amylase is strongly inhibited by heavy metal ions like Hg^{+2}, Pb^{+2} and Cu^{+2} implies that *P.minor* β-amylase A_2 needs sulfhydryl groups for its activity like other β-amylases (Bernfeld, 1955; Serafimova *et al.*, 1996).

3.3.6. Effect of sulfhydryl reagents and cyclodextrins

Plant β-amylase was inhibited by thiol binding reagents (Tkachuk and Tipples, 1966; Thoma *et al.*, 1971; Lizotte *et al.*, 1990; Serafimova *et al.*, 1996). The sulfhydryl reagents: *N*-ethylmaleimide, *p*-HMB and DTNB greatly reduced *P. minor* β-amylase A_2 activity (Table 4). In contrast, iodoacetamide had only marginal inhibition effect. This pattern of sulfhydryl reagent selectivity was similar to that reported for *B. polymyxa* (Sohn *et al.*, 1996) and pea epicotyl (Lizotte *et al.*, 1990) β-amylases. If β-amylase cysteinyl sulfhydryls were necessary for catalysis, all exposed cysteinyl sulfhydryl groups would be reactive towards low concentration of any sulfhydryl

Table (3): Effect of different cations on *P. minor* β-amylase A_2 activity. Enzyme (2.0 units) was preincubated for 15 min at 37°C with listed cations at final concentrations indicated prior to substrate addition. Activity without adding cations was taken as 0.0% inhibition. Each reading represents the average of three replicates.

Cations	Final concentration (mM)	Inhibition (%)
None	---	0.0
Ni^{2+}	1	0.0
Ag^{+}	2	32.8
Cu^{2+}	1	86.6
Fe^{3+}	2	0.0
Hg^{2+}	1	100
Mg^{2+}	2	6.2
Mn^{2+}	2	5.7
Pb^{2+}	1	91.4
Zn^{2+}	1	52.5

reagent (Lizotte *et al.*, 1990). *P. minor* β-amylase A_2 inhibition by sulfhydryl reagents could be due to binding of noncatalytic cysteinyl sulfhydryl, causing changes in the alignment of catalytic amino acids as is proposed for sweet potato (Thoma *et al.*, 1971; Chang *et al.*, 1996) and pea epicotyl (Lizotte *et al.*, 1990) β-amylases or by steric hinderance.

Table (4): Effect of sulfhydryl reagents and cyclodextrins on *P. minor* β-amylase A_2 activity. Enzyme was preincubated for 15 min at 37°C with listed reagents prior to substrate addition. Activity without adding reagents was taken as 0.0% inhibition.

Sulfhydryl reagents	Final concentration (mM)	Inhibition (%)
Control	--	0.0
N-Ethylmaleimide	2.0	81.7
Iodoacetamide	2.0	14.8
p-HMB	1.0	96.6
DTNB	2.0	91.3
α- Cyclohexaamylose	10.0	70.7
β-Cycloheptaamylose	10.0	15.2

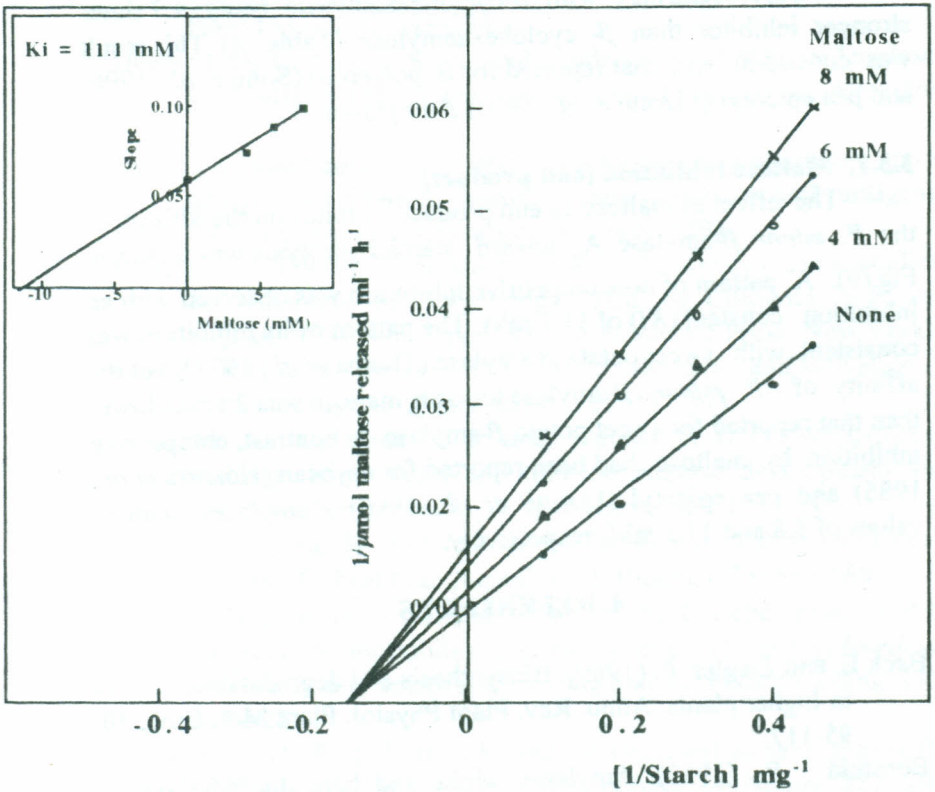

Fig. (9): Inhibition of *P minor* β-amylase A_2 with maltose. Plots of reciprocal of initial velocities versus reciprocal concentrations of starch. Reaction mixtures contained in 1.0 ml: 50 mM citrate-phosphate buffer, pH 5.5, 0.2 units of enzyme and maltose was asses in the concentrations indicated.The inhibition constant (*K I*) of maltose as the noncompetitor of starch was estimated from the replot, shown in the inset to be 11.1 mM.

P. minor β-amylase A_2 was also inhibited by Schardinger dextrins (cyclodextrins), with α-cyclohexaamylose being 4.7 times stronger inhibitor than β- cyclohexaamylose (Table 4). This result was consistent with that reported for *B. polymyxa* (Sohn *et al.*, 1996) and pea epicotyl (Lizotte *et al.*, 1990) β-amylases.

3.3.7. Maltose inhibition (end product)

The effect of maltose as end product inhibitor on the affinity of the *P. minor* β-amylase A_2 toward starch hydrolysis was shown in Fig.(9). A pattern of noncompetitive inhibition was observed with an inhibition constant (*Ki*) of 11.1 mM. The pattern of its inhibition was consistent with sweet potato β-amylase (Thoma *et al.*, 1971), but the affinity of *P. minor* β-amylase towards maltose was 2 times lower than that reported for sweet potato β-amylase. In contrast, competitive inhibition by maltose had been reported for soybean (Nomura *et al.*, 1986) and pea epicotyl (Lizotte *et al.*, 1990) β-amylases with *Ki* values of 5.8 and 11.5 mM, respectively.

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تنقية إنزيم الـ β- amylase من بذور حشيشة الكنارى *Phalaris minor* و دراسة خواصه

ماجدة عبد النبي محمد

المركز القومي للبحوث - الجيزة

ملخص

تم تنقية أيزو انزيم البيتا-أميليز A_2 و الذى يحتوى على معظم نشاط الانزيم من بذور حشيشة الكنارى *Phalaris minor* الى درجة التجانس. و قد قدر الوزن الجزيئ للإنزيم و كان حوالى 53000 و51000 دالتون لكل من انزيم A_2 الأصلى و الانزيم المجزئ مما يوضح ان الإنزيم أحادى الشكل و قد دلت النتائج على أن انزيم البيتا-أميليز ليست لديه القدرة على تحليل البيتا-ديكسترين المحدود و تحرير الصبغة الزرقاء من نشا الأزور. و قد وجد أن الإنزيم له أس هيدروجينى أمثل حاد عند 5.5 كما قدرت قيمة ثابت ميكائيل له بـ6.7 مجم/مل$^{-1}$ و ذلك باستخدام نشا البطاطس الذائب كمادة وسيطة. و قد أظهرت النتائج أن الجلوكانات المتوسطة التفرع هى أفضل مواد وسيطة لإنزيم *Phalaris minor* بيتا-أميليز A_2 و ذلك بالمقارنة بالجلوكانات الأقل تفرعا أو غير المتفرعة مثل الأميلوزات أو عديدة التفرع مثل الجليكوجينات. و قد وجد أن الفلزات ثنائية التكافؤ مثل الرصاص و النحاس و الزئبق و مركبات السلفهيدريل مثل حمض الباراهيدروكسى مركيورى بنزويك (*p*- HMP) لها تأثير تثبيطى علي الإنزيم مما يوضح طبيعة الإنزيم السلفهيدريلية. و قد وجد أن مالتوديكسترينات الشيردنجر تسبب تثبيطا جزيئيا للإنزيم حيث وجد أن الألفا-سيكلوهكسا أميلوز له نشاط تثبيطى أقوى من البيتا-سيكلوهيبتا أميلوز. و قد وجد أن الإنزيم يحدث له تثبيطا غير متنافس بمادة تفاعل الإنزيم الناتجة و هي المالتوز، و كانت قيمة ثابت التثبيط 11.1 ملى مولار. و قد دلت هذه الدراسة التي تتعلق بالخواص الحفزية لإنزيم ***P. minor*** البيتا-أميليز علي أهميته كإنزيم محلل للنشا. و قد قورنت هذه النتائج مع مثيلاتها المنشورة سابقا لانزيمات البيتا-أميليز النباتية ذات الأهمية الصناعية.

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