Identification and Characterization of α-Amylase from *Yemeni Bean (Dolichos Lablab* L.) Seeds

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Abstract

The α-amylase from local Yemeni bean (*Dolichos Lablab*) seeds was partially purified by conventional methods of protein purification such as pH and thermal precipitation, ammonium sulphate fractionation and ion-exchange chromatography on DEAE-Cellulose. Native Polyacrylamide Electrophoresis revealed that *cashed* α-amylase had isoform AmyI and AmyII. The enzyme AmyII showed the following characteristics: optimum pH 7.0, optimum temperature, K_m value for hydrolyzing starch was 1.25 mg. The affinity between substrate and enzyme was detected only for glycogen and starch compared with other carbohydrates tested, where glycogen had more affinity than starch. Enzyme activity was stimulated by Ca^{2+}, Ni^{2+} and Cd^{2+}, inhibited by Zn^{2+} and Hg^{2+}. In conclusion, this α-amylase from Yemeni seeds have interesting characteristics such as low km value, neutral pH optimum, high optimum temperature, high affinity toward starch and glycogen and activation by some metal as calcium. Therefore, these meet the prerequisites need for food industry.

Keywords: α-Amylase activity; Legumes; Characterization; *Dolichos Lab-lab* seeds.

Introduction

Legumes are the most important plant food material, for the outstanding reason that they are the concentrated cheap sources of protein for the vegetarian population. Pulses are important sources of protein in the diets of millions of people in the world [1]. However their contribution to the nutrition of the consumer is limited, principally due to poor digestibility and antinutritional factors [2]. To the best of our knowledge, this is the first report on the kinetic properties of α-amylase in the Yemeni bean (*cashed*).

Yemeni bean (*Dolichos lablab*) both in tender green and mature dry stages is consumed after cooking in Yemen, India and parts of South America. Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process. Alpha amylase (endo-1,4-Dglucose-D glucohydrolase 3.2.1.1.) belongs to the family of endo amylases that randomly cleave the 1,4-D glycoside linkage between adjacent glucose units in the product chain retaining the α-anomeric configuration in the product [3]. It is therefore important to increase protein utilizing by all ways and means the increasing world demand for food and the need for feed protein led to the search for a nonconventional protein source to conventional

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protein source. Carbohydrate hydrolyzing enzymes play main roles in the carbohydrate digestion and absorption. The enzymes degrade poly- and oligosaccharides to monosaccharides before they can be absorbed. α-Amylases–ubiquitous in nature–have been isolated, purified and characterized from a number of animal, plant, fungal as well as bacterial sources [4]. Beans α-amylases have gained importance due to their suitability for biotechnological applications in supplementary foods, breweries and starch saccharification [5,6]. However, no study was carried out on activity of *Dolichos Lablab* seeds also called (in Yemen) *cashed.*

The number of identified isoenzymes depends on the cultivars studied and the sensitivity of the resolving method used [7,8,9,10].

The present investigation was initiated to *Lablab* seeds for α- amylase activity, Partially purify and to characterized α-amylase activity. This information is important for industrialists, who may be looking for a cheap source the enzyme.

Materials and Methods

Chemicals:

All chemicals were of analytical grade and purchased from Sigma Inc., USA. Soluble potato starch, dextrose, glycogen, dextrin and pectin were also obtained from Sigma Co.

Plant Material:

Yemeni bean (cashed) seeds , a local variety in Republic of Yemen, was purchased from local market.

Extraction of Seed Proteins

Cashed seeds dry were powdered and 100g of the powder were defatted with chilled acetone (1:10, wt/vol) for 2 hrs. Supernatant was separated by 30,000 r.p.m for 20 min at 4°C and dried in a speed vacuum.

Amylase Assay:

α-Amylase activity was determined by measurement of maltose released from starch according to the method of [11].The samples were incubated at 37°C for 30 min in tubes containing 1% starch, 100 mM phosphate buffer, pH 7.0, appropriate amount of enzyme and distilled water to give a final volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml dinitrosalicylic acid reagent, followed by incubation in a boiling water bath for 10 min followed by cooling. The absorbance was recorded at 540 nm. The enzymatically liberated reducing sugar was calculated from a standard curve using maltose. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol reducing sugar as maltose per hour under the standard assay conditions.

Protein Determination:

Protein was quantified by the method of [12] with bovine serum albumin as standard.

Preparation of Crude Extract:

The α-amylase extracted from 3g defatted flour with 100 mM phosphate buffer pH 7·0 for 3 h. at 4°C (1:10 w:v ratio). The suspension was centrifuged for 30 min.at 10,000 rpm at 4°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 for the estimation of α-amylase and protein.

Effect of pH and temperature on α-amylase activity:

The effect of pH on the α-amylase activity was measured by assaying the activity at different pH values, using the following buffers: 50 mM sodium acetate buffer (pH 3.6 - 6.5) and 50 mM phosphate buffer (pH 7.0 - 8.5). The effect of the temperature on the α-amylase activity was determined assaying the enzyme at temperatures from 25 to 70ºC.

Partial purification of cashed α-amylase:

For the partial purification of *cashed* α-amylase enzyme. The crude extract which was extracted at pH 7.0 was to pH 5.5 using 1 M CH_3COOH in order to precipitate out proteins. Then thermal treatment was given at a temperature for 10 min. and cooled (4°C). Then thermally treated solution was centrifuged at 10,000 rpm for 20 min. Supernatant was submitted to fractionation by ammonium sulfate precipitation (80% saturation). The precipitate was recovered by centrifugation at 10,000 rpm for 30 min at 4 °C and dissolving the pellet in 100 mM phosphate buffer pH 7·0. The suspension was dialyzed against 1.0 L of the same buffer overnight at 4 °C. The dialyzed material (designated as ammonium sulphate fraction) was fractionated by ion exchange chromatography on DEAE-Cellulose column (20 × 2 cm) with 50 mM phosphate buffer pH 7·0, and then , the column was washed with two bed volumes of buffer. The bound enzymes were eluted by stepwise from the column with different concentration of NaCl (0.1M and 0.2M).

The active fractions were pooled. Activity was determined in the eluted solution. and the partially purified cashed seed extracts were stored at −20 °C until use.

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).**

Effect of substrate concentration on α-amylase activity:

To determine the optimum concentration of substrate for maximum enzyme activity, starch solution of various concentration (0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 2.5%) were used for the assay and enzyme concentration remained constant.

Polyacrylamide gel electrophoresis (PAGE)

The α-amylase activity was also detected by electrophoresis on 7% Polyacrylamide gels with 20 mM Tris-glycine (pH 8·2) buffer [13]. The protein bands

were stained with Coomassie brilliant blue. The enzyme activity in different bands were detected by incubating the gel for 1 h at 37°C in 1% starch solution in phosphate buffer (pH 7·0) and then stained with 10 mM iodine in 14 mM KI for 5 min.

Kinetic Constant:

Kinetic parameters of the amylase for starch as substrate was determined at pH 7.0 and 37ºC. The values of Michaelis constants (Km) and the maximum velocity (Vmax) were determined from Lineweaver Burk plot.

Substrate Specificity:

To determine the substrate specificity of the enzyme, starch, glycogen, dextrin, dextrose and pectin were used as substrates during the assay. Activity assayed under standard assay conditions.

Effect of various metal ions on the activity on α-amylase:

Effect of various metal ions on the activity on α-amylase was examined by incubating the enzyme solution at room temperature in the presence of 3mM and 5 mM of the respective ion for 20 minutes and at the end of incubation period, aliquots were withdrawn and assayed. In all of the above cases, the α-amylase activity is expressed as a percentage of the control enzyme activity (100%).

Results

Determination of enzyme activity:

The amylase enzyme gave 100% hydrolytic activity when treated with the substrate in absence of EDTA, but no hydrolytic property was found if the substrate solution was pre-mixed with 1×10^{-2} M , 5×10^{-2} M and 10×10^{-2} M EDTA, an inhibitor of α-amylase. This finding clearly revealed that the amylase was of α-type. Studies showed that amylase activity is present in Yemeni cashed seeds. The activity of dry extract from cashed seeds was (10432 units/g), using starch as a substrate**.**

Partial Purification of α-amylase:

The results of partial purification of α-amylase from Yemeni cashed is summarized in table 1. From the elution profile of the chromatography on DEAE–Cellulose (Figure 1), two peaks with α-amylase activity was separated: the fractions eluted with 0.1 M sodium chloride amyI and the fraction eluted with 0.2 M NaCl (amyII). The specific activity was found to be 13152.22 and 18975.25 units/mg protein with a fold purification of 4.03 and 5.82 for amyI and amyII, respectively. The characterization of amyII with highest specific activity was achieved.

Characterization of partially purified alpha amylase enzymes (amyII):

Kinetic analysis of enzyme activity at optimum pH value gave linear reciprocal Michealis-Menten (Lineweaver-Burk) plots, enabling estimation of Km and Vmax values for hydrolysis of starch were obtained (Figure 2). The apparent K_m value and Vmax value of amyII for hydrolyzing starch was 1.25 mg/0.5 ml (0.25 %) and 4.0 mM.

Table 1: Purification Scheme of α-amylase from Yemeni cashed seeds .

Sample	Total activity Units	Total protein(mg)	Specific activity (units/mg protein)	Fold purification	Recovery %
Crude extract	10432	3.2	3260	1	100
pH and heated treated	8880.4	1.6	5550.25	1.7	85.1
80% Ammonium sulphate	6831.1	0.95	7190.60	2.21	65.48
DEAE-Cellulose column: 0.1 M NaCl	2367.4	0.18	13152.22	4.03	22.69
0.2 M NaCl	4554.06	0.24	18975.25	5.82	43.65

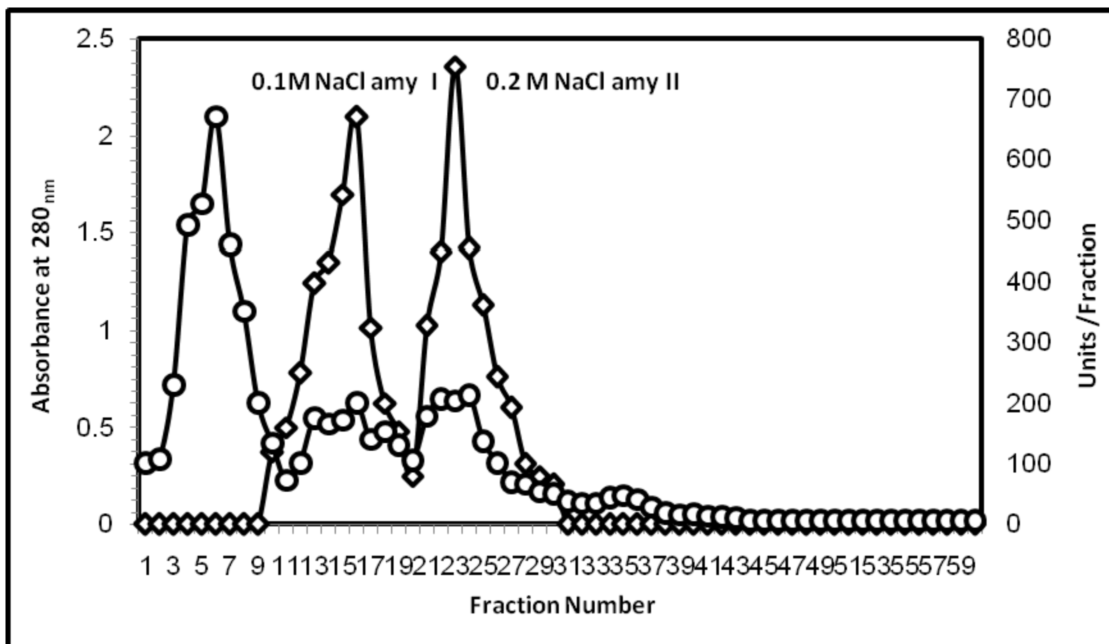

Figure 1: Ion-exchange chromatography on DEAE-Cellulose Column of the Yemeni cashed α-amylase. Absorbance at 280 $_{nm}$ **(** O) α-amylase activity (◇).

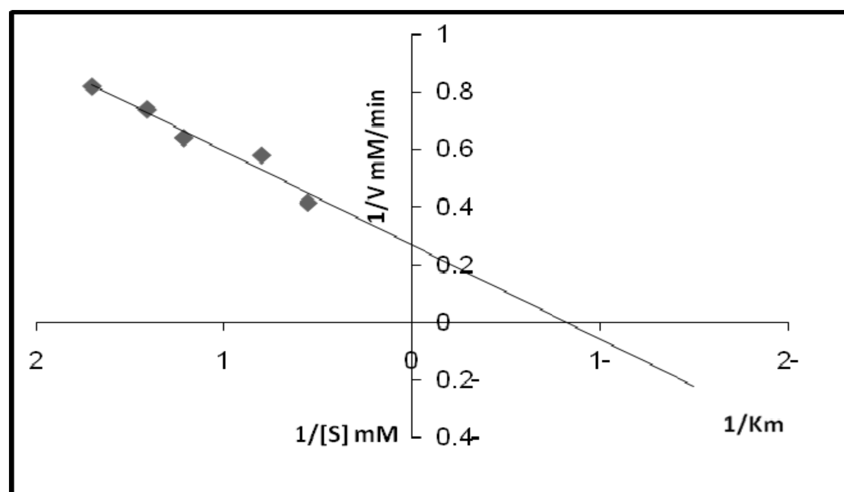

Figure. 2: Linewearver-Burk double reciprocal plot for the determination of K_m value of partially purified Yemeni cashed (amyII).

Optimum pH is required for maximum enzyme activity. α-Amylase (amyII) is an neutral enzyme. Results showed that the maximum specific activity was obtained at pH 7.0 (Figure 3). The optimum temperature was at 50°C (Figure 4) under the standard reaction conditions. The activity increased with increasing temperature to 30°-50°C, followed by a sharp decline to 80°C where complete inhibition in the activity was found. Enzyme activity was evaluated with different substrate (soluble starch) concentrations. As per the data presented in figure 5, maximum activity was 18912.90 U/mg protein with 1% starch as the substrate concentration.

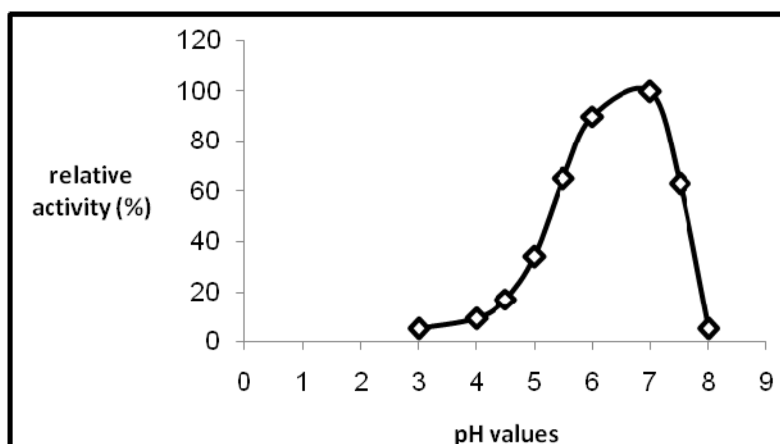

Figure 3: pH optimum of Yemeni cashed α-amylase (amyII).

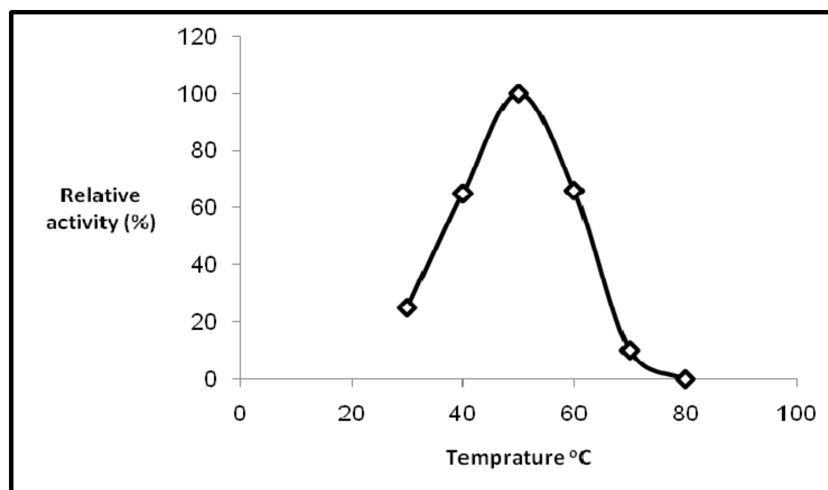

Figure 4: Temperature optimum of Yemeni cashed α-amylase (amyII).

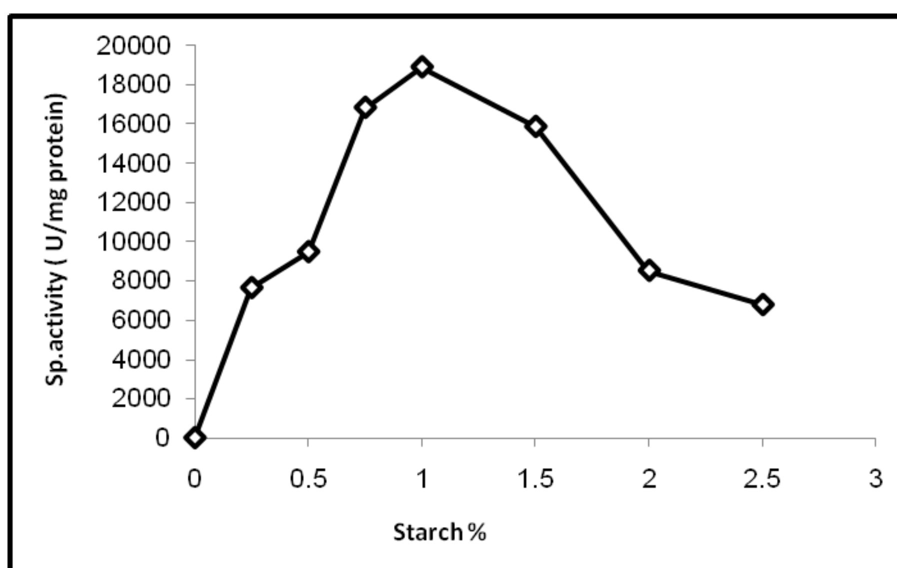

Figure 5: Effect of substrate (starch) concentration on cashed amyII activity.

Substrate specificity was measured for starch, glycogen, dextrin, dextros and pectin (Table 2). AmyII hydrolyzed starch (100 %) at a rate much lower than that of glycogen (174%), whereas the enzyme did not hydrolyze dextrin, dextrose and pectin.

Effect of metal ions such as Ca^{2+} , Zn^{2+} Ni^{2+}, Hg^{2+} and Cd^{2+} at 3 mM concentration were tested for amylase (amyII) activation/inhibition effect and the results are given in (Table 3). Ca^{2+}, Cd^{2+} and $Ni^{2+}$are found to have activating effect as indicated by increased activity. Zn^{2+} and Hg^{2+}had partial inhibition effect on activity .

Zymogram pattern in the native gel revealed that *cashed* α-amylase had 2 isoform AmyI and AmyII on activity staining (Figure 6 A), with protein bands on protein staining for 80 % saturation fraction (Figure 6 B).

Table 2: Relative activity of Yemeni cashed amyII toward different substrates

Substrate	% Relative activity
Starch	**100**
Glycogen	**174**
Dextrin	**4.5**
Dextrose	**4**
Pectin	**1**

Table 3: Effect of metal cations on Yemeni cashed α-amylase (amyII)

Metal cations	% Relative activity
Ca^{+2}	**185**
Ni^{+2}	**127**
Hg^{+2}	**85**
Cd^{+2}	**154**
Zn^{+2}	**65**

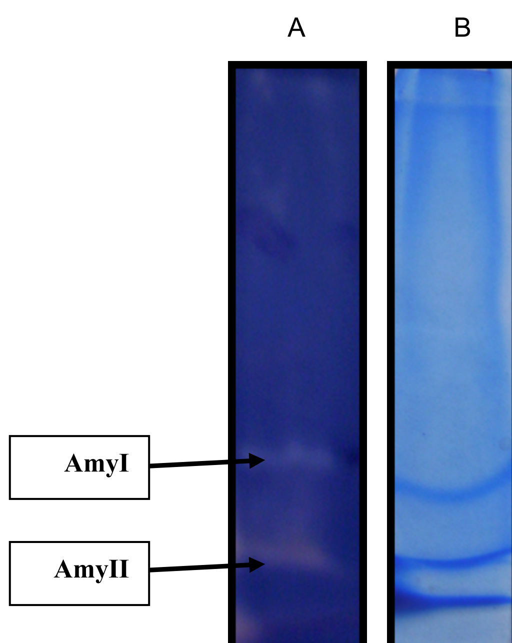

Figure 6: Electrophoretic analysis of *cashed* α-amylase

Discussion

The commercial use of α-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases [13]. The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. Several amylases have

been purified from different plant source using conventional as well as classical methods. Ion exchange chromatography is a tool for the separation of proteins on the basis of charge. DEAE–cellulose matrix has been to purify most of the cereal amylases [14, 15], whereas in few cases CM-cellulose has also been employed [16].

K_m value was reported for α- amylase from tuber *Pachyhizus erosus* (0.29% starch) [17]. The lower km value was reported for α-amylases from wheat Sakha 69 (0.57 mg and 1.33 mg starch/ml) [18] and mung beans (1.6 mg/ml) [19]. However, maize and millet α-amylases had the higher K_m values (12.5 mg and 5.8 mg/ml, respectively) [6].

Results showed that the maximum specific activity was obtained at pH 7.0. These results obtained data agree with the finding of [20] who found that the optimum of pH α-amylase for vine shoot internodes was neutral pH optima. [21] reported that the optima of different α-amylase of wheat have broad pH optima range from 5.0 to 7.0.

Acidic pH optima were reported from *Vigna angularis* L. [22], wheat Sakha 69 [18], finger millet [23], shoots and cotyledons of pea (*Pisum sativam* L.) seedlings [24] and mung beans [19]. Slightly alkaline pH optimum was detected for α-amylase from *P.* erosus tuber (pH 7.3) [17].

The activity increased sharply with gradual increase in temperature up to 50ºC while it gradually declined with further rise in temperature, indicating loss in the active conformation of the enzyme. These results agree with that reported by [25]. [26] found that the maximum activity of a thermostable purified α-amylase was observed at 50ºC from microbial source. Moreover, [27] reported that, the optimum temperature for purified α-amylase was 55ºC. The cashed amylase was about 60 % active at 60ºC. A broad temperature optima (40-50°C) were detected in wheat [28].The lower temperature optima (37°C) reported for α-amylases from rice [29] and *P. erosus* tuber [17].

Starch and glycogen are polysaccharides which are used by plant and animals for storing glucose for future use. Starch consists of amylose (α-1,4-linkage) and may be amylopectin (α-1,6-linkage), but glycogen must the two linkages. These results indicated that cashed α-amylase enzyme had highest activity toward α-1,6-linkage. For tuber α-amylase, high-molecular-mass substrates containing the α-1,4-linkage were better substrates for the enzyme. The relative rate of hydrolysis of the polymeric substrate decreased with decreasing percentage of α-1,4-linkages and increasing percentage of α-1,6-linkages in the substrate, suggesting that the enzyme prefers high molecular-mass, amylose type material as the substrate. It hydrolyzed amylose at rates similar to those obtained with soluble starch, but it was considerably less active on amylopectin and showed no effect on maltose and maltotetraose [17].

The inactivation by these metals may be due to their binding to either catalytic residues or by replacing the Ca^{2+} from the substrate binding site of the enzyme [23,30]. It is well known that plant and animal á-amylases are metalloenzymes that contain a Ca^{2+}-binding domain which is important for the stabilization of the tertiary

structure [31,32,20]. All plant α-amylases appear to contain loosely bound Ca^{2+} compared to microbial enzymes and its removal results in both irreversible as well as reversible inactivation resulting in the loss of thermal stability [33, 32]. Amylase activity of Ca^{2+} ions enhancement of is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation. In addition, calcium is known to have a role in substrate binding [34]. It has also been documented that binding of Ca^{2+} to amylase is preferred over other cations such as Mg^{2+} [35].

Native starch-polyacrylamide gel electrophoresis revealed 2 isozymes of α-amylase-Amy-1 and Amy-2. Several á-amylases have been purified and characterized from different plants by using conventional as well as classical methods. The main problem encountered in the purification of the enzymes is their occurrence in multiple forms as isoenzymes. However, this problem does not occur in dicotyledonous plants for which the number of isoforms does not exceed two in most cases [22].

However, the number of α-amylases identified in different source species varied from 1 to 8 isoforms e.g., finger millet [36] maize [37] and Wheat Variety (Balady) [21].

The result of this study ,in correlation with data on other enzymes produced by this family, suggest that α- amylase Yemeni bean *Dolichos Lablab* is one of the better species obtained of those thermostable enzyme that are useful in many industrial fields.

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References

[1] Millward, D.J., *J. Nutr.*, 2004, 134, 1588S-1596S.
[2] Davila, M.A.; Sangronis, E.; Granito, M., *Arch. Latinoam Edn., Nutr.*, 2003, 53: 348-354.
[3] Sasi, A.; Baghyaraj, R.; Yogananth, N.; Chanthuru, A, Ravikumar, M., *Res. J. Biol. Sci.* 2008, 1, 50-57.
[4] Kumar, R.S.S.; Singh, S.A.; Rao, A.G.A., *Biochimie*, 2009, 91, 548-557.
[5] Muralikrishna, G.; Nirmala M., *Carbohydr. Polym*, 2005, 60, 163-173.
[6] Adewale, I.O.; Agumanu, E.N.; Otith-Okoronkwo, F.I., *Carbohyd. Polym.*, 2006, 66, 71-74.
[7] Kruger, J.S., *Cereal Chemistry*, 1979, 56, 298-302.
[8] Khan, A.A.; Verbeek, R.; Waters, E.C.; Onckelen, H.A., *Plant Physiology*, 1973, 51: 641-645.
[9] Marchylo, B.A.; LaCroix, L.J.; Kruger, J.E., *Canadian Journal of Plant Science*, 1980, 60, 433-443.
[10] Hill, R.D.; McGregor, A.W., in: Pomeranz, Y. (Ed.), "Advances in Cereal Science and Technology", 9, AACC, St Paul, USA, 1988, pp: 217-261.
[11] Bernfeld, P., *Adv. Enzymol.*, 1951, 12: 379–428.
[12] Bradford, M.M., *Analyt. Biochem.*, 1976, 72: 248–254.
[13] Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami, V.K.; Chauhan, B., *Process Biochemistry*, 2003, 39, 1-18.
[14] Marchylo, B.; Kruger, J.E.; Irvine, G.N., *Cereal Chem.*, 1976, 53: 157–173.
[15] Warchaleswiski, J.R.; Tkachuk, R., *Cereal Chem.*, 1978, 55, 146–156.
[16] MacGregor, A.W., *Inst. Brew.*, 1977, 83, 100–103.
[17] Noman, A.S.M.; Hoque, M.A.; Sen, P.K.; Karim, M.R., *Food Chem.*, 2006, 99: 444-449.
[18] Fahmy, A.S.; Mohamed, M.A.; Mohamed, T.M.; Mohamed, S.A., *Bull. NRC, Egypt*, 2000, 25, 61-80.

[19] Tripathi, P., Leggio, L.L.; Johanna, J.; Mansfeld, R., Ulbrich-Hofmann, A.M.; Kayastha, *Phytochem*., 2007, 68, 1623-1631.
[20] Berbezy, P.; Legendre, L.; Maujean, A., *Plant Physiol and Biochem.*, 1996, 34, 353–361.
[21] Saleh A.; Abdulrahman M.; Al-Malki, L.; Taha, A., Kumosani, *Aust. J. Basic & Appl. Sci.*, 2009, 3(3), 1740-1748.
[22] Mar, S. S.; Mori, H.; Lee, J. H.; Fukuda, K.; Saburi, W., Fukuhara, A.; Okuyama, M., Chiba, S.; Kimura, A., *Biosci. Biotechnol. Biochem*. 2003, 67 (5), 1080-1093.
[23] Nirmala, M.; Muralikrishna, G., *Carbohydr. Polym.*, 2003, 54, 43–50.
[24] Beers, E.; Duke, S., *Plant Physiol.*, 1990, 92, 1154-1163.
[25] Egwin, E.C.; Oloyede, O.B., *Nig. Cereal Biokem.*, 2006, 18, 15-20.
[26] Chakraborty, K.; Bhattacharyya, B. K.; Sen, S. K., *Folia Microbiol (Praha)*, 2000, 45(3), 207-210.
[27] Khoo, S. L.; Amirul, A. A.; Kamaruzaman, M.; Nazalan, M.; Azizan, M. N., *Folia Microbiol (Praha)*, 1994, 39(5), 392 – 398.
[28] Tkachuk, R.; Kruger, J.E., *Cereal Chem*., 1974, 51, 508–529.
[29] Abe, R.; Chib A.Y.; Nakajima, T., *Biologia Bratislava*, 2002, 57, 197–202.
[30] Elarbi, M.B.; Khemiri, H., Jridi, T.; Hamida, J.B., *C.R. Biologies* (In Press).
[31] Vallee, B.L.; Stei, E.A.; Sumerwell, W.N.; Fischer, E.H., *J. Biol. Chem.*, 1959, 234, 2901-2905.
[32] Greenwood, C.T.; MacGregor, A.W., *J. Inst. Brew*., 1965, 71, 405-417.
[33] Thoma, J.A.; Sparlin, J.E.; Dygert, S.,. "Plant and animal amylases". In P. Boyer (Ed.), (Vol. 5). The enzymes, New York: Academic press. 1971, pp: 115-189.
[34] Sprinz, C., In: "Food Chemistry". Belitz, H.D.; Gross, W., Eds. Springer Verlag, Berlin, Heidelberg, New York, 1999, PP.92–151.
[35] Bush, D.S.; Sticher, L.; Hwystee, R.V.; Wegner, D.; Jones, R.L., *J. Biol. Chem*., 1989, 264, 19392–19398.
[36] Nirmala, M.; Muralikrishna, G., *Phytochem*., 2003, 62, 21-30.
[37] Donn, A.; Warner; Marilyn, J. G.; Clarence, A. K., *Cereal Chem.* 1991, 68(4), 383-390.