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Biochemical Properties of α-Amylase from Peel of *Citrus sinensis* cv. Abosora

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Abstract α-Amylase activity was screened in the peel, as waste fruit, of 13 species and cultivars of Egyptian citrus. The species *Citrus sinensis* cv. Abosora had the highest activity. α-Amylase AI from Abosora peel was purified to homogeneity using anion and cation-exchange, and gel filtration chromatographies. Molecular weight of α-amylase AI was found to be 42 kDa. The hydrolysis properties of α-amylase AI toward different substrates indicated that corn starch is the best substrate. The α-amylase had the highest activity toward glycogen compared with amylopectin and dextrin. Potato starch had low affinity toward α-amylase AI but it did not hydrolyze β-cyclodextrin and dextran. Apparent Km for α-amylase AI was 5 mg (0.5%) starch/ml. α-Amylase AI showed optimum activity at pH 5.6 and 40 °C. The enzyme was thermally stable up to 40 °C and inactivated at 70 °C. The effect of mono and divalent metal ions were tested for the α-amylase AI. Ba^{2+} was found to have activating effect, where as Li^{+} had negligible effect on activity. The other metals caused inhibition effect. Activity of the α-amylase AI was increased one and half in the presence of 4 mM Ca^{2+} and was found to be partially inactivated at 10 mM Ca^{2+}. The reduction of starch viscosity indicated that the enzyme is endoamylase. The results suggested that, in addition to citrus peel is a rich source of pectins and flavanoids, α-amylase AI from orange peel could be involved in the development and ripening of citrus fruit and may be used for juice processing.

Keywords α-Amylase · *Citrus sinensis* cv. Abosora · Peel · Purification · Properties

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Introduction

Amylases (EC 3.2.1.1) are enzymes capable of hydrolyzing starch. They are produced by animals, plants, and microorganisms [1]. They can specifically cleave the *O*-glycosidic bonds in starch, a storage polysaccharides present in seeds, tubers of various plants. Starch composed of α-1, 4-linked α-D-glucose units formed by amylose and amylopectin. These two starch components differ from each other in the degree of branching. Amylose is a mainly linear polysaccharide whereas the structure of amylopectin is highly α-1, 6-branched [2]. Amylases can be classified into endoamylases, (α-amylases), exoamylases (β-amylases, glucoamylase), and debranching enzymes (pullulanses) based on their mode of action [3]. Amylases from different sources have been studied in great depth. For example, in germinating cereal grains, α-amylases are the most abundant starch-degrading enzymes. The enzymes are secreted by aleurone cells into the starchy endosperm where they degrade the starch grains [4]. Starch degradation by amylases is the basis for several industrial applications such as the preparation of glucose syrup, bread making, warp sizing of textile fibers, the clarification of haze formed in beer or in fruit juice, and also in the field of detergents [1, 3].

Peels are the major by-products obtained during the processing of various fruits and these were shown to be a good source of polyphenols, carotenoids, and other bioactive compounds which possess various beneficial effects on human health [5–7]. Citrus peel is a rich source of pectins and flavanoids such as hesperidins, eriocitrin, and nobiletin [8–11]. Very few reports are available with regard to enzymes in citrus peel, where Mohamed et al. [12] reported the presence of peroxidase in citrus peel. Saby John et al. [13] also reported the presence of peroxidase in mango peel.

Citrus fruit is one of the commercial crops in the Egyptian market. Peels are available in large quantities as a by-product in citrus-processing industry. Most knowledge about starch degradation by amylases is based on seeds and leaves currently used as models. However, information about the citrus amylase is scarce. Our first approach was to focus on the investigation of peel α-amylase in some Egyptian citrus species and cultivars. We have found that the peel of *Citrus sinensis* cv. Abosora had the highest α-amylase activity. The enzyme has been purified and the biochemical properties has been investigated.

Materials and Methods

Plant Materials

All of the studied 13 different citrus species and cultivars were obtained from the Citrus Department, Horticulture Institute research, Agriculture Centre, Cairo, Egypt.

Amylase Assay

Amylase activity was assayed by the dinitrosalicylate (DNS) method of Miller [14]. Three sets of reaction mixtures (blank, control, and sample), prepared for the assay of amylase activity, contained 1 ml 1% starch solution in 50 mM sodium acetate buffer, pH 5.6, and a suitable amount of enzyme. Assays were carried out at 40 °C for 1 h. Then 0.5 ml of DNS reagent was added to each tube and it was vortexed. The tubes were heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of maltose per minute under standard assay conditions.

Purification of α- Amylase from *Citrus sinensis* cv. Abosora

All operations were performed at 4–8 °C unless otherwise specified. Twenty grams of freshly chopped orange peel were blended in 0.05 M sodium acetate buffer, pH 5.6 containing 1 M sodium chloride. The extract was filtered, dialyzed against 20 mM Tris-HCl buffer, pH 7.2, and centrifuged at 12,000×*g* for 15 min, where the supernatant was designated as the crude extract. The crude extract was loaded on a TEAE-cellulose column (4×1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h, and collected in 3-ml fractions. The column was thoroughly washed with the equilibrated buffer until the UV absorbance of the column eluate returned to the base line. The bound protein was eluted with a stepwise gradient of NaCl ranged from 0.0 to 0.4 M in the same buffer. Fractions exhibiting α-amylase activity were eluted at 0.0, 0.1, 0.2, and 0.3 M NaCl, where most of the active fractions eluted at 0.0 M NaCl. The pooled unbound fractions (0.0 M NaCl) was dialyzed against 20 mM sodium acetate buffer, pH 5.6. The dialysate was loaded on a carboxymethyl (CM)-cellulose column (6×0.8 cm i.d.) previously equilibrated with 20 mM sodium acetate buffer, pH 5.6 at a flow rate of 30 ml/h, and collected in 3-ml fractions. The column was washed with the same buffer until the absorbance of the column eluate returned to the base line. The bound protein was eluted with a stepwise gradient of NaCl ranged from 0.0 to 0.4 M in the same buffer. Fractions exhibiting α-amylase were eluted at 0.0, 0.1, and 0.2 M NaCl and designated as α-amylases AI, AII, and AIII, respectively. α-Amylase AI with the highest activity was concentrated through lypholization and reconstituted at the least volume of the same buffer and loaded on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer, pH 5.6, and collected in 3-ml fractions at a flow rate of 20 ml/h. The α-amylase AI was eluted with the same buffer.

Protein Determination

Protein was estimated by the method of Bradford [15] using bovine serum albumin as the standard.

Molecular Weight Determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90×1.6 cm i.d.) was calibrated with cytochrome c (14,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000), and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (v_0). Subunit molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to Laemmeli [16] method. SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,200) were used for the calibration curve.

pH Optimum

Amyalse activity was determined at various pH using different buffers, sodium citrate (pH 3.6–5.6), sodium acetate (pH 4.0–5.6), sodium phosphate (pH 6.0–8.0), and Tris-HCl (7.2–9.0) at 50 mM concentration. The maximum activity was taken as 100%, and percent relative activity were plotted against different pH values.

Temperature Optimum

Amylase activity was determined at a temperature range of 10–80 °C (with an interval of 10 °C). The maximum activity was taken as 100%, and percent relative activity were plotted against different temperatures.

Thermal Stability

The enzyme was incubated at a temperature range of 10–80 °C for 15 min prior to substrate addition. The percent relative activity was plotted against different temperatures.

Effect of Metal Ions

The enzyme was incubated with 2 mM solution of salts of metal ions (chlorides of Ba^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and Li^{+}) for 15 min prior to substrate addition. The enzyme activity without metal ions was taken as 100%, and percent relative activity was determined in the presence of metal ions.

Effect of $CaCl_2$

The enzyme was incubated with different concentrations of $CaCl_2$ (0.5–10 mM) at 40 °C for 1 h, and the enzyme activity in the presence of $CaCl_2$ was plotted as percent relative activity against different $CaCl_2$ concentrations.

Determination of Change in Specific Viscosity (η)

Viscometric assays were done in an Ostwald viscometer containing of reaction mixture (9 ml of 1% starch in 0.05 M sodium acetate buffer, pH 5.6 and 1 unit of enzyme). Measurements were made at room temperature in a glass tube viscometer. Loss in viscosity was determined at 30 min intervals for 180 min.

Results and Discussion

In most plants, α-amylase can be found in pollens, seeds, stems, roots, and other tissues [17], where very little information has been reported about the presence of α-amylase in any peel of fruits. In the present study, α-amylase activity was screened in the peel of 13 species and cultivars of Egyptian citrus (Table 1). The activities of the α-amylase were detected in all the examined species and cultivars and ranged from 10.3 to 163 units/g peel, and their specific activities ranged from 12.5 to 956 units/mg protein. These results suggested that citrus peel α-amylase could be involved in the development and ripening of citrus fruit. α-Amylase is an enzyme of cell wall-degrading enzymes which are responsible for the development and ripening of apricot [18]. Peroni et al. [19] reported that amylases are involved in starch degradation during mango ripening. α-Amylase from citrus peel could be also used for increasing the quantity and quality of citrus juice, where α-amylases are commercially used in juice processing [3]. The peel of *Citrus sinensis* cv. Abosora had the highest activity (163 units/g peel) and specific activity (956 units/mg protein) among the examined species.

Table 1 Screening of orange peel α-amylase from Egyptian citrus species and cultivars.

Citrus species	Units/g peel	(mg protein/g peel)	Specific activity (units/mg protein)
Citrus sinensis			
cv. Abosora	163	0.17	956
cv. Khalely Ahmer	57	0.422	135
cv. Balady Fakir	20	0.296	68
cv. Sifi	91.4	0.588	155
cv. Balady Aswani	68	0.308	221
cv. Banati Balady	108	0.451	239
cv. Balady Daem Elhaml	38.5	0.807	47.7
Citrus jumbhiri (Adalia)	10.3	0.820	12.5
Citrus reticulata			
cv. Balady Cleopatra	45.7	0.472	96.8
cv. Balady	48.9	0.222	220
Citrus mites	161	0.645	249
Citrus paradise	78.6	0.427	184
Citrus aurantium	10.8	0.403	26.8

The peel of *Citrus sinensis* cv. Abosora has been taken to be a suitable source for the purification and characterization of α-amylase. The overall scheme employed in the purification of *Citrus sinensis* α-amylase was summarized in Table 2. Chromatography of the crude enzyme on TEAE-cellulose gave one peak of activity before addition of NaCl and three active peaks at 0.1, 0.2, and 0.3 M of NaCl, respectively (Fig. 1). The unbound peak (0.0 M NaCl) had the highest activity of α-amylase and was applied on a CM-cellulose column (Fig. 2). The profile of the column revealed one active peak at 0.0 M NaCl (unbound α-amylase AI) and two active peaks (bound α-amylases AII and AIII) at 0.1 and

Table 2 Purification scheme of α-amylase from *C. sinensis* cv. Abosora peel.

Purification step	Total protein (mg)	Total activity (unit)[a]	Specific activity (units/mg protein)	Fold Purification	Recovery (%)
Crude extract	27.5	26,286	956	1	100
Chromatography on TEAE-Cellulose					
0.0 M NaCl	9.95	19,688	1,979	2.07	74.9
0.1 M NaCl	3.93	663	168.7	0.176	2.5
0.2 M NaCl	3.69	93.5	25.3	0.026	0.35
0.3 M NaCl	4.56	56.7	12.4	0.013	0.21
Chromatography on CM-Cellulose					
0.0 M NaCl (AI)	4.11	14,430	3,511	3.67	54.9
0.1 M NaCl (A II)	1.694	652	385	0.4	2.48
0.2 M NaCl (AIII)	2.21	442	200	0.21	1.68
Chromatography on Sephacryl S-200					
AI	1.68	13,416	7,986	8.4	51

[a] One unit of α-amylase activity was defined as the amount of enzyme that liberated 1 μmol of maltose/min

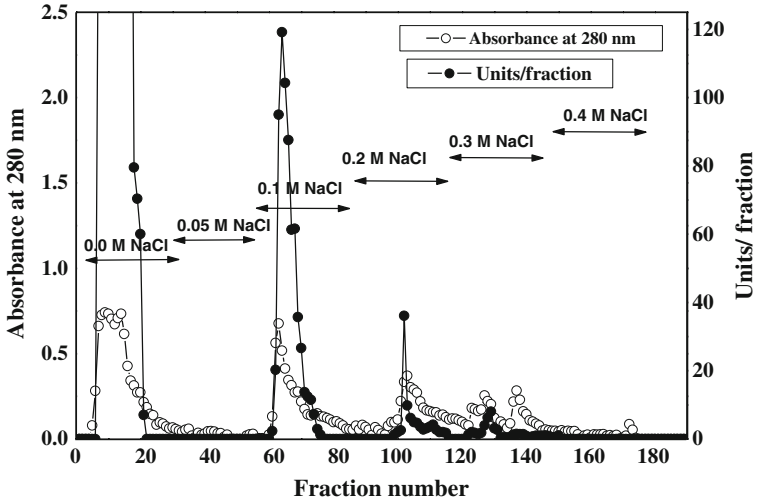

Fig. 1 A typical elution profile for the chromatography of *C. sinensis* cv. Abosora peel α-amylase on TEAE-cellulose column (4×1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions

0.2 M NaCl, respectively. The unbound α-amylase AI had the highest activity and was applied on a Sephacryl S-200 column (Fig. 3). This final step provided about 8.4 fold purification of the enzyme over the crude extract. The purified enzyme was homogenous on SDS-PAGE giving a single protein band (Fig. 4). Generally, in plant by ionexchange and gel filtration chromatographies, a number of isoenzymes of α-amylases were separated such as three α-amylases from malted finger millet were purified using DEAE-Sephacyl and Sephacryl S-200 columns [19] and Noman et al. [20] used two ion exchange columns (DEAE- and CM-cellulose) to purified *Pachyrhizus* erosus L. tuber α-amylase.

By using Sephacryl S-200 column, the native molecular weight of orange peel α-amylase AI was found to be 42 kDa. However, SDS-PAGE of the enzyme revealed a single protein band, suggesting that the enzyme consisted of a single polypeptide chain. Reference to the relative mobility of the molecular weight marker proteins, run in parallel with this enzyme on SDS-PAGE (Fig. 4), gave also a molecular weight of 42 kDa for the

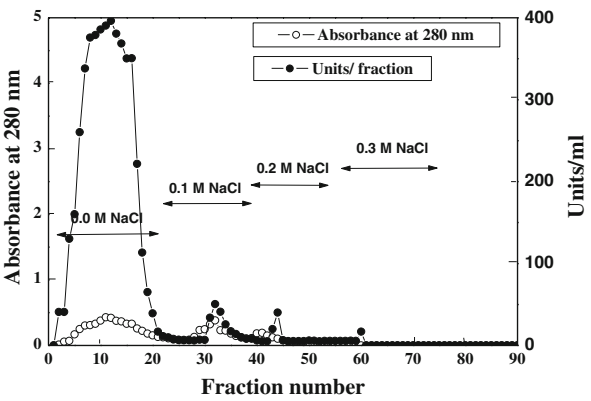

Fig. 2 A typical elution profile for the chromatography of *C. sinensis* cv. Abosora peel α-amylase TEAE-cellulose fraction 0.0 M NaCl on CM-cellulose column (6×0.8 cm i.d.) previously equilibrated with 20 mM acetate buffer pH 5.6 at a flow rate of 30 ml/h and 3 ml fractions

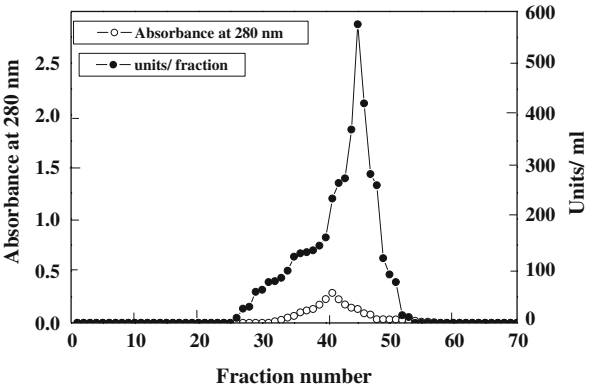

Fig. 3 Atypical elution profile for the chromatography of *C. sinensis* cv. Abosora peel α-amylase AI CM-cellulose fraction on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer, pH 5.6 mM at a flow rate of 20 ml/h and 3 ml fractions

enzyme. The molecular weight of α-amylase AI is in the same range of 38–45 kDa as previously reported for α-amylases from other sources [20–22].

The hydrolysis properties of α-amylase AI toward different substrates were investigated as shown in Table 3. The activity for corn starch was taken as 100% activity. The α-amylase had the highest activity toward glycogen (Glu α 1,4Glu-Glu 1, 6 Glu) compared with amylopectin (Glu α 1,4Glu-Glu 1, 6 Glu) and dextrin (Glu α 1, 4 Glu). Potato starch had low affinity toward α-amylase AI, but it did not hydrolyze β-cyclodextrin and dextran. The differences observed in the hydrolysis of substrates from different sources by α-amylase AI could be attributed to different chemical characteristics of these substrates, e.g., variations in the amylose/amylopectin ratio, organizations of glucose within the cluster structures of glycogen, amylopectin and dextrin, chain length distribution of starch, and degree of branching. These

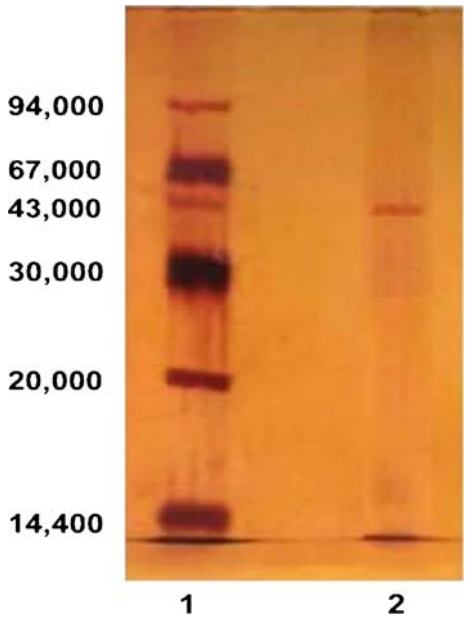

Fig. 4 SDS-PAGE for homogeneity and molecular weight determination of *C. sinensis* cv. Abosora peel α-amylase AI. *1* Protein markers. *2* Sephacryl S-200 α-amylase AI

Table 3 Relative activities of *C. sinensis* cv. Abosora peel α-amylase AI toward substrates.

Substrate	Relative activity (%)
Starch (corn)	100
Glycogen	81
Amylopectin	73
Dextrin	67
Starch (potato)	12
β-Cyclodextrin	0.0
Dextran	0.0

findings tend to suggest that high-molecular mass substrates containing the α-1,6 linkage were the better substrate for the enzyme except potato soluble starch. These results are similar to substrates specificity for *Pachyrhizus erosus* L. tuber α-amylase reported by Noman et al. [20]. Nirmala and Muralikrishna [23] reported that the three α-amylases from malted finger millet had different affinity toward different sources of cereal starches.

Apparent Km for α-amylase AI, determined from Lineweaver-Burk plot, was 5 mg (0.5%) starch/ml (Fig. 5). This Km value was near to the Km values reported for α-amylases from ragi *Eleusine coracana* (0.59%–1.43% starch) [23] and tuber *Pachyhizus erosus* (0.29% starch) [20]. The low Km value was reported for α-amylase from wheat Sakha 69 (0.57 mg and 1.33 mg starch/ml) [24].

α-Amylases are generally stable in the pH range from 5.5–8.0 but exceptions exist on both sides, mainly in the enzymes of microbial origin [1]. In this investigation, the effect of the pH on the *Citrus sinensis* α-amylase AI activity was analyzed by carrying out assays at different pHs, using four different buffers (sodium citrate, sodium acetate, sodium phosphate, and Tris-HCl) ranging from 3.5 to 9.0. α-Amylase AI showed optimum activity at pH 5.6 in sodium acetate (Fig. 6). Similar optimum pH was observed for α-amylases from malted finger millet [25] and from shoots and cotyledons of pea (*Pisum sativium L.*) seedlings [21]. Noman et al. [20] reported the optimum pH 7.0 for α-amylase from *Pachyrhizus erosus* L. tuber.

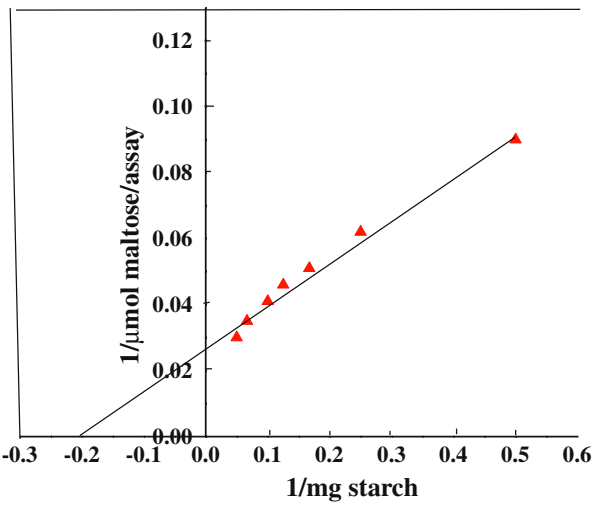

Fig. 5 Lineweaver-Burk plot relating *C. sinensis* cv. Abosora peel α-amylase AI reaction velocities to starch as substarte concentration. The reaction mixture contained in 1 ml, 50 mM sodium acetate buffer pH 5.6, suitable amount of enzyme and concentrations of starch ranging from 2 to 20 mg. Each point represents the average of two experiments

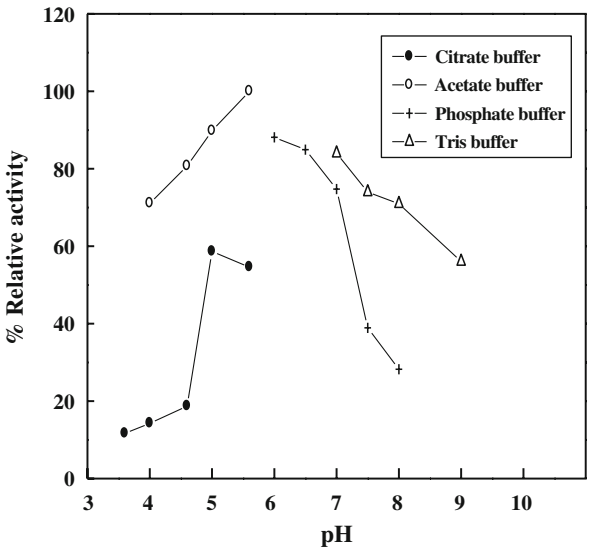

Fig. 6 pH optimum of *C. sinensis* cv. Abosora peel α-amylase AI. The reaction mixture contained in 1 ml 1% starch, suitable amount of enzyme, and 50 mM sodium citrate buffer (pH 3.6–5.6), sodium acetate buffer (pH 4.0–5.6), sodium phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (7.2–9.0). Each point represents the average of two experiments

The α-amylase AI maintained about 50% activity over a temperature range from 10–50 °C with the optimum at 40 °C (Fig. 7). The activity increased sharply with gradual increase in temperature up to 40 °C while it gradually declined with further rise in temperature, indicating loss of the conformational arrangement of the active site. The enzyme was lost 80% of its activity at 60 °C. These results are similar to those previously reported for α-amylases from *Pachyrizus erosus* L. tuber [20] and S. Oryzae [26]. The α-amylase AI was thermal stable up to 40 °C and inactivated at 70 °C (Fig. 8). Nirmala and Muralikrishna [25] reported also that three α-amylases from malted finger millet inactivated around 70 °C.

The effect of mono and divalent metal ions at 2 mM concentration were tested for the α-amylase AI and the results are given in Table 4. Ba^{2+} was found to have activating effect

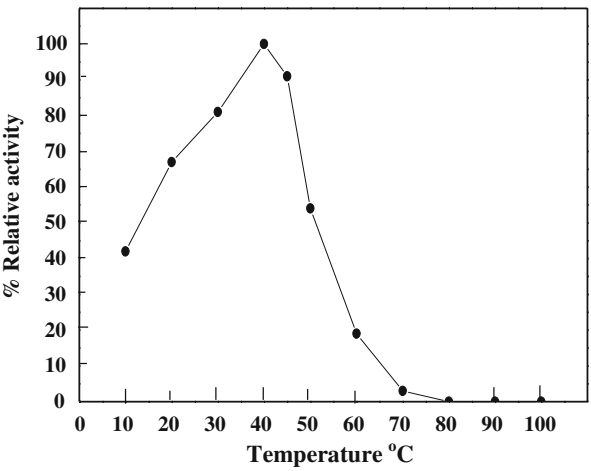

Fig. 7 Optimum temperature of *C. sinensis* cv. Abosora peel α-amylase AI. The enzyme activity was measured using the standard assay method as previously described. Each point represents the average of two experiments

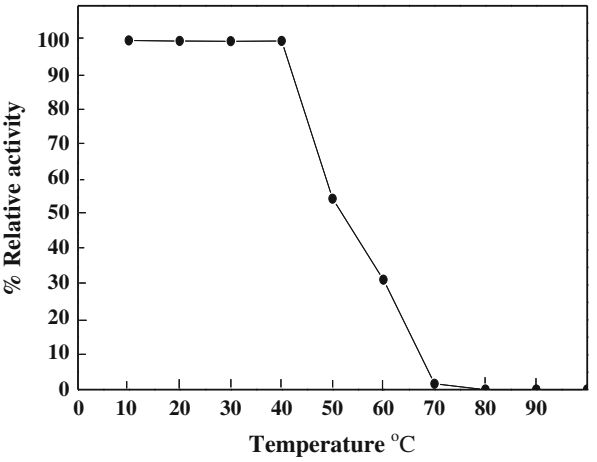

Fig. 8 Effect of temperature on the thermal stability of *C. sinensis* cv. Abosora peel α-amylase AI. The reaction mixture contained in 1 ml, 1% starch, 50 mM sodium acetate buffer pH 5.6, and suitable amount of enzyme. The reaction mixture was preincubated at various temperatures for 15 min prior to substrate addition, followed by cooling in an ice bath. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments

as indicated by increase a 35% activity, whereas Li^{+} had negligible effect on activity. However, the inactivating effect of Mg^{+2} and Ni^{+2} was found to be partial, where Mn^{+2} caused strong inhibition for the enzyme. The addition of Zn^{+2}, a known inhibitor of plant α-amylase [27], to α-amylase AI resulted in a 95% loss of activity and this strong inhibition by Zn^{2+} clearly revealed that the enzyme is α-type. Shaw and Ou-Lee [17] reported that α-amylase from germinated rice seeds was strongly inhibited by Cu^{2+} as seen in our investigation. Only Hg^{+2} completely abolished the α-amylase AI activity. Nirmala and Muralikrishna [23] reported Hg^{2+} completely inactivated the three amylases of malted finger millet. The inhibitory effects of the divalent cations might be due to the competitions for calcium-binding sites on the α-amylases while monovalent cations and Mg^{+2} might poor competitors for calcium binding [17]. The inhibition by tested 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.

Activity of the α-amylase AI was increased one and half in the presence of 4 mM Ca^{2+} and was found to be partially inactivated at 10 mM Ca^{2+} (Fig. 9). This result suggesting that calcium is required for the optimum activity and stability of enzyme [20]. Calcium is known to have a role substrate binding [28]. It has also been documented that binding of Ca^{+2} to amylase is preferred over other cations such as Mg^{2+} [29]. Cereal α-amylases are

Table 4 Effect of metal ions on *C. sinensis* cv. Abosora peel α-amylase AI.

Metal	Relative activity (%)
None	100
Ba^{+2}	135
Li^{+}	94
Mg^{+2}	82
Ni^{+2}	73
Mn^{+2}	12
Cu^{+2}	7
Zn^{+2}	5
Hg^{+3}	0.0

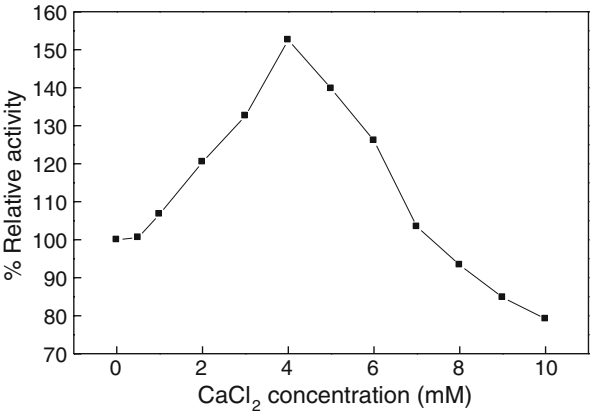

Fig. 9 Effect of different concentration of calcium ion on *Citrus sinensis* cv. Abosora peel α-amylase AI

known to be metalloenzymes containing at least one Ca^{2+} per molecule [1], and its number may go to up to ten [30]. All the plant α-amylases appear to have loosely bound Ca^{2+} compared to microbial enzymes and its removal results in both irreversible as well as reversible inactivation leading to the loss of thermal stability [31].

Starch-degrading enzymes can be classified into two main groups based on their mode of action: (1) endoamylases and (2) exoamylases. Endoamylases, also known as "liquefying" enzymes and are termed as α-amylases, which cleave α-1,4 glycosidic bonds in amylose, amylopectin, and related polysaccharides such as glycogen. As the name suggests, endoamylases hydrolyze the bonds located in the inner regions of the substrate resulting in rapid decrease of the viscosity of the starch solution as well as decrease in iodine staining power [32]. Exoamylases, also known as "saccharifying" enzymes and are termed as β-amylases and glucoamylases, which cleave α-1,4 glucosidic bonds in amylose, amylopectin, and glycogen from the non-reducing end by successive removal of maltose/glucose in a stepwise manner. In this study, the action mode of the enzyme (exo or endo) was determined by using viscometric assay. The viscosity of starch was decrease with incubation time indicated that α-amylase AI is endoamylase (Fig. 10).

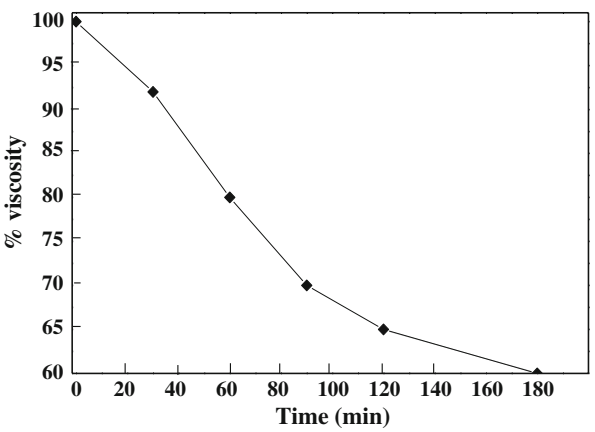

Fig. 10 Changes in viscosity of starch by *C. sinensis* cv. Abosora peel α-amylase AI

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