http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences, 4 (1): 98-102, 2001

Purification, Characterization and Effect of Physico-chemical Agents on the Stability of Amylase from Mango-pulp

M. Mahbubar Rahman[1] and Nurul Absar
BCSIR, Laboratories, Rajshahi and Department of Bio-chemistry
Rajshahi University, Rajshahi, Bangladesh

Abstract: The α-amylase from mango-pulp of Himsagar variety was purified by successive ion-exchange chromatography on DEAE-cellulose followed by CM-cellulose. The purified enzyme was found to be homogenous as judged by polyacrylamide gel electrophoresis. The molecular weight of a -amylase was estimated to be 54,500 by gel filtration and 55,200 by SDS-PAGE respectively. The enzyme showed the following characteristics : Neutral sugar content 4.8%, optimum pH 6.8, optimum temperature 37°C and Km value 0.33% against starch as substrate and absorption maximum at 279nm in aqueous solution. The activity of the enzyme was increased in the presence of metallic salts such as Ca^{2+}, Mg^{2+} and Mn^{2+} while Fe^{2+}, Zn^{2+} and Cu^{2+} inhibited the activities moderately. The effect of urea, guanidine-HCl and acetic acid on the activities of α-amylase were discussed.

Key words: Mango, α-amylase, MW, characteristics, stability

Introduction

Starch is the principal storage carbohydrate in plant cells and mango-pulp contains high percentage of starch (Gangwar and Tripathi, 1973). Amylase an important industrial enzyme is used in starch industry (Liquefaction of starch for production of glucose, fructose and maltose), baked goods, brewing, paper, textiles, detergent and sugar industries (Crueger and Crueger, 1990).

The hydrolysis of starch during fruit ripening by hydrolytic enzymes has been reported for pear (Pech and Latche, 1972), mango (Mattoo and Modi, 1969a). avocado (Pesis *et al*., 1978) and tomato (Davies and Cocking, 1967).

Amylases have been purified from many sources such as organisms (Takahashi *et al*., 1981), poplar leaves (Witt and Sauter, 1996) and Pears fruit (Pech *et al*., 1973). Amylase in the mango-pulp plays an important role during the development of mango (Tandon and Kalra, 1983). The activity of amylase in mango-pulp was found to be changed during development (Rahman *et al*., 1997; Mattoo and Modi, 1969b). This paper describes the purification and some characteristics of mango-pulp amylase. Further, effect of physico-chemical agents on the activity of amylase was performed to obtain information about stability of the enzyme.

Materials and Methods

Himsagar variety of mango was collected from a selected tree at the Horticultural Research Station of Rajshahi. DEAf-cellulose, CM-cellulose and SDS were the products of Sigma chemical Co. USA. Acetic acid and urea were the products of British Drug house (BDHJ. poole, England. Guanidine-HCl was the product of Bio-Rad laboratories, Richmond California, USA. All other regents used in the study were of analytical grade. Unless specified all the experimental conditions are maintained at 4°C.

Preparation of crude enzyme extract: The mango pulp (200 gm) was grinded with cold 0.05 M phosphate buffer, pH 7.4 in a mortar with pestle and finally crushed into paste using a homogenizer. The filtrate was collected by filtration through double layer of muslin cloth and finally centrifuged at 8,000 g for 15 min 4°C. The clear filtrate was concentrated to about 1 /8th of the original volume by commercial sucrose at 4°C. The concentrate was dialyzed against 50 mM phosphate buffer, pH 7.4 for over night and clarified again by centrifugation at 4°C. The clear supernatant obtained was used as crude enzyme extract.

Purification of amlyase: The crude enzyme extract was loaded onto the DEAE-cellulose column chromatography which was previously equilibrated with 50 mM phosphate buffer, pH 7.4 at 4°C. The protein was eluted from the column with the same buffer by stepwise elution containing increasing concentration of NaCI. Absorbance of each fraction at 280 nm, protein concentration and amylase activity of each fraction were measured.

CM-cellulose chromatography: The amylase contammg fraction obtained from DEAE-cellulose was applied to CM-cellulose column chromatography after dialysis against 20mM phosphate buffer, pH 6.5. The protein was eluted from the column with the same buffer by step wise elution containing different concentration of NaCI. Absorbance at 280 nm, protein concentration and amylase activity of each fraction were measured.

Polyacrylamide disc gel electrophoresis: The purity of the enzyme containing fraction was detected by polyacrylamide disc gel electrophoresis at room temp on 7.5% gel, pH 8.3 as described by Ornstein (1964).

Protein concentration: The concentration of protein determined following the method of Lowry *et al*. (1951) using BSA as standard.

Assay of amylase activity: Amylase activity was assayed by the method described in Laboratory manual in Biochemistry (Jayaraman, 1981) using starch as substrate. The activity was measured by estimating the release of maltose calculated from the standard curve prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1 mg of maltose in 15 minutes at 37°C. Specific activity of the enzyme is expressed as the enzyme unit per mg of protein.

Determination of molecular weight: The molecular weight of amylase was determined by Gel filtration on Sephadex G-150 and 80S-polyacrylamide gel electrophoresis as described by Andrews (1965) and Weber and Osborn (1969) respectively. The molecular weight of the enzyme was also determined by SDS-PAGE after reducing the amylase with -mercaptoethanol in the presence of 0.1% SDS.

Ultraviolet absorption: The ultraviolet absorption spectrum of the enzyme was recorded in aqueous solution with a Shimadzu Model UV-180 Double Beam Spectrophotometer at room temperature.

Determination of Km value: Michaelis constant (Km) of the purified amylase was determined by Lineweaver-Burk's double reciprocal plot. The initial velocity was equal to the amount of product formed per unit time. The initial velocity (V_i) is determined by measuring the amount of one theproduct (maltose) at various time intervals (Robyt and White, 1990).

Estimation of total sugar: Total sugar was estimated by Phenol sulphuric acid method (DuBois *et al.*, 1956).

Determination of pH optimum and pH stability: The activity of the amylase at different pH values (2-10) was measured following the procedure as described above. Starch solution was prepared in the respective buffer of different pH and was used as substrate. Stability of the enzyme at various pH values were examined by incubating the reaction mixture in the buffer for 15 minutes and then adjusted to pH 6.7. The activities were assayed. The activity determination of optimum temperature and thermostability of the amylase solution at pH 6.7 was incubated at various temperature for 1 5 minutes. After cooling the heated enzyme solution in running tap water, the residual activity was assayed.

Treatment with urea, guanidine-HCl, acetic acid and various salts: To the amylase solution of 0.5 ml (0.25-0.30 mg/ml) were added urea, guanidine-HCl, acetic acid and various salts at different concentrations and incubated for 10 minutes at 20°C. The mixtures ware again incubated with the substrate for 15 minutes at 37°C and the enzyme activity was assayed.

Results

Figure 1 shows the ion exchange chromatography on DEAE- cellulose of crude enzyme extract prepared from mango- pulp. It was found that the components were separated in to 2 major fractions (F-1, F-3) and 3 minor fractions (F-3, F-4 and F-5). Of these fractions, fraction F-1 was eluted by the buffer only while the other fractions were eluted from the column with the buffer containing different concentrations of NaCl. Further, only F-1 fraction contained the amylase activity. This enzyme active fractions as indicated by solid line was pooled, dialyzed against 20 mM phosphate buffer, pH 6.5 for overnight at 4°C and purified further by ion-exchange chromatography on eM-cellulose. As shown in Fig. 2, the components of fraction F-1 were absorbed completely on the CM-cellulose column and eluted from the column as one sharp major peak (F-1 b) which was eluted by the buffer containing 0.1 M NaCl and the two other minor broad peaks, F-1 a and F-1 c which were also eluted by same buffer containing 0.05 M and 0.15 M NaCl respectively. It was found that only the fraction F-1b contained amylase activity. The area, as indicated by solid bar was pooled, dialyzed against the same buffer for over night at 4°C and rechromatographed on eM-cellulose column under identical conditions. The fraction was eluted only as a sharp single peak and its purity was checked by polyacrylamide disc gel electrophoresis. The fraction might be contained pure amylase enzyme as it gave single band on polyacrylamide gel (Fig. 3).

Table 1 summarizes the data pertaining to the purification of mango-pulp amylase. As presented in table, the specific activity of amylase was increased at each purification step. It might be significant that although the yield of amylase was found to be about 16% but the enzyme was purified with an increase in purification fold of about 40 fold.

Table 1: Summary of data on the course of purification of amylase

Fraction	Total protein (mg)	Total activity (units)	specific activity (units/mg	Yield (%) (fold)	Purification
Crude extract	238.00	284.5	1.19	100	1
DEAE-cellulose ion exchange chromatography	3.2	66.76	20.86	23.46	17.52
CM-cellulose ion exchange chromatography	1.06	48.6	45.84	17.08	38.72
Rechromatograph on CM-celtulose	0.98	46.75	47.70	16.43	40.08

The purified 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 3×10^{-2} M EDT A, an inhibitor of α-amylase (Garcia and Lajolo, 1988). Further the hydrolytic activity of the purified enzyme was found to be unchanged in the presence of 2×10^{-2} M $HgCl_2$ (SH-dependent, β-amylase inhibitor). These finding clearly revealed that the purified amylase was of α-type.

The molecular weight of the α-amylase was determined by comparing their elution volume on Sephadex G-150, with those of the marker proteins, Tryspin inhibitor (MW-12028). Carbonic anhydrase (MW-29000). Brovin serum albumin (MW-66,000), β-Galactosidase (MW-116,000) and Catalase (MW-2,20,000) under identical experimental condition. The molecular weight was estimated to be 54,500. The molecular weight of α-amylase, purified from mango-pulp was also determined by 50S-polyacrylamide gel electrophoresis using the same maker proteins as those used for gel filtration under reducing and non-reducing conditions. The molecular weight of the enzyme was found to be unchanged under both the conditions indicating that the enzyme contained only one subunit and calculated to be about 55,200.

The purified α-amylase in aqueous solution gave absorption maximum around 279 nm and minimum around 242 nm respectively.

The activity of α-amylase was analysed using different concentrations of starch as substrate and the extrapolated Km value was estimated to be 0.33% (Fig. 4-6).

As given in Table 2, the α-amylase gave about 100% activity when soluble starch, amylose and amylopectin were used as substrates. On the other hand, no hydrolytic activity was observed when the enzyme was incubated with maltose and maltortriose.

Table 2: Substrate specificity of the purified α-amylase from mango pulp

Substrate	Relative activity%
Soluble starch	100.0
Amylopectin	94.8
Amylose	88.5
Maltose	0.0
Maltotriose	0.0
Starch grains {potato)	5.2
Starch grain {corn)	15.7

The purified α-amylase is glycoprotein in nature as they gave pinkish red colour with phenol-sulphuric acid and the amount of neutral sugar present in the enzyme was calculated to be 4.8%.

Table 3: Effect of calcium, urea, guanidine-HCl and acetic acid on the activities of a-amylase

Concentration of $CaCl_2$ (M)	0.00	0.001	0.002	0.005	0.01	0.05	0.10	0.20	0.30	0.50
Relative activities (%) of α-amylase	100.00	105.38	109.45	113.12	119.25	124.08	129.62	133.44	137.86	141.52
Concentration of urea (Molar)	0.00	1.00	2.00	4.00	6.00	8.00				
Relative activities (%) of α-amylase	100.00	92.24	80.35	45.74	17.12	0.00				
Concentration of guanidine-HCl	0.00	0.50	1.00	1.50	2.00	2.50				
Relative activities(%) of α-amylase	100.00	85.50	50.62	27.12	6.24	0.00				
Concentration of acetic acid (%)	0.00	2.50	5.00	10.00	15.00	20.00				
Relative activities (%) of α-amylase	100.00	96.44	80.28	58.22	27.50	0.00				

Optimum temperature and thermostability: As shown in Fig. 7, the activity of the enzyme was also greatly influenced with the changes of temperature. The activity of α-amylase was increased rapidly with the rise of temperature and the maximum activity was observed at 37 °C. With further rise of temperature, the activity of the enzyme was decreased gradually and the enzyme lost about 80% of its activity at 75°C. Further the purified α-amylase was found to be stable between 10 to 40°C (Fig. 8).

Effect of calcium, urea guanidine-HCl and acetic acid: The effect of calcium, urea, guanidine-HCl and acetic acid on the activities of α-amylase purified from mango-pulp were presented in Table 3.

The activity was gradually increased with the increase in concentration of calcium and the activity was increased more than 41% in the presence of 0.5 M $CaCl_2$.

As shown in the Table 3, the activity of α-amylase decreased gradually after treatment with increasing concentration of urea, guanidine-HCl and acetic acid and the activity of the enzyme was destroyed completely in the presence of 8 M urea, 2.5 M guanidine-HCl and 20% acetic acid.

Effect of various metallic salts: Table 4 represented the effect of various metallic salts on the activity of α-amylase. It is evident from the results that the presence of Mg^{2+} and Mn^{2+} increased the activity of the enzyme while the other metallic salts such as K^{2+}, Na^{+}, Hg^{2+} produced no inhibitory on the α-amylase but the presence of Fe^{+}, Zn^{2+} and Cu^{2+} reduced the activity of the enzyme remarkably.

Discussion

The amylase enzyme purified from the crude extract of mango-pulp, is glycoprotein in nature as they gave pinkish red colour in presence of phenol sulphuric acid. Although the enzyme was purified with an increase of purification fold of 40 but the yield was found to be about 16% only. This decrease in yield may be due to denaturation of enzyme during the lengthy purification procedures or some other reasons. Witt and Sauter (1996) reported the recovery of popular leave amylase about 11%.

The molecular weight of mango-pulp α-amylase reported in this investigation are in relative agreement with molecular weight estimated for the enzyme from other sources. The molecular weight of α-amylase have been reported to be 51,000-52,000 from *Aspergillus niger* and 58,000-61,000 from *Aspergillus niger* (Crueger and Crueger, 1990) and 44,000 from poplar leaves (Witt and Sauter, 1996). Further it can be concluded from SDS electrophoresis result that like amylase from other sources, mango-pulp α-amylase also contained only one subunit.

Table 4: Effect of various metallic salts on the activity of α-amylase

Test salts	Concentration (Molar)	α-amylase
None	-	100.00
$MgCl_2$	0.001	102.55
	0.002	105.28
$ZnCl_2$	0.001	82.16
	0.002	73.84
$CuCl_2$	0.001	88.64
	0.002	76.24
$MnCl_2$	0.001	112.76
	0.002	122.30
NaCl	0.001	100.00
	0.002	99.36
KCl	0.001	100.00
	0.002	100.00
$HgCl_2$	0.001	100.00
	0.002	99.25
$FeCl_2$	0.001	64.36
	0.002	58.45

The Km value of α-amylase was estimated to be 0.33% using starch as substrate, which was correlated with that reported for α-amylase (Km-0.25-0.8%, Jian, 1988).

Different disaccarides and polysaccharides were tested as substrates for α-amylase and it is clear from the results that the rate of hydrolysis is highest in case of soluble starch followed by amylopectin and amylose. Very similar substrate specificities for poplar leave α-amylase was also reported (Witt and Sauter, 1996).

The activity of mango-pulp α-amylase was found to be affected greatly with the changes of pH as well as temperature and the optimum pH and temperature reported in the study were very similar to that reported for α-amylase from poplar leaves (pH 6.5, Witt and Sauter, 1996) and banana-pulp (pH 6.9 and 38°C, Mao and Kinsella, 1981). The activity of α-amylase was abolished completely by the denaturing agents 8.0 M urea and 2. 5M guanidine-HCl suggesting more sensitivity of guanidine-HCl than urea. These denaturing agents may sequentially destroy the secondary/tertiary structure of the enzyme which result complete destruction of the activity of enzyme at higher concentration.

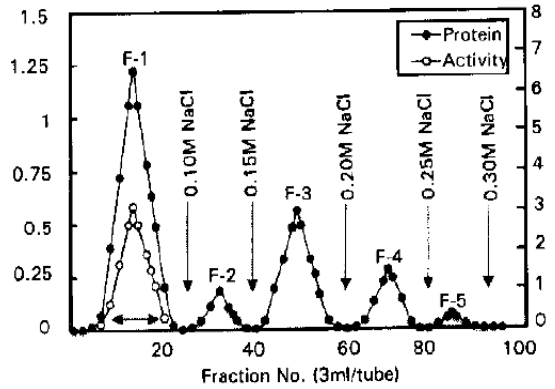

Fig. 1: Ion-exchange chromatography on DEAE-cellulose. Enzyme extract (238 mg) was applied to the column (2.0 × 24 em) prewashed with 50 mM phosphate buffer, pH 7.4 at 4°C

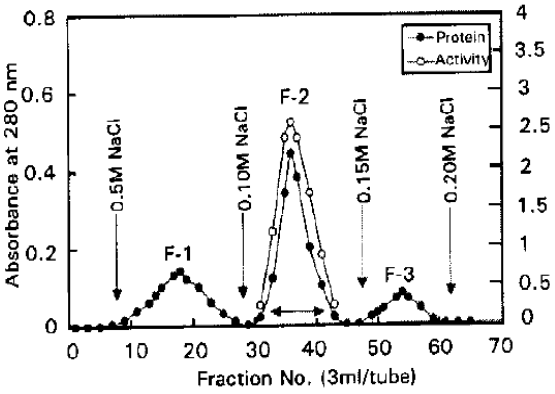

Fig. 2: Ion-exchange chromatography on CM-cellulose. Enzyme extract (3.2 mg) obtained by DEAE-cellulose chromatography was applied to the column (1.25 × 16cm) prewashed with 20 mM phosphate buffer, pH 6.5 at 4°C

Fig. 3: Polyacrylamide disc gel electrophoretic pattern of the purified enzyme

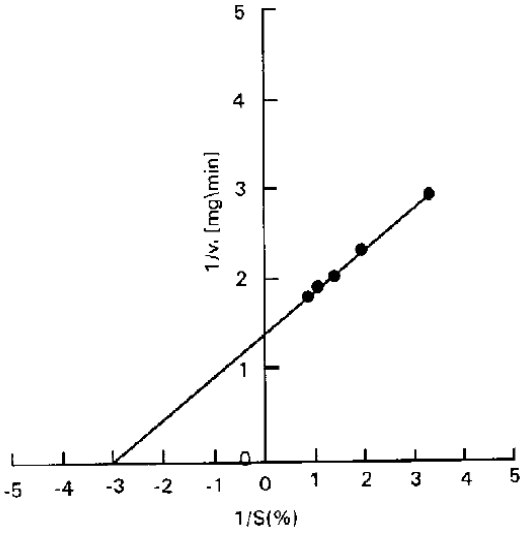

Fig. 4: Lineweaver-Burk's double reciprocal plots for the determination of Km value of α-amylase

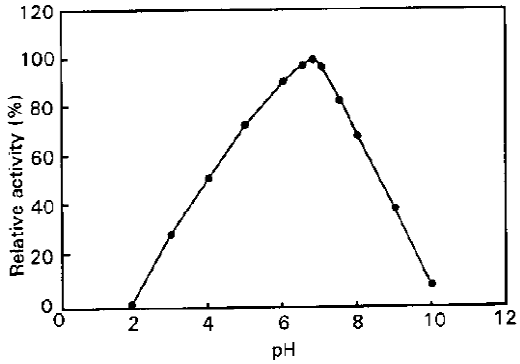

Fig. 5: Effect of pH on the activities of α-amylase. The buffers used were as follows:
pH 2.0-2.5 →KCl-HCl
pH 3.0-4.0 →AcONa-HCl
pH 4.5-5.5 →AcONa-CH_3COOH
pH 6.0-8.0 →NaH_3PO_4-Na_2HPO_4
pH 8.5-9.0 →$Na_2B_4O_2$-HCl
pH 9.5-10.0 →$Na_2B_4O_2$-Na_2CO_3

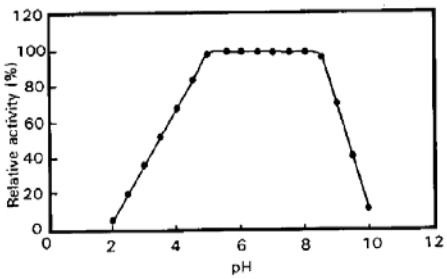

Fig. 6: Effect of pH on the stability of α-amylase. The buffers used were same as described in legends to figure-5

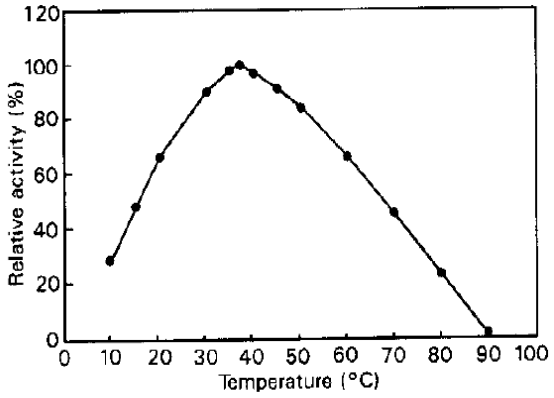

Fig. 7: Effect of temperature on the activities of α-amylase. The enzyme solution was incubated at various temperatures as represented in the figure for 15 minutes and the activities were assayed as described in "materials and methods". The highest activity was expressed as 100%

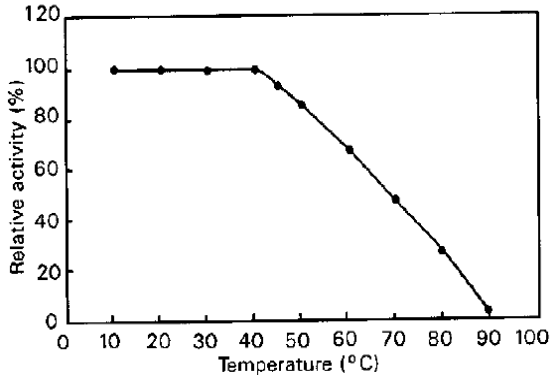

Fig. 8: Effect of temperature on the stability of α-amylase

The activity of the enzyme increased significantly in the presence of divalent cation Ca^{2+} and Mn^{2+} suggesting the involvement of these divalent ions in maintaining the active conformation of the enzyme. The role of divalent ion was also confirmed from the results that the activity of mango pulp amylase was abolished completely in the presence of EDTA.

References

Andrews, P., 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J., 96: 595-606.

Crueger, W. and A. Crueger, 1990. Biotechnology: A text book of Industrial Microbiology. Sinauer Associates Inc., Sunderland, USA., pp: 191-193.

Davies, J.W. and E.C. Cocking 1967. Protein synthesis in tomato fruit locule tissue: The sites of synthesis and the pathway of carbon into protein. Planta, 76: 285-305.

DuBois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350-356.

Gangwar, B.M. and R.S. Tripathi, 1973. A study on physico-chemical changes during growth, maturity and ripening in mango. Punjab Hortic. J., 13: 230-236.

Garcia, E. and F.M. Lajolo, 1988. Starch transformation during banana ripening: The amylase and glucosidase behavior. J. Food Sci., 53: 1181-1186.

Jayaraman, J., 1981. Laboratory Manual in Biochemistry. Wiley Eastern Ltd., New Delhi, India, pp: 53.

Jian, J.L., 1988. Fundamental of Biochemistry. 3rd Edn., S. Chand and Co., New Delhi, India.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Mao, W.W. and J.E. Kinsella, 1981. Amylase activity in banana fruit: Properties and changes in activity with ripening. J. Food Sci., 46: 1400-1403.

Mattoo, A.K. and V.V. Modi, 1969a. Ethylene and ripening of mangoes. Plant Physiol., 44: 308-310.

Mattoo, K. and V.V. Modi, 1969b. Biochemical aspects of ripening and chilling injury in mango fruit. Proceedings of the Conference Trop Subtrop, (CTS'69), London, pp: 111-114.

Ornstein, L., 1964. Disc electrophoresis-I: Background and theory. Ann. N. Y. Acad. Sci., 121: 321-349.

Pech, J.C. and A. Latche, 1972. Activities of enzymes involved in sugar metabolism in passe-crassane pears during cold storage. J. Sci. Food Agric., 23: 1499-1502.

Pech, J.C., G. Bonneau and J. Fallot, 1973. Purification partielle et proprietes des amylases de la poire. Phytochemistry, 12: 299-305.

Pesis, E., Y. Fuchs and G. Zauberman, 1978. Starch content and amylase activity in avocado fruit pulp. J. Am. Soc. Hortic. Sci., 103: 673-676.

Rahman, M.M., N. Absar and M.A. Gofur, 1997. Comparative study on changes in the activities of amylase and invertase of different varities of mango at different maturity levels. Indian J. Plant Physiol., 2: 245-246.

Robyt, T.F. and D.J. White, 1990. Biochemical Techniques: Theory and Practice. Waveland Press, Chicago, IL.

Takahashi, T., N. Inokuchi and M. Irie, 1981. Purification and characterization of a glucoamylase from *Aspergillus saitoi*. J. Biochem., 89: 125-134.

Tandon, D.K. and S.K. Kalra, 1983. Changes in sugars, starch and amylase activity during development of mango fruit cv Dashehari. J. Hortic. Sci., 58: 449-453.

Weber, K. and M. Osborn, 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., 244: 4406-4412.

Witt, W. and J.J. Sauter, 1996. Purification and characterization of α-amylase from poplar leaves. Phytochemistry, 41: 365-372.