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A Comparative Analysis on the Purification and Characterization of Amylase from the Flesh of Healthy and Disease-affected Moringa Fruit (*Moringa oleifera* Lam)

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Abstract: Two different amylase from the flesh of healthy and fruit-rot disease-affected Moringa fruit were purified by successive chromatography of the crude enzyme extract on DEAE-cellulose followed by CM-cellulose and were purified 46- and 50-fold respectively. Both the enzymes appeared to be homogeneous as judged by polyacrylamide gel electrophoresis. Molecular weights of amylases from healthy and diseased Moringa estimated by gel filtration were 59 kDa and 66.5 kDa respectively. The purified enzymes were classified as α-amylase and consist of a single polypeptide chain. The amylase from healthy and diseased Moringa flesh showed the following characteristics: pH optima, 6.8 and 6.4; temperature optima, 38°C and 40°C; K_m value, 0.28 and 0.22% for starch as substrate respectively.

Key words: Amylase, Moringa fruit.

Introduction

Starch is the principal storage polysaccharide in plant cell. Investigation of the enzymatic mechanism of starch degradation in several plant tissues led to the assumption that α-amylase play a major role in attack on starch granules in vivo (Steup, 1988, Beck and Zeigler, 1989). A variety of α-amylase, mostly from microbial, mammal, and cereal sources were well characterized. These enzymes belong to a large family of Ca^{2+} -proteins, which show several structural features (MacGvegor, 1993, Svensson, 1994). Endo-amylase from higher plants other than cereals were less well characterized although leaf and seed α-amylase were purified from several species.

Moringa (*Moringa olifera* Lam), is a popular vegetables in Bangladesh. It grows throughout most of the tropics and has several industrial and medicinal usage. It is locally known as Sajina. There are two varieties of this vegetables in Bangladesh one of which has high production in summer. A bulk of the vegetables is affected by some diseases if it rains in that season. It was found that the amylase content of Moringa increased remarkably after infection with disease and normally the flesh of Moringa contained higher amount of amylase in mature stage as compared to that in immature and ripen stages (Rahman, 1998). Karim *et al.* (1995) also reported that the highest amylase activity in tomato was found at mature stage. From a comparative point of view, in this study, we purified and characterized amylase from healthy and disease affected Sajina.

Materials and Methods

Moringa fruits were collected at harvest maturity from the campus of Rajshahi University, Bangladesh. Sephadex G-150, DEAE-cellulose and CM-cellulose were the products of Sigma Chemical Co. USA. All other reagents were of analytical grade.

Preparation of enzyme : Unless mentioned otherwise, all the operations were performed at 4°C. Water-cleaned Moringa flesh (about 100 gm) were cut into pieces and ground into paste in a mortar with 20 ml of cold 0.1M phosphate buffer, pH 6.7, and finally homogenized into a slurry using a tissue homogeniger. The slurry was filtered with double layer of cheese cloth and the filtrate was further clarified by centrifugation at 6,000 rpm for 15 minutes. The clear supernatant was concentrated to 1/6 of its original volume by commercial sucrose. The concentrate was then dialyzed against 25 mM phosphate buffer, pH 7.5 for 24 hours. The dialysate was then centrifuged at 7,000 rpm for 6 minutes and the clear supernatant was used as crude enzyme extract.

DEAE-cellulose chromatography : The crude enzyme preparation was loaded onto a DEAE-cellulose column pre-equilibrated with 25 mM phosphate buffer, pH 7.5 and the protein was eluted from the column using the same buffer with stepwisely increasing concentration of NaCl. Enzyme activity and protein concentration were monitored at one fraction (3 ml) intervals.

CM-cellulose chromatography : The active fractions obtained from DEAE-cellulose chromatography were collected and dialyzed against 25 mM phosphate buffer, pH 6.5 for 24 hours. After centrifugation, the clear supernatant was loaded onto a CM-cellulose column pre-equilibrated with the same buffer. The separation was achieved by stepwise elution of protein from the column with the same buffer with increasing concentration of NaCl. Enzyme activity and protein concentration were monitored at one fraction (3 ml) intervals.

Amylase activity: Activity of amylase was measured according to the procedure as described in Laboratory Manual in Biochemistry (Jayaraman, 1981) using 1% starch solution in 0.1M phosphate buffer, pH 6.7 as substrate. One unit of amylase activity was defined as the amount required for liberating 1 μg of maltose per minute at 37°C.

Determination of protein concentration : Protein concentration was routinely determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.* (1951) using BSA as standard.

Polyacrylamide disc gel electrophoresis : Purity of the enzyme at every step of purification was monitored by polyacrylamide disc gel electrophoresis following the method as described by Ornstein (1964) on 7.5% gel at pH 8.3.

Molecular weight determination : Molecular weight of the purified amylase under non-denaturing condition was determined by gel filtration on Sephadex G-150 column (0.9 x 90 cm) as described by Andrews (1965). Trypsin (20 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), β-galactosidase (160 kDa) and β-amylase (200 kDa) were used as marker protein. Furthermore, molecular weight under denaturing condition as well as the sub-

unit structure was determined by SDS-PAGE using the method of Weber and Osborn (1969). Before electrophoresis, the sample was treated with 0.1% SDS in presence or absence of 1% β-mercaptoethanol and heated at 100°C for 3 min. Molecular weight markers used were the same as those for gel filtration. Commassie brilliant blue was used as staining reagent.

Results and Discussion

Purification of enzyme: Almost identical experimental conditions were maintained to compare the elution profile of amylase from healthy and disease-affected Moringa flesh during the purification steps. Fig. 1, 2 show the ion exchange chromatography of crude enzyme extract on DEAE-cellulose. It was found that the crude enzyme extracts from healthy one as well as that from diseased Moringa flesh were separated into one major fraction and a few minor fractions. Both fractions containing amylase activity were eluted by the initial buffer solution, while the other fractions were eluted by buffer with stepwisely increasing concentrations of

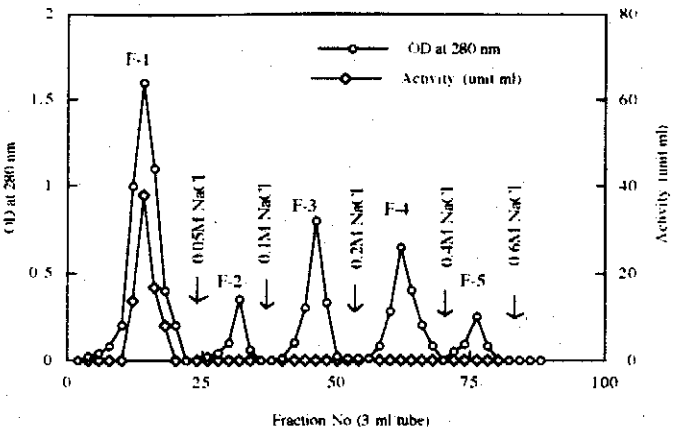

Fig. 1: Stepwise elution profile of amylase from healthy Moringa on DEAE-cellulose column. Crude enzyme extract (165 mg protein) was applied to a column pre-equilibrated with 25 mM phosphate buffer, pH 7.5 and eluted with the same buffer containing different concentration of NaCl.

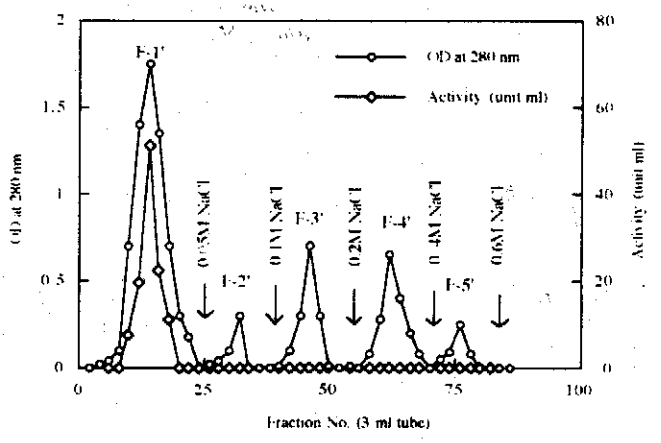

Fig. 2: Stepwise elution profile of amylase from diseased Moringa on DEAE-cellulose column. Crude enzyme extract (168 mg protein) was applied to a column pre-equilibrated with 25 mM phosphate buffer, pH 7.5 and eluted with the same buffer containing different concentration of NaCl.

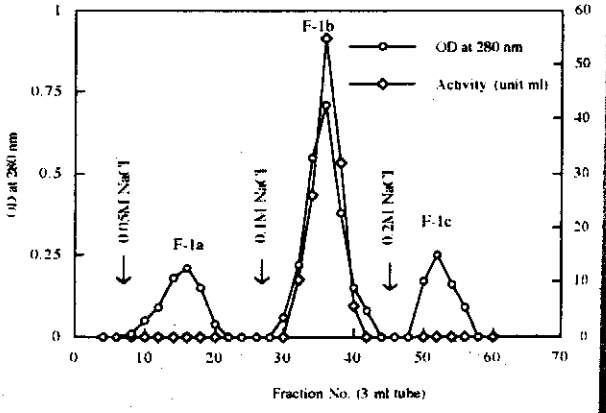

Fig. 3: Stepwise elution profile of F-1 fraction on CM-cellulos column. Protein (3.55 mg) was applied to a column pr equilibrated with 25 mM phosphate buffer, pH 6.5 an eluted with the same buffer containing differe concentration of NaCl.

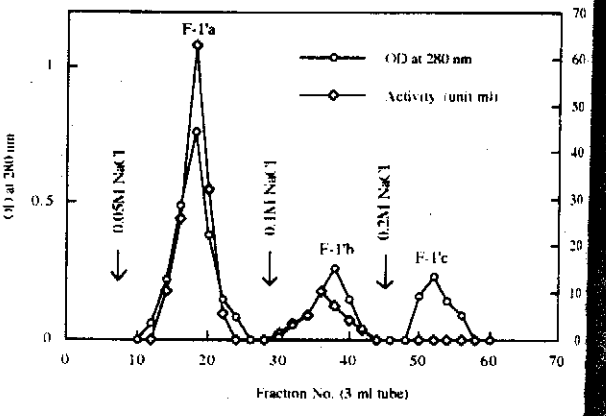

Fig. 4: Stepwise elution profile of F-1' fraction on CM-cellulo column. Protein (3.64 mg) was applied to a column p equilibrated with 25 mM phosphate buffer, pH 6.5 a eluted with the same buffer containing differe concentration of NaCl.

NaCl. The active fractions (F-1 and F-1') were pooled separate dialyzed against 25 mM phosphate buffer, pH 6.5 for 24 ho and purified further by ion exchange chromatography on C cellulose. As shown in Fig. 3, 4, both the fractions were bound the column and eluted as three fractions from the column w different concentrations of NaCl. Significantly, the elution profi of the active fractions from the healthy and diseased Mori enzyme extract were found to be different. In CM-cellul chromatography, amylase containing fraction from healthy Mori flesh extract was eluted from the column at 0.1 M N concentration while that from diseased Moringa flesh extr eluted at 0.05 M NaCl, though traces of activity were also noti in 0.1 M NaCl. Again, the concentration of the enzyme extrac from diseased flesh was found to be slightly higher than that f healthy one. As shown in Fig. 5, the amylase containing fracti F-1b and F-1'a might be contained pure enzyme as they g single band on polyacrylamide gel.

Table 1: Summary of purification of amylase from healthy Moringa flesh.

Steps	Total protein (mg)	Total activity) (units)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	165.00	312	1.9	100.00	1.00
DEAE-cellulose	3.55	145	40.5	46.50	21.60
CM-cellulose	0.98	85	86.6	27.20	45.80

Table 2: Summary of purification of amylase from diseased Moringa flesh.

Steps	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	168.00	325	1.9	100.00	1.00
DEAE-cellulose	3.64	157	43.1	48.3	22.3
CM-cellulose	1.02	98	96.6	30.2	49.8

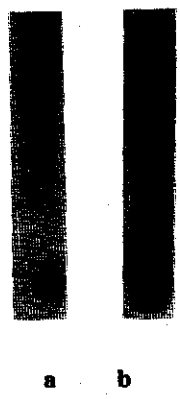

Fig. 5: Polyacrylamide disc gel electrophoretic pattern of the protein on 7.5% gel.
a : F-1b fraction (Pure amylase for healthy Moringa).
b : F-1'a fraction (Pure amylase for diseased Moringa).

Fig. 6: SDS-polyacrylamide disc gel electrophoresis of amylase under reducing and non-reducing conditions on 10% gel.
a,c : Absence of β-mercaptoethanol (F-1b, F-1'a)
b,d : Presence of β-mercaptoethanol (F-1b, F-1'a)

Table 1 and Table 2 show the data on purification steps for amylase from healthy and diseased Moringa flesh. The specific activity of the enzyme was found to increase in each step.

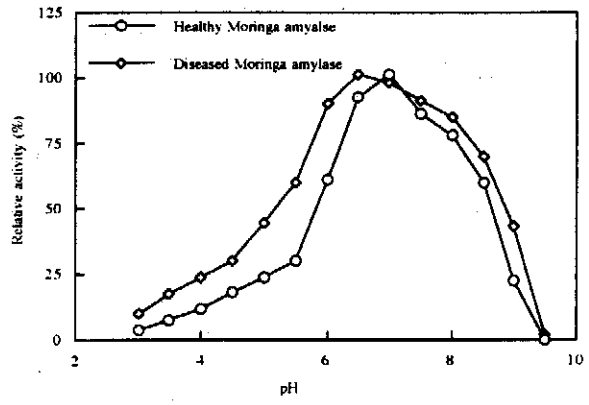

Fig. 7: Effect of pH on Moringa amylase activity.

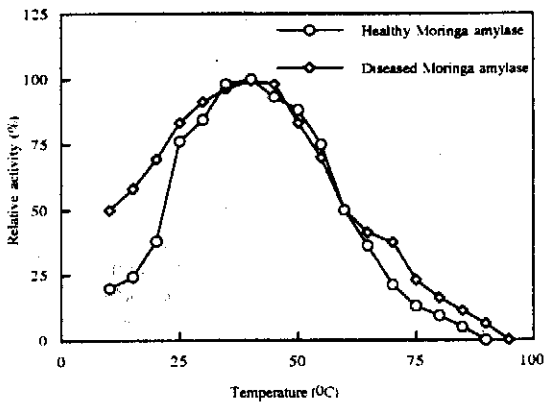

Fig. 8: Effect of temperature on Moringa amylase activity.

Although the yield was low, the purification was achieved about 46- and 50-folds for healthy and diseased Moringa flesh enzymes respectively. The decrease in yield may be due to denaturation of the enzyme during lengthy purification procedures or for some other reasons.

Characterization of enzyme : The purified enzyme from both the sources gave 100% hydrolytic activity when incubated with the substrate in absence of EDTA, but the hydrolytic property was completely lost when the substrate was pre-incubated with 30 mM EDTA, an inhibitor of α-amylase (Gracia and Lajola, 1988). Further, the hydrolytic property of the enzymes were found to remain unaffected in the presence of 10 mM cysteine or 20 mM $HgCl_2$, an inhibitor of β-amylase. These results clearly indicated that the purified amylase from both the sources were of α-type.

The molecular weights of amylase purified from healthy and diseased Moringa flesh, as determined by gel filtration, were estimated to be 59 and 66.5 kDa respectively. SDS polyacrylamide gel electrophoresis of the enzyme either in presence or in absence of reducing agent yielded a single band (Fig. 6) demonstrating that the enzyme consisted of a single polypeptide chain with a molecular weight of 60 and 67 kDa respectively. Molecular weight of the purified Moringa α-amylase from both sources was found to be very similar with that reported for *Aspergillus niger* (MW 58-61 kDa) (Cruger and Cruger, 1990).

Starch hydrolyzing activity of the purified enzymes were determined at 37°C as a function of pH. As shown in Fig. 7, the enzymes gave a characteristic bell-shaped curve and the maximum hydrolytic activity was demonstrated at pH 6.8 for healthy and 6.4 for diseased Moringa amylase. Further, the decrease in activity was found gradual in acidic pH and more rapid on the alkaline side (Fig. 7).

On incubating in 25 mM phosphate buffer (pH 6.7) with substrate for 15 min at varying temperature, the activity of the enzyme was observed to increase gradually and α-amylase from healthy source gave maximum activity at temperature 38°C, while that from diseased source gave maximum activity at temperature 40°C (Fig. 8). The enzymes lost the activity completely at or above 90°C.

The kinetic parameter, K_m for amylase from healthy and diseased Moringa flesh was determined to be 0.28 and 0.22% respectively using starch as substrate. From the data it can be concluded that the enzyme from diseased Moringa flesh has higher affinity for the substrate than that purified from healthy ones.

Moringa fruit in its mature stage has appreciably high amylase activity which is necessary from the metabolic point of view. Remarkable increase in amylase activity in diseased Moringa drew our attention whether this enzyme is the same as that in healthy one or is produced by the invading microorganism. Our comparative study showed clear diferences in the properties of the two purified enzymes from healthy and diseased Moringa. It might be concluded from the present findings such as MW, pH-profile, optimum temperature, and K_m values that α-amylase from healthy Moringa flesh was quiet different from that purified from diseased one. Further the pH-activity and temperature-activit curve demonstrated that the enzyme from diseased source slightly more stable than that from healthy source. This stud might bear some importance in further investigation on t diseases that affect Moringa.

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