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Comparative Analysis on the Purified Amylases from Healthy and Diseased Sugarcane Juice

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Abstract: Amylase was purified from the juice of healthy and diseased sugarcane, Ishurdi (Isd)-20 by successive chromatographies on DEAE-Sephadex A-50 and Sephadex G-150 to a homogeneous state as confirmed by polyacrylamide disc gel electrophoresis (PAGE). The molecular weights of the enzyme from healthy and diseased sugarcane juices were estimated as 55.8 and 56.3 kDa, respectively by gel filtration. The purified amylases were of α -type and not a glycoprotein in nature. The purified amylases showed the following characteristics, respectively; K_m values- 0.27% and 0.23%; optimum pH 6.4 and 6.3; and optimum temperature 39 and 40°C for starch as substrate. Ca^{2+} , Mg^{2+} , and Mn^{2+} increased amylase activities remarkably. The activities of amylases were increased slightly by Zn^{2+} . On the other hand Cd^{2+} and Fe^{3+} increased moderately. Na^+ had almost no effect on the amylase activity. Ag^+ and K^+ produced a little inhibitory effect but Cu^{2+} inhibited amylase activities moderately. EDTA and acetic acid almost completely ceased amylase activities. Both amylases were found to contain 70% activity in presence of 8M urea.

Key words: Sugarcane, amylase, red-rot and *C. falcatum*

Introduction

Starch is the principal storage polysaccharide in plant cells. Investigation of the enzymatic mechanism of starch degradation in several plant tissues led to the assumption that α -amylase plays a major role in attack on starch granules *in vivo* (Steup, 1988). A variety of α -amylases, mostly from microbial, mammal and cereal sources were well characterized and the enzymes belong to a large family of Ca^{2+} -proteins which show several structural features (Svensson, 1994). Amylase is an industrially important enzyme which hydrolyzes starch into glucose, maltose and dextrin and used in liquefaction of starch for production of glucose, fructose and maltose, baked goods, brewing, textiles, detergents and sugar industries (Crueger and Crueger, 1990).

Sugarcane is only the sugar producing crop in Bangladesh and plays very important role in national economy. A reasonable portion of total populations of Bangladesh are associated with sugarcane cultivation and sugar industries for their living hood. Annually 0.2 million tons of sugar and 0.3 million tons gur are produced from sugarcane. Some other commercial products such as industrial alcohol, paper and paper-board are also manufactured from by-products of sugarcane. But the yield of sugar is quite low in Bangladesh because a lot of sugarcane fields are adversely affected by various diseases especially red rot causing pathogen *Colletotrichum falcatum* Went. Our previous experimental results (data not shown) showed that the amylase content of sugarcane juice increased remarkably when infected with the pathogen *Colletotrichum falcatum*. Sharma and Seema Wahab (1975) reported that the amylase activity was increased in *Luffa cylindrica* when infected with *Pythium aphanidermatum*. From a comparative point of view, we have purified and characterized amylase from healthy and diseased sugarcane juices.

Materials and Methods

Sugarcane was collected from Harian, Rajshahi, Bangladesh. DEAE-Sephadex A-50 and Sephadex G-150 were the products of Sigma Chemicals Co., USA.

Preparation of crude enzyme extract: Unless mentioned otherwise, all the operations were performed at 4°C. The nodal tissue from sugarcane (200 gm) were cut into small pieces and ground in a mortar with 80 ml of 50 mM cold Tris-HCl buffer, pH 7.4 and finally crushed into paste using a homogenizer. The suspension was then filtered through double layers of cheese cloth and the filtrate was clarified further by

centrifugation at 6,000 rpm for 15 minutes. The clear supernatant was concentrated to about one fourth of the original volume by commercial sucrose and dialyzed against 50 mM Tris-HCl buffer (pH 7.4) for 24 hours. This dialyzate was then centrifuged at 7,000 rpm for 6 minutes and clear supernatant was used as crude enzyme extract.

DEAE-Sephadex column chromatography: The crude enzyme extract was loaded onto a DEAE-Sephadex A-50 column pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $CaCl_2$ and the protein was eluted with a gradient of 1M NaCl in the same buffer at a flow rate of 20-30 ml hr^{-1} . Enzyme activity and protein concentration were determined at one fraction (3 ml) intervals.

Sephadex G-150 column chromatography: The active fractions from DEAE-Sephadex chromatography were collected and dialyzed against 50 mM Tris-HCl buffer, pH 7.4 for 24 hours. After centrifugation, the clear supernatant was loaded onto a Sephadex G-150 column pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and the protein was eluted with the same buffer at a flow rate of 10-20 ml hr^{-1} . Enzyme activity and protein concentration were monitored at one fraction (3 ml) intervals.

Protein concentration and amylase activity: Protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as standard. Amylase activity was assayed by the method as described by Mahadevan and Sridhar (1982). 1% starch solution was used as substrate. The amylase activity was measured by estimating the amount of glucose released. One unit of amylase activity was defined as "the amount of enzyme required for liberating 1 mg of glucose per minute at 37°C".

Polyacrylamide disc gel electrophoresis: Purity of enzymes at each step of purification were checked by polyacrylamide disc gel electrophoresis (PAGE) following the method as described by Ornstein (1964) on 7.5% gel (pH 8.5).

Molecular weight determination: Molecular weight of the purified amylase was determined by gel filtration on Sephadex G-150 column (0.9 × 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 molecular weight marker. Marker

proteins and unknown proteins were applied separately onto Sephadex G-150 column under identical conditions. Commaesie brilliant blue was used as staining reagent.

Effect of pH and temperature: The activities of amylases were measured at different pH values (3.0-9.5) at 37°C by the procedure of Mahadevan and Sridhar (1982). 1% starch solution was used as substrate and the activities of the amylases were measured at different temperatures (10-90°C) using 0.1 M acetic acid-sodium acetate buffer (pH 8.7) following the procedure of Mahadevan and Sridhar (1982).

Effect of various metal ions and certain compounds: The effect of different metals and compounds on enzyme activity was tested using, different concentrations of metallic salts or compounds according to Mahadevan and Sridhar (1982).

Results and Discussion

Purification of amylase: Almost identical experimental conditions were maintained to compare the elution profile of amylases from healthy and diseased sugarcane juices during the purification steps. Fig. 1 and 2 showed the ion exchange chromatographies of crude enzyme extract on DEAE-Sephadex A-50. Both fractions containing amylase activity were eluted with 1M NaCl containing initial buffer solution. The active fractions (F-1 for healthy and F-1' for diseased sugarcane juices) were pooled separately and further purified by Sephadex G-150 gel filtration chromatography. As shown in Fig. 3 and 4, both the fractions were eluted as two peaks. The elution profiles of the active fractions from both sources were found to be almost same. But the concentration of the enzyme extracted from diseased sugarcane juice was found to be higher than that of the healthy ones. As shown in Fig. 5, the amylase containing fractions F-1b and F-1'b might contain pure enzyme, as they gave single band on polyacrylamide gel electrophoresis.

Tables 1 and 2 show the data on purification steps for amylases from healthy and diseased sugarcane juices. The specific activity of the enzyme was found to increase in each step. Although the yield was low, the purification was achieved about 40.76 and 42.15-folds for healthy and diseased sugarcane juices, respectively. The decrease in yield may be due to denaturation of the enzyme during lengthy purification procedures or for some other reasons.

Characterization of amylase: The purified enzyme from both the sources gave 100% hydrolytic activity in absence of EDTA but no activity was found when the substrate solution was

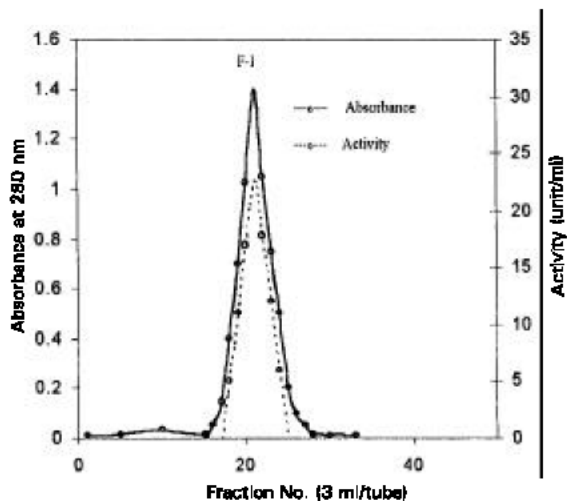


Fig. 1: Stepwise elution profile of amylase from healthy sugarcane juice on DEAE-Sephadex A-50 column. Crude enzyme extract (78 mg protein) was applied to column pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.4 and eluted with the same buffer containing 1M NaCl.

premixed with 30 mM EDTA, an inhibitor of α -amylase (Garcia and Lajolo, 1988). Further, the hydrolytic properties of the enzymes were found to remain unaffected in the presence of 10 mM cysteine or 20 mM HgCl₂, an inhibitor of β -amylase. The results clearly showed that the purified amylases from both healthy and diseased sugarcane juices were of α -type. The purified amylases were not a glycoprotein in nature as it gave no colour with phenol sulfuric acid test (Crueger and Crueger, 1990). Molecular weight of the amylases purified from healthy and diseased sugarcane juices were estimated to be 55.8 and 56.3 kDa by gel filtration, respectively (Fig. 6). Molecular weights of the purified sugarcane α -amylase from both the sources were very close to that reported for *Aspergillus oryzae* (51-52 kDa) and *Aspergillus niger* (58-61 kDa) by Crueger and Crueger (1990).

As shown in Fig. 7, α -amylase activity of healthy and diseased sugarcane juices gave a characteristic bell-shaped curve against pH. Very similar pattern of pH profile has been

Table 1: Summary of purification of amylase from healthy sugarcane juice

Steps	Total protein (mg)	Total activity units	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	78	118	1.51	100	1
DEAE-Sephadex A-50 chromatography	1.75	54	30.34	45.8	20.10
Sephadex G-150 gel Filtration chromatography	0.65	40	61.54	33.90	40.76

Table 2: Summary of purification of amylase from diseased sugarcane juice

Steps	Total protein (mg)	Total activity units	Specific Activity (unit/mg)	Yield (%)	Purification fold
Crude extract	102	164	1.61	100	1
DEAE-Sephadex A-50 chromatography	2.11	77	36.49	46.95	22.67
Sephadex G-150 gel Filtration chromatography	0.84	57	67.86	34.76	42.15

Table 3: Effect of various metallic salts on the activity of amylase

Various metallic salts	Concentration (Molar)	Relative activities (%)	
		Healthy	Diseased
CuCl ₂	0.000	100.00	100.00
	0.001	71.64	73.17
	0.002	65.67	67.07
KCl	0.005	61.20	64.63
	0.000	100.00	100.00
	0.001	86.57	85.37
AgCl	0.002	89.55	91.46
	0.005	82.09	89.02
	0.000	100.00	100.00
NaCl	0.001	97.87	96.72
	0.002	93.62	93.44
	0.005	85.10	88.52
CaCl ₂	0.000	100.00	100.00
	0.001	99.07	99.21
	0.002	98.08	98.41
MnCl ₂	0.005	98.00	98.89
	0.000	100.00	100.00
	0.001	108.55	107.15
CdCl ₂	0.002	122.35	118.75
	0.005	140.30	142.38
	0.000	100.00	100.00
ZnCl ₂	0.001	127.45	135.38
	0.002	137.25	138.46
	0.005	144.62	145.68
FeCl ₃	0.000	100.00	100.00
	0.001	103.92	104.61
	0.002	109.80	109.23
MgCl ₂	0.005	117.64	120.00
	0.000	100.00	100.00
	0.001	117.65	123.07
ZnCl ₂	0.002	141.17	147.69
	0.005	154.90	163.08
	0.000	100.00	100.00
FeCl ₃	0.001	102.11	103.56
	0.002	105.32	106.19
	0.005	108.75	109.50
FeCl ₃	0.000	100.00	100.00
	0.001	111.39	110.86
	0.002	117.45	118.38
	0.005	126.65	125.25

Table 4: Effect of urea, EDTA and acetic acid on the activity of amylase

Concentration (Molar)	Relative activities (%)	
	Healthy	Diseased
a-Effect of urea		
0	100.00	100.00
1	92.60	92.00
2	87.03	89.33
4	83.33	85.33
6	75.92	80.00
8	70.37	73.35
b-Effect of EDTA		
0.000	100.00	100.00
0.001	47.37	46.15
0.002	36.84	34.62
0.005	31.58	30.77
0.010	24.56	20.51
0.100	10.53	12.80
0.200	7.02	8.97
0.500	2.63	1.92
c-Effect of acetic acid Concentration (%)		
0	100.00	100.00
1	35.48	33.78
2.5	24.19	24.32
5	17.74	18.92
10	12.90	13.51
20	8.87	9.46
30	4.83	4.07

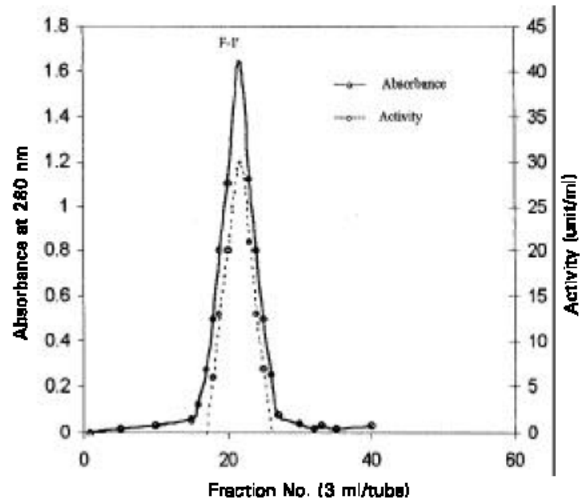


Fig. 2: Stepwise elution profile of amylase from diseased sugarcane juice on DEAE-Sephadex A-50 column. Crude enzyme extract (102 mg protein) was applied to column pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.4 and eluted with the same buffer containing 1M NaCl.

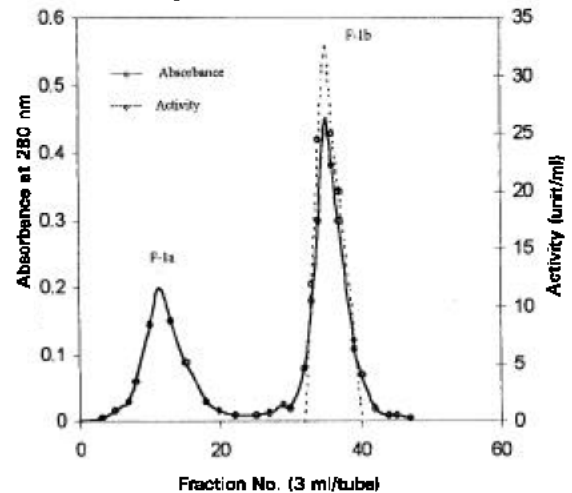


Fig. 3: Stepwise elution profile of F-1 fraction on Sephadex G-150 column. Protein (1.75mg) was applied to a column pre-equilibrated with 50mM Tris-HCl buffer, pH 7.4 and eluted with the same buffer.

reported for the amylases from banana pulp (Mao *et al.*, 1981). The maximum activity was demonstrated at pH 6.4 and 6.3 for healthy and diseased sugarcane juice amylases, respectively. Further, the activities were decreased gradually in the acidic pH but rapidly in the alkaline pH. This observation indicated that the purified enzyme from both sources were relatively stable in acidic region than that of alkaline. The acidic pH for α -amylase have been reported in *Alternaria alternata* (Chung and Hwang, 1998) and in shoots and cotyledons of pea seedlings (Beers and Duke, 1990). The activity of the enzymes were increased gradually with temperature and the maximum activity was observed at 39°C for healthy and 40°C for diseased sugarcane juice. Further, the

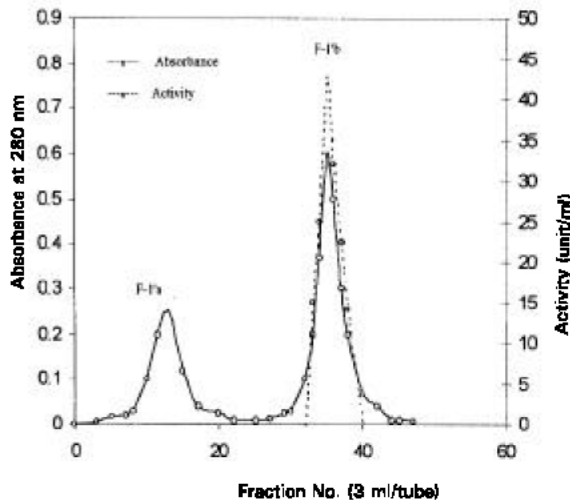


Fig. 4: Stepwise elution profile of F-1 fraction on Sephadex G-150 column. Protein (2.11 mg) was applied to a column pre-equilibrated with 50mM Tris-HCl buffer, pH 7.4 and eluted with the same buffer.

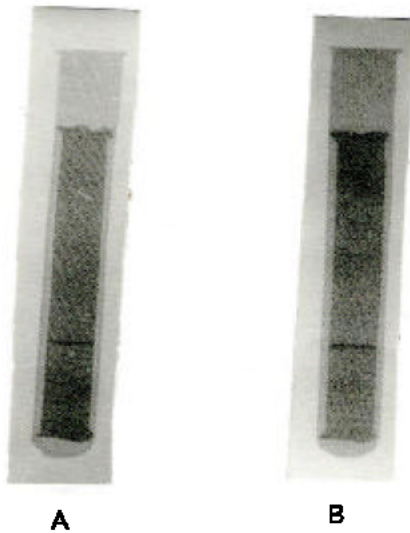


Fig. 5: Polyacrylamide disc gel electrophoretic pattern of the protein on 7.5% gel.
A) F-1b fraction (Pure amylase for healthy sugarcane juice)
B) F-1'b Fraction (Pure amylase for diseased sugarcane juice)

activity decreased gradually with the increase in temperature and was abolished completely at or above 90°C (Fig. 8). The optimum temperature of 38°C for amylase of crude banana was reported by Mao and Kinsella (1981). Chung and Hwang (1996) also reported the optimum temperature of 40°C for *Alternaria alternata* α -amylase. The kinetic constants, K_m of the purified enzymes were estimated to be 0.27 and 0.23% for healthy and diseased sugarcane juices, respectively using 1% starch as substrate. K_m value 0.08-0.25% have been reported for amylases against starch as substrate in different

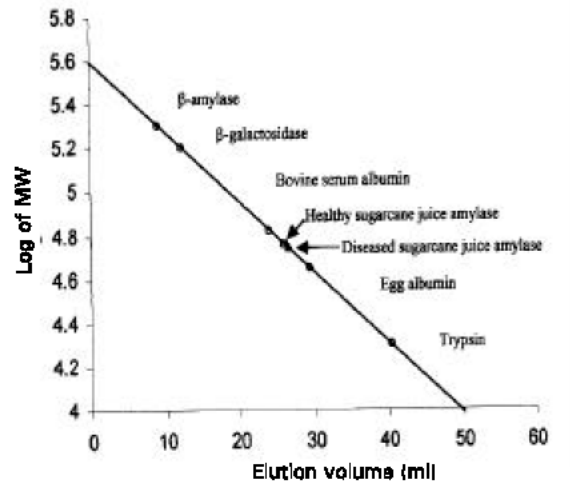


Fig. 6: Standard curve for the determination of molecular weight of enzymes by gel filtration.

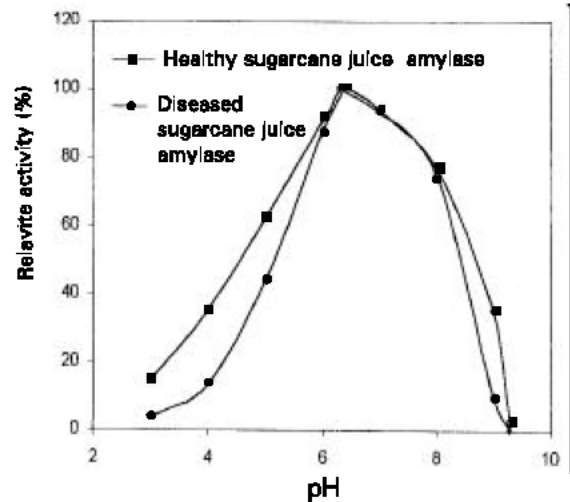


Fig. 7: Effect of pH on sugarcane juice amylase activity

sources (Jain, 1988).

Table 3 shows the effect of various metallic salts on the activity of amylases purified from healthy and diseased sugarcane juices. It was gradually increased with the increase in concentration of calcium. Kato *et al.* (1967) reported that the removal of calcium resulted in the loss of activity. It was evident that the presence of K^+ , Ag^+ , Cu^{2+} and Na^+ reduced the activities of amylases while the presence of Cd^{2+} , Mg^{2+} , Fe^{3+} , Zn^{2+} and Mn^{2+} increased the amylase activities. Greenwood and Milne (1968) reported the similar inhibitory effects of Silver and Copper on the amylase activity. The inhibitory effect of Silver on amylase activity was also reported by Chung and Hwang (1996). Na^+ had almost no effect on the amylase activity while Ag^+ and K^+ produced a little inhibitory effect. Cu^{2+} decreased the amylase activities moderately. About 50% increased activities were found in the presence of 0.005M Mg^{2+} and Mn^{2+} . The activity of amylases was increased slightly by Zn^{2+} whereas increased moderately with Cd^{2+} and Fe^{3+} . The

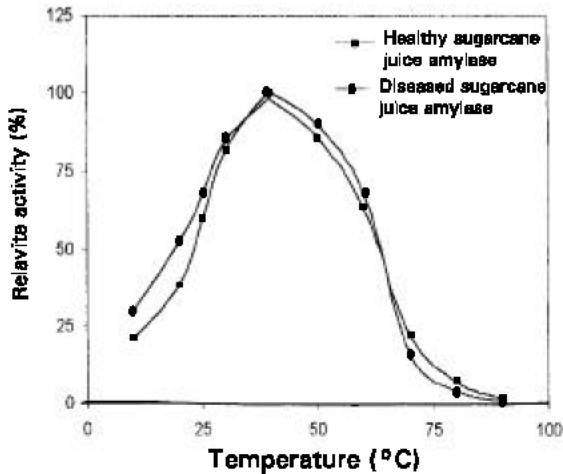


Fig. 8: Effect of temperature on sugarcane juice amylase activity.

activity of purified amylases was highly affected by certain compounds. As shown in Table 4, the activity of purified enzymes decreased in presence of urea, EDTA and acetic acid. The activities of amylase were gradually decreased with the increasing concentration of these compounds.

Remarkable increase in amylase activities in diseased sugarcane juice drawn our attention whether this enzyme is the same as that in healthy ones or is produced by the invading microorganisms, *C. falcatum*. Although a very negligible difference was observed in some properties of both the enzymes such as MW, pH, temperature, K_m etc., the protein elution profile (Fig. 1,2,3 and 4) and the inhibition or acceleration pattern of the enzymes by some metal ions and certain compounds were almost the same (Table 3 and 4). The difference in molecular weight may be due to some specific interaction with the matrix components of Sephadex G-150 gel. It was concluded that α -amylase from healthy sugarcane juice was not different from that of the diseased sugarcane juice. The higher concentration of amylase in diseased sugarcane juice was probably due to the secretion of the enzyme by the invading pathogen for their metabolic purposes.

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