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Purification and Properties of Invertase from Mango Fruit

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Abstract: A mango invertase was purified from the flesh of Himsagar variety to an electrophoretically homogeneous state, by successive ion-exchange chromatography on DEAE-Cellulose and CM-Cellulose column chromatography. The molecular weight of the purified enzyme was 68 kDa in gel filtration chromatography and 65.5 kDa on SDS-polyacrylamide gel electrophoresis. However, SDS-polyacrylamide gel electrophoresis revealed four bands, indicating that the enzyme was a tetramer. The enzyme was a glycoprotein as it gave yellow-orange colour in the presence of phenol sulphuric acid. The optimum pH for the enzyme was 4.5 and the enzyme was found to be stable from pH 2.5 to 8. The optimum temperature for enzyme was 75°C and the enzyme was stable between 10 - 75°C. The K_m for sucrose was 5.25 mM (pH 4.5). The activities of enzyme were remarkably enhanced by Cu^{++}, K^{+}, Ca^{++} whereas, completely ceased by Hg^{++} and sodium dodecylsulfate.

Key words: Himsagar, *Mangifera indica*, properties, invertase

Introduction

Invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) has been isolated from several plant tissues, especially those engaged in active growth and development (Rees, 1974). Plant invertases have been examined in a number of plants (Doehlert and Felker, 1987; Faye *et al*., 1981; Hatch *et al*., 1963; Krishnan *et al*., 1985) and in yeast (Neumann and Lampen, 1967). This enzyme hydrolyzes sucrose and related sugars, thereby providing hexoses which can be utilized for the energy and carbon requirements of the cell. Plant invertases are classified as acid and alkaline invertases on the basis of their pH-optima. They are also classified as soluble and bound invertases on the basis of their intracellular localization. Soluble invertases purified from Semillon (Nakanishi and Yokotsuka, 1990) and Ohanez (Arnold, 1965) grapes were stable and active under acidic conditions. The variety Himsagar mango is well known for its taste and quality in Bangladesh and it was reported that this variety contains the highest invertase activity at mature stage (Aynul, 1998). In this study, the invertase was purified and characterized from the flesh of Himsagar produced in northern region of Bangladesh.

Materials and Methods

Himsagar variety of mango was collected from selected lice at the Horticulture Research Station in Rajshahi, Bangladesh. DEAE-Cellulose, CM-Cellulose and SDS were the products of Sigma Chemical Co., USA. All other reagents used in this study were of analytical grade. Unless specified, all the experimental conditions were maintained at 4°C.

Preparation of crude enzyme extract: The mango flesh (200 gm) was ground with cold 0.1M phosphate buffer (pH 7.0) and finally crushed into a paste, using homogenizer. The filtrate was collected by filtration through double layer of muslin cloth and centrifuged at 6000 rpm for 15 minutes. The clear filtrate was concentrated to about $1/8^{th}$ of the original volume by commercial sucrose and dialyzed over night against 20 mM phosphate buffer (pH 7.4). The dialyzate was clarified by centrifugation at 7,000 rpm for 6 minutes. The clear supernatant obtained was used as crude enzyme extract.

DEAE-Cellulose column chromatography: The crude enzyme extract was loaded onto DEAE-Cellulose column which was previously equilibrated with 20 mM phosphate buffer (pH 6.5). The protein was eluted from the column with the same buffer by stepwise elution with increasing concentration of NaCl. Enzyme activity and protein concentration were monitored at one fraction (3 mL) intervals.

CM-Cellulose column chromatography: The active fractions from DEAE-Cellulose chromatography were collected and dialyzed against 20 mM phosphate buffer (pH 6.5) for 24 hours. After centrifugation, the clear supernatant was loaded onto a CM-Cellulose column, pre-equilibrated with 20 mM phosphate buffer (pH 6.5) and the protein was eluted stepwise by the same buffer containing different concentrations of NaCl. Enzyme activity and protein concentrations were measured at one fraction (3 mL) intervals.

Polyacrylamide disc gel electrophoresis: Purity of the enzyme containing fraction at each step was monitored by polyacrylamide disc gel electrophoresis as described by Ornstein (1964) on 7.5% gel at pH 8.5.

Protein concentration and enzyme assay: The concentration of protein was determined following the method of Lowry *et al*. (1951). Invertase activity was assayed as described by Mahadevan and Sridhar (1982), using sucrose as a substrate. The liberated reducing sugar was estimated by dinitrosalisylic acid method (Miller, 1972).

Determination of molecular weight: Molecular weight of the purified amylase was determined by gel filtration on Sephadex G-150 column (0.9×90 cm^2) and SDS-polyacrylamide gel electrophoresis (Andrews, 1965; Weber and Osborn, 1969). 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 under identical conditions.

Effect of pH on stability of enzyme: The activities of invertase at different pH values (2-10) were measured according to Mahadevan and Sridhar (1982). Sucrose solution (2.5%) was made in 0.1M acetic acid-sodium acetate buffer of different pHs and were used as substrate for invertase. Stability of the enzyme at various pHs were examined by incubating the reaction mixture for 15 minutes and the activities were assayed at 37°C.

Effect of temperature on stability of enzyme: The activities of invertase at different temperatures (10 - 90°C) were measured according to Mahadevan and Sridhar (1982). To examine the thermostability, the enzyme solutions at pH 5 were incubated at various temperatures for 15 minutes. After cooling, the activities were assayed at 37 °C.

Effect of metallic salts on invertase activity: Enzyme activities were determined in the presence of 1mM metallic salts as described by Mahadevan and Sridhar (1982).

Results and Discussion

Purification of enzyme: Fig. 1 shows the ion-exchange chromatography of crude enzyme extract on DEAE-Cellulose column. It was found that the crude enzyme extracts from mango flesh were eluted into one major fraction and a few minor fractions. Only, the major fraction, F-2 contained the invertase activity and was eluted by buffer solution with stepwise increasing concentrations of NaCl. The active fraction, F-2 was pooled separately, dialyzed against 20 mM phosphate buffer (pH 6.5) for 24 hours and purified further by ion-exchange chromatography on CM-Cellulose. The proteins were eluted as major peak F-2a and two minor peaks F-2b and F-2c (Fig. 2). The active fraction, F-2 was eluted by the initial buffer solution while the same buffer with increasing concentrations of NaCl eluted the other peaks. The invertase containing fraction, F-2a might contain pure enzyme as it gave single band on polyacrylamide gel electrophoresis (Fig. 3). Table 1 showed the data on purification steps of invertase from mango flesh. The specific activity of the enzyme was found to be increased in each step. Although the yield was low, the purification was achieved about 53 folds for mango invertase. The decrease in yield may be due to denaturation of the enzyme during lengthy purification procedures or for some other reasons. Porntaveewat *et al.* (1994) and Konno *et al.* (1993) found the recovery about 19.5% from Muscat Bailey grapes and about 14.1% from the tomato fruit, respectively.

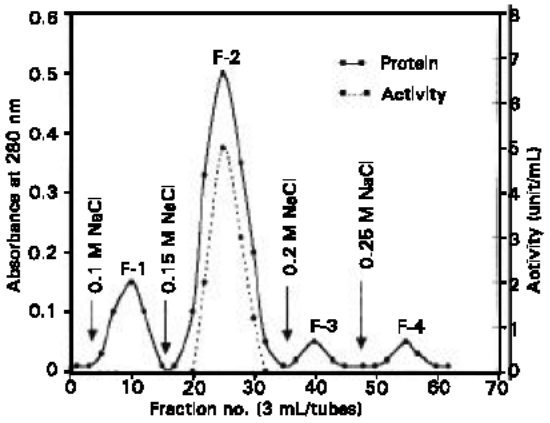

Fig. 1: Stepwise elution profile of invertase from mango flesh on DEAE-Cellulose column. Crude enzyme extract (224 mg) was applied to a column pre-equilibrated with 20 mM phosphate buffer (pH 6.5) and eluted with the same buffer containing different concentration of NaCl.

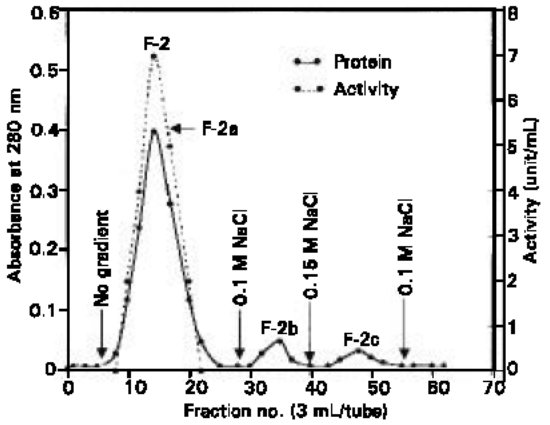

Fig. 2: Stepwise elution profile of F-2 fraction on CM-Cellulose column. Protein (2.86 mg) was applied to a column pre-equilibrated with 20 mM phosphate buffer (pH 6.5) and eluted with the same buffer containing different concentrations of NaCl.

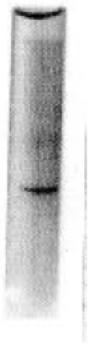

Fig. 3: Polyacrylamide disc gel electrophoretic pattern of F-2a fraction on 7.5% gel.

Fig. 4: SDS-polyacrylamide disc gel electrophoretic pattern of F-2a fraction in presence of β-mercaptoethanol at room temperature on 7.5% gel.

Characterization of invertase: The purified enzyme was a glycoprotein in nature, as it gave yellow-orange colour with phenol sulphuric acid test (Crueger and Crueger, 1990). Other investigators using similar methods have also reported plant invertase to be glycoprotein (Faye *et al.*, 1981; Krishnan *et al.*, 1985). The molecular weight of mango flesh invertase was estimated to be 68 and 65.5 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively (Fig. 5 and 6). The molecular weight of the enzyme in SDS-polyacrylamide gel electrophoresis was slightly lower than that

Table 1: Summary of purification of invertase from mango fruit

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	224	218	0.97	100	1
DEAE-Cellulose ion-exchange	2.86	54	18.88	24.77	19.46
CM-Cellulose ion-exchange chromatography	0.821	42	51.15	19.26	52.73

Table 2: Effect of various metallic salts and chemicals on the activity of invertase

Salts and chemicals (1 mM)	Relative activity (%)
None	100
$CdCl_2$	60
$CuCl_2$	128
$FeCl_2$	47
KCl	118
NaCl	98
$MnCl_2$	92
$MgCl_2$	97
$CaCl_2$	138
$ZnCl_2$	89
$HgCl_2$	0
Sodium dodecylsulfate	0
β-mercaptoethanol	62

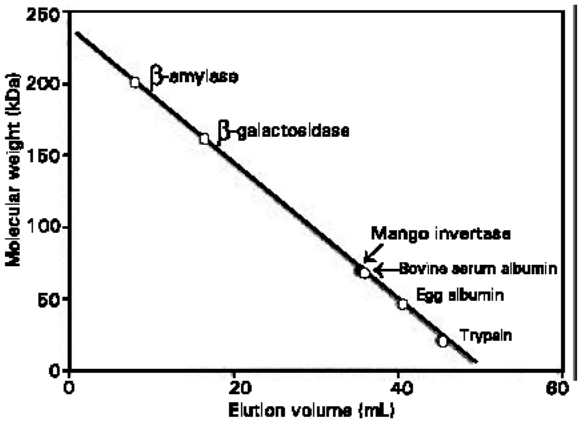

Fig. 5: Standard curve for the determination of molecular weight of enzyme by gel filtration.

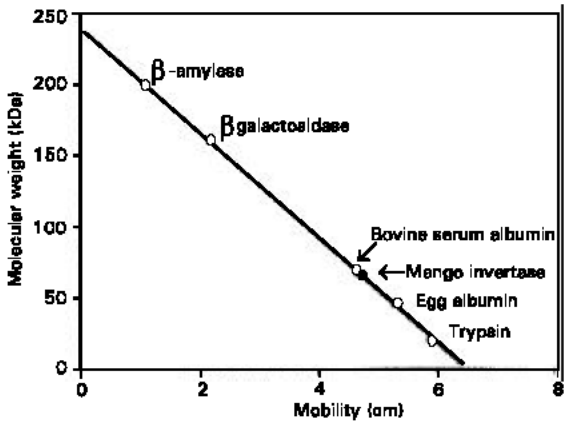

Fig. 6: Standard curve for the determination of molecular weight of enzyme by SDS-polyacrylamide gel electrophoresis.

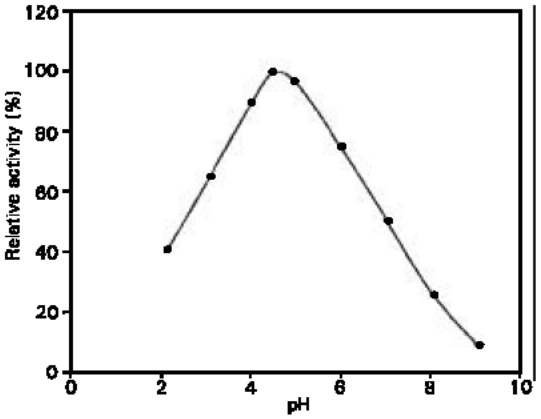

Fig. 7: Effect of pH on the activity of mango invertase.

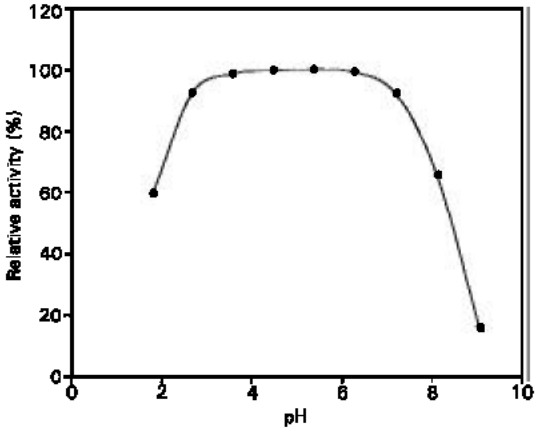

Fig. 8: pH stability of the enzyme.

of gel filtration due to some specific interaction with gel components or electrical disturbances during electrophoresis. SDS-polyacrylamide gel electrophoresis of the enzyme in presence of β-mercaptoethanol yielded four bands (Fig. 4) demonstrating the enzyme consisted of four subunits. This is in good agreement with the work of other laboratories, where plant invertase have been reported to exist in multimeric forms with apparent molecular weight ranging from 48.5 to 450 kDa (Faye *et al.*, 1981). Sucrose hydrolyzing activity of the purified enzyme was determined at 37°C as a function of pH. The purified enzyme gave maximum activity at around pH 4.5 (Fig. 7). Ishikawa *et al.* (1989) reported very similar pattern of pH profiles for invertase in grape berries. Nakanishi *et al.* (1991) found that the optimum pH for grape juice invertase was 4.0. The enzyme was found to be stable at pH values between 2.5 - 8 (Fig. 8). This result clearly demonstrated that mango flesh invertase was more stable in acidic pH region. Nakanishi *et al.* (1991) showed same pH (2.0 - 8.0) stability for grape juice invertase.

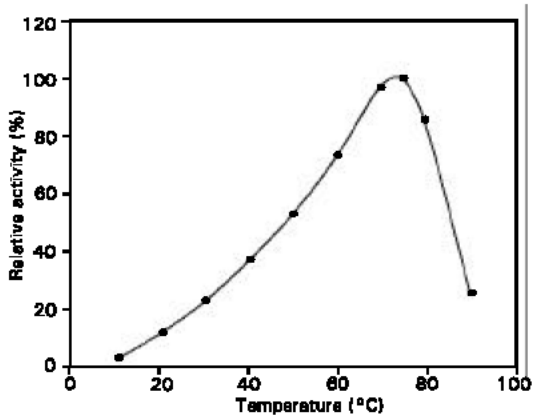

Fig. 9: Effect of temperature on the activity of mango invertase.

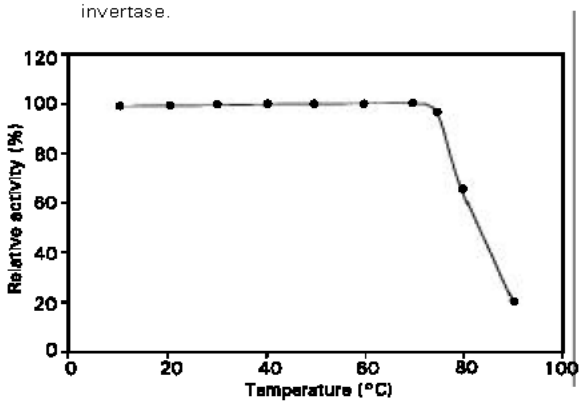

Fig. 10: Temperature stability of the enzyme.

The invertase activity of mango was found to be affected profoundly by temperature. As shown in Fig. 9, the activities of invertase were increased gradually and the maximum activities were observed at around 75°C. The enzyme invertase was found to be stable at the temperature betwveen 10 - 75°C (Fig. 10). Nakanishi *et al*. (1991) reported optimum temperature of 75°C for grape juice and also found the similar thermostability in wine invertase.

The kinetic constant, K_m for mango flesh invertase against sucrose as substrate was estimated to be 5.26 mM. Konno *et al*. (1993) and Isla *et al*. (1995) reported K_m value of 4.35 mM for invertase of tomato fruit and 6.6 mM for *Oryza sativa*, respectively, using sucrose as a substrate.

The activity of mango invertase was completely inhibited by 1mM mercuric chloride and sodium dodecylsulfate (Table 2). Nakanishi *et al*. (1991) also observed a similar inhibitory effect of Hg^{++} and sodium dodecylsulfate in wine invertase. The activity of invertase was greatly reduced by Cd^{++}, Fe^{++} and β-mercaptoethanol and remarkably enhanced by Cu^{++}, K^{+} and Ca^{++} ions (Table 2). Manganese chloride and zinc chloride inhibited the enzyme activity moderately, while a very little inhibition was found by sodium chloride and magnesium chloride (Table 2).

From the above results it was concluded that the invertase purified from mango flesh was in electrophoretically homogeneous state and it was tetrameric in nature.

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