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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF TOMATO INVERTASE

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Abstract: The enzyme invertase (β-D-fructofuranoside fructohydrolase; E.C. 3.2.1.26) was isolated and partially purified from healthy tomato juice. The purification involved buffer extraction, DEAE-cellulose and Sephadex G-75 chromatography. The purity of the enzyme preparation was determined by SDS-PAGE. The enzyme was purified 29.9 fold with 23.19% yield, giving a final specific activity 87.62 U/mg. The molecular weights of the purified enzymes measured by gel filtration chromatography and SDS-PAGE were found to be 54 kDa and 49 kDa, respectively. The purified invertase was a glycoprotein with 17.5% sugar. The optimum pH of the purified enzyme was 5.5 and the activity was stable at pH 3.5-7.5. The enzyme showed maximum activity at 35ºC and was found to be stable at the temperature ranged from 10ºC to 35ºC. The K_m value of this enzyme for sucrose was 4.5 mM at pH 5. Tris, glucose and fructose reduced invertase activities poorly while urea, EDTA, acetic acid, Zn^{2+} and Cd^{2}+ decreased moderately. Ag+ and Al^{3}+ produced a slight inhibitory effect on invertase activity. Ca^{2+} had almost no effect on tomato invertase activity. Mn^{2+}, Mg^{2+}, K+, Na+ and Ba^{2+} increased invertase activity slightly, while Cu^{2+} accelerate invertase action moderately. Hg^{2+} almost completely ceased the tomato invertase activity.

Keywords: *Lycopersicon esculentum;* Solanaceae; tomato; invertase; partial purification; characterization.

Introduction

Sucrose is the most abundant transportable free carbohydrate in the plant kingdom. It is formed by plants but not by higher animals. Sucrose is a major intermediate product of photosynthesis. In many plants, it is the principal form in which sugar is transported from the leaves to the portions of plants via their vascular systems. Sucrose serves as an important storage carbohydrate in plants, especially sink organs as tuber, root, fruit and seed. During germination, sucrose is a readily degradable source of energy. In storage organs, invertase (β-D-fructofuranoside fructohydrolase; E.C. 3.2.1.26), hydrolyzes sucrose to yield glucose and fructose.

Invertase was one of the enzymes isolated from yeast more than a century ago [1]. The enzyme occurs widely in many plants, microorganisms and animal sources [2,3,4]. The expression and distribution of plant invertase have been especially well documented, because it plays an important role in sugar metabolism [5,6,7,8]. Strong invertase activity has been detected in ripe grape berries and the accumulation of sugar in the fruit during maturation has been shown to correlate with high level of invertase activity [9]. Grape invertase isolated from certain white grapes has been shown to be present in both soluble and bound forms [10,11]. Soluble invertase purified from Semillon [12] is stable under acidic conditions. Peter and Stephanie [13] have

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reported that invertase activity is positively correlated with reducing sugar accumulation in tomato. Invertase protein is present throughout fruit development in tomato. However, the increase in activity during ripening is due to changes in the activation state of the protein rather than the amount of protein present [1 arified by centrifugation at 8000 r.p.m. for 15 minutes at 4°C. The clear supernatant was collected and concentrated to about 1/8th of the original volume using dialysis tube and dialyzed against deionized water for 12 h followed by 20 mM acetic acid-sodium acetate buffer (pH 5) for 24 hours at 4°C. The dialyzed extract was clarified by centrifugation at 7000 r.p.m. for 10 minutes at 4°C to remove any insoluble materials present. The clear supernatant obtained was used as the crude enzyme extract.

Purification of the invertase enzyme

Step-1: DEAE-cellulose column chromatography

The crude enzyme extract was applied to a DEAE-cellulose column (24×2.1 cm, flow rate 30 ml/h) at 4°C previously equilibrated with 20 mM acetic acid-sodium acetate buffer (pH 5.0) and eluted with increasing concentration (gradient) of NaCl (0.05-0.3 M).

Step-2: Shephadex G-75 column chromatography

The active fractions from DEAE-cellulose column chromatography were pooled, concentrated and applied to a column of Shephadex G-75 (100×0.9 cm) at 4°C, pre-equilibrated with 20 mM acetic acid-sodium acetate buffer (pH 5.0). The protein was eluted with the same buffer at a flow rate of 9 ml/h. The peak with the invertase activity was checked for purity using 10% SDS-PAGE (75 µl protin was loaded) according to the method of Laemmli [15]. The gel was stained with Coomassie Brilliant Blue. The purified enzyme thus obtained was used for physicochemical analysis.

Enzyme assay

Invertase activity was assayed following the method of Mahadevan and Sridhar [16]. The invertase activity was determined by measuring the amount of glucose formed by invertase catalyzed sucrose hydrolysis at pH 5.0. The assay mixture contained 375 µl of enzyme solution, 750 µl of 0.1M acetic acid-sodium acetate buffer pH 5.0 and 375 µl of 2.5% sucrose solution. The reaction was performed at 37°C for 15 minutes. The assay reaction was stopped by the addition of 1.5 ml of 3, 5-dinitrosalicylic acid reagent [17]. The mixture was heated for 5 minutes in a boiling waterbath. After the color had developed, 0.5 ml of 40% sodium potassium tartrate was added when the contents of the tubes were still warm. A control tube was also prepared in which the enzyme was denatured by heating before the addition of the substrate. The absorbance of the solution was measured at 575 nm. The enzyme activities were obtained from a calibration curve prepared by following the same procedure with D-glucose as standard. One unit of invertase activity was defined as the amount required for liberating 1 µg of glucose and fructose from the breakdown of sucrose per minute at 37°C.

Determination of protein concentration and molecular weight

Protein concentration was determined colorimetrically by Lowry method [18] using bovine serum albumin as a standard. The molecular weight of the purified tomato invertase was determined by Shephadex G-75 gel filtration through a column (100×0.9 cm) at 4°C, equilibrated with 20 mM acetic acid-sodium acetate buffer (pH 5.0) as described by Andrews [19]. Trypsin inhibitor (MW-20 kDa), bovine serum albumin (MW-67 kDa) and β-galactosidase (MW-116 kDa) were used as molecular weight markers. The molecular weight of the purified enzyme was also determined by using

10% SDS-PAGE as previously described [15] using the same molecular weight markers used in gelfiltration.

Determination of K_m value of tomato invertase

The initial value is equal to the amount of product formed per minute. The initial velocity (V_o) was determined by measuring the amount of one of the products (glucose or fructose) at various time intervals [20].

Test for glycoprotein and estimation of sugar

Phenol, in the presence of sulfuric acid, can be used for quantitative calorimetric micro determination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois [21]. The method was also employed for detecting the presence of sugar in protein.

Determination of optimum pH and pH stability of tomato invertase

The invertase activities from pH 2 to 10 were measured at 37°C following the procedure described by Mahadevan and Sridhar [16]. Sucrose solution (2.5%) was made in the above-mentioned buffer of different pH and was used as substrates for the determination of invertase activities. Stability of the invertase activities at the selected pH range was examined by incubating the reaction mixture for 15 minutes at 37°C.

Determination of optimum temperature and temperature stability of tomato invertase

The invertase activities from 10 to 80°C were measured at pH 5 according to Mahadevan and Sridhar [16]. To examine the heat stability of tomato invertase, the enzyme solution (pH 5) was pre-incubated at various temperatures for 30 minutes and cooled at 0°C for 5 minutes. The residual activities of the treated enzymes were assayed under the standard assay conditions.

Effect of various chemicals on the activity of tomato invertase

The enzyme activities were determined in the presence of various concentrations of chemicals following the procedure described by Mahadevan and Sridhar [16].

Effect of various metallic salts on the activity of tomato invertase

The enzyme activities were determined in the presence of various concentrations of metallic salts following the procedure described by Mahadevan and Sridhar [16].

Results and Discussion

Purification of the enzyme

The concentrated dialyzed crude enzyme extract was passed over a column of DEAE-cellulose previously equilibrated with 20 mM acetic acid-sodium acetate buffer of pH 5.0 at 4°C. The crude enzyme extract was first eluted with the buffer and then with increasing concentration of NaCl (0.05-0.3M). The proteins were eluted as two major peaks and two minor peaks. Only the major peak, which was eluted with the buffer containing 0.05 M NaCl contained the invertase activity with specific activity 9.75 U/mg, purification 3.33 fold and a yield of 31.37% (Table 1). The active fractions were pooled separately and the purity was checked by SDS-PAGE (Fig. 1a). The fraction gave more than one band, indicating that it contained some other proteins. Purification by gel filtration using Sephadex G-75 of the active fractions from DEAE-cellulose chromatography gave single peak, in which invertase activity was regular with specific activity 87.62 U/mg, purification 29.9 fold and yield 23.19% (Table 1). The single band obtained by

SDS-PAGE (Fig. 1a) indicated that the protein was a single polypeptide.

Table 1. Purification profile of invertase from tomato

Step	Total protein (mg)	Total activity (units)	Specific activity (units/ mg)	Yield (%)	Purifi-cation (fold)
Crude extract	1082.6	3173.3	2.93	100	1
DEAE-cellulose ion exchange	102.13	995.5	9.75	31.37	3.33
Sephadex G-75 gel filtration	8.4	736	87.62	23.19	29.9

Properties of the purified invertase

The molecular weight of the purified tomato invertase estimated by gel filtration on Sephadex G-75 was 54 kDa. Very similar molecular weight of invertase has been reported (50 kDa) in tomato [25] by gel filtration. SDS-PAGE, on the other hand, gave a mol. Wt 49 kDa (Fig. 1b). This is slightly lower than that obtained by gel filtration and this might be due to some specific interactions with gel components or electrical disturbance during electrophoresis. The present value (49 kDa) is very close to the molecular weight of 51 kDa reported by Endo *et al.* [8] but lower than the molecular weight of 56 kDa observed by Peter and Stephanie [13] in tomato invertase.

The K_m value of tomato invertase against sucrose as substrate was found to be 4.5 mM at pH 5.0 in acetic acid-sodium acetate buffer. Konno *et al.* [21] reported 4.35 mM K_m value for tomato invertase. A value of 5.25 mM K_m value was reported for mango flesh invertase [26]. Isla *et al.* [27] observed K_m value of 6.6 mM for *Oryza sativa* invertase using sucrose as substrate.

The purified tomato invertase was glycoprotein in nature and it contained 17.5% of sugar. Peter and Stephanie [13] and Nakagawa *et al.* [2] have also shown that purified tomato invertase is a glycoprotein. Invertase from grape berries has been reported to contain about 25% carbohydrate [11]. Geracimo and Jhon [28], using sucrose as a standard, have reported that the purified potato invertase is a glycoprotein with 10.9% carbohydrate.

The enzymic activity of the invertase was determined at different pH values at 37°C. As shown in Fig. 3, the pH for maximal activity was 5.5, with 29% and 24% activities at pH 3.0 and 8.0, respectively. Very similar pattern of pH profiles has been reported for cherry invertase [29], invertase of *Oryza sativa* [27], grape berries [30] and tomato invertase [8]. The enzyme was fount to be stable at pH values of 3.5-7.5 (Fig. 2). This result demonstrates that the tomato

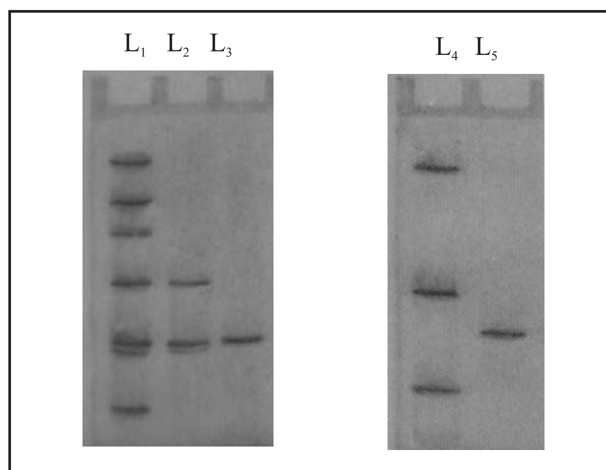

Fig. 1. (a) SDS-PAGE of partially purified tomato invertase at various stages of purification (L_1: Crude enzyme extract, L_2: Fraction from DEAE-Cellulose column, L_3: Fraction from Sephadex G-75 column).
(b) SDS-PAGE of purified tomato invertase (L_4: Molecular weight markers; Trypsin inhibitor MW-20 kDa, bovine serum albumin MW-67 kDa and β-galactosidase MW-116 kDa, L_5: Fraction from Sephadex G-75 column).

invertase is more stable in the acidic pH region. Peter and Stephanie [13] have reported that the activity of buffer-insoluble tomato invertase is optimum at pH 5.0 and stable from pH 3.5-7.0. The effects of temperature on the activity and stability of the purified invertase are shown in Fig. 3. The enzyme displayed maximal activity at 35°C. Further, the activities gradually decreased with the rise of temperature and there was a drastic drop in the activity above 50°C. The enzyme was found to be stable at temperature between 10-35°C. Finger *et al.* [31] have reported the optimum temperature of 35°C for acid invertase from *Beta vulgaris* roots. They also noted that the rapid inactivation of this enzyme occurs at 40°C and complete inactivation occurs at 55°C.

The effect of various chemicals and metallic salts on the activity of tomato invertase is cited in the Table 2. Tris, glucose and fructose reduced invertase activity poorly. Alexander [32] found that acid invertase is 25% inhibited by 0.05 M tris at pH 5.5. Tomato invertase was inhibited by its product glucose and fructose. Fructose is stronger inhibitor (15.67%) than glucose (14%) at 0.05 M. Darren *et al.* [24] have found that sugarcane neutral invertase is inhibited about 27% and 37% by its products glucose and fructose, respectively. On the other hand, moderate inhibition effects on tomato invertase activities at 0.05 M have been found by urea (32.67%), EDTA (48.15%), acetic acid (52%), Zn^{2+} (44.50%) and Cd^{2+} (37%). Darren *et al.* [24] have observed almost complete inhibition of sugarcane neutral invertase with $ZnCl_2$. The invertase activities were decreased slightly by $AgNO_3$ and $AlCl_3$. Plant invertase is characteristically inhibited by heavy metals and reagents that react with sulfhydryl groups [3,10,33,34]. The activity of invertase decreased drastically with the increased concentration of $HgCl_2$ and was completely inhibited by 0.05 M $HgCl_2$. Similar type of inhibition has been reported for sugarcane neutral invertase [24] and mango invertase [26]. Complete inhibition of invertase by $HgCl_2$ is consistent with the reports of Pressy and Avants [34] and Schwimmer *et al.* [35]. Calcium had almost no effect on tomato invertase activity, which receives support from the works of Darren *et al.* [24] and Hari *et al.* [29]. Mn^{2+}, Mg^{2+}, K^{+}, Na^{+} and Ba^{2+} increased invertase activities slightly, while Cu^{2+} accelerated invertase activity moderately.

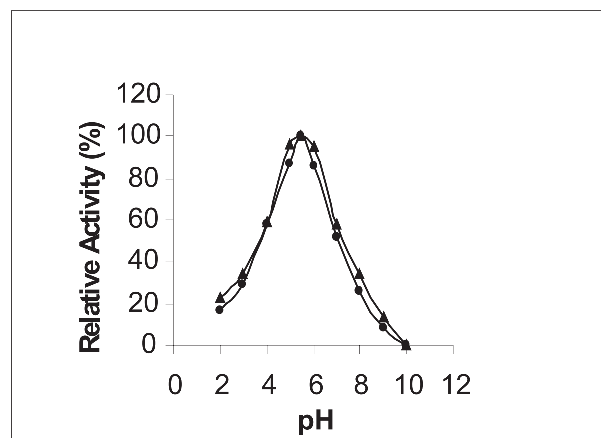

Fig. 2. Effect of pH on the activity (●) and stability (▲) of purified tomato invertase.

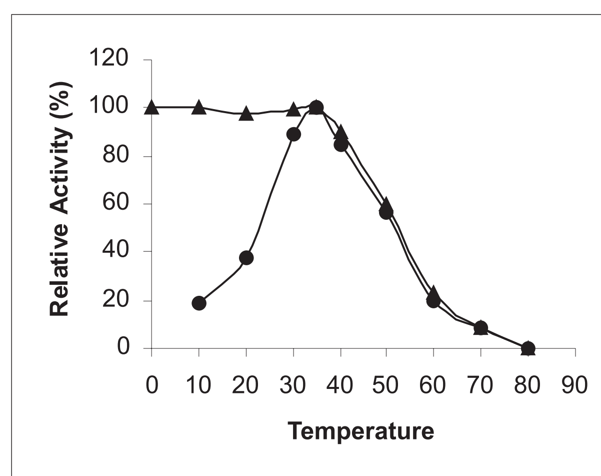

Fig. 3. Effect of temperature on the activity (●) and stability (▲) of purified tomato invertase.

Table 2. Effect of metal ions and chemicals on enzymic activity of tomato invertase.

Additives	Concentration (mM)	% Relative activity*
Tris	5	85.00 ± 1.63
Glucose	5	86.00 ± 0.81
Fructose	5	84.40 ± 3.90
Urea	5	67.33 ± 1.40
EDTA	5	51.85 ± 1.57
Acetic acid	5	48.00 ± 1.04
$ZnSO_4$	5	55.50 ± 1.75
$CdCl_2$	5	70.37 ± 1.50
$AgNO_3$	5	87.00 ± 0.79
$AlCl_3$	5	82.00 ± 0.98
$HgCl_2$	5	3.70 ± 0.93
$CaCl_2$	5	98.10 ± 1.33
$MnCl_2$	5	105.00 ± 2.11
$MgCl_2$	5	120.37 ± 1.55
KCl	5	114.81 ± 1.12
NaCl	5	115.80 ± 2.05
$BaCl_2$	5	114.80 ± 2.89
$CuCl_2$	5	137.00 ± 3.05

*Invertase activity without added compounds was taken as 100% activity.

Conclusion

We have partially purified and characterized invertase from inexpensive raw plant source, the pulp of tomato *(Lycopersicon esculentum* Mill). Further complete purification will enable explosive use of invertase in various commercial sectors. Extensive research can be carried out to enhance the activity of invertase using various metal ions.

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