Biochemistry

PURIFICATION AND SOME PROPERTIES OF INVERTASES FROM ACHRAS SAPOTA FRUIT

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SUMMARY: Invertase (E.C. 3.2.1.26) from the Achras sapota fruit was 6.5% and at temperature 20°C. The activity of both invertases were found heat labile and were completely inactivated at 45°C and 70°C within 10 minutes. Invertase I and II activities were increased in the presence of $MnCI_2$, $CoCI_2$ and $CaCI_2$ but completely inhibited with EDTA due to chelation with metal ions. Raffinose and stachyose were hydrolyzed at the rate of 59.52% and 33% with invertase I and 88.88% and 83.33% with invertase II relative to sucrose whereas cellobiose, inulin and maltose were not hydrolyzed with Invertase I and II.

Key Words: Achras sapota, Invertase, Purification.

INTRODUCTION

Commercially fructose and glucose syrups are produced from sucrose by the action of invertase (Fructofuranoside Fructo-hydrolase (E.C. 3.2.1.26). Invertase which occur in higher plant tissues are mostly extracellular or in soluble form. Invertases have been detected, isolated and characterized from several higher plant tissues that are engaged in growth, development and sugar storage (1-3). In plant tissues invertases are usually classified as acid, neutral or alkaline depending on the basis of the pH range required for their maximum activity. The acid invertase is widely distributed in plants such as beet root (4), carrot, potato and red beet (5). *Ricinus communis* leaves (6), date fruit (7) and *Oxystelma esculantum* tuber (8). Whereas acid and neutral type of invertases have been detected in sugar cane (9) and musk melo (Cucumis melo L.) fruit (10,11). However, both acid and alkaline invertases have been isolated from soybean nodules (12) and *Lupinus angustifoltus* root nodules (13).

Very little work has been carried out to purify and characterize invertases from plant tissues. Ranwala *et al.* (14) have separated acid and neutral types of invertases by DEAE cellulose chromatography to a homogenous state and characterized acid invertase. We are here presenting the data on purification and some properties of acid invertases from *Achras sapota* fruit.

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MATERIALS AND METHODS

Achras sapota: (Chicu fruit) was purchased from local market of Hyderabad, as fresh as possible. All the reagents were used of analytical grade. Sucrose was the product of BDH Chemicals and 3.5 -dinitrosalicylic acid, Sephadex G-100 and DEAE A-50 were purchased from Sigma Chemicals.

Enzyme preparation: *Achras sapota* fruit pulp was peeled and the edible portion was chopped. The chopped material was then soaked in acetone and kept at 4°C for 6 hours. Acetone was removed and the remaining material was dried. Enzyme solution was prepared as described previously (15).

Purification of Invertase: To 100 ml of crude invertase enzyme solution, two volume of cold acetone was added and kept overnight at 4°C. The precipitates were collected by the centrifugation at 6000 x g for 15 minutes at 4°C (Kubota refrigerated centrifuge). The resulting precipitates were dissolved in small volume of 0.1 M citrate-phosphate buffer pH 3. The undissolved material was removed by centrifugation. The enzyme sample was dialyzed over night against 0.1 M citrate-phosphate buffer pH 3. The dialyzed material was loaded on column (2.5 x 17 cm) of DEAE Sephadex A-50 already equilibrated with 0.1 M citrate-phosphate buffer pH 3. The enzyme was eluted with pH gradient (citrate-phosphate buffer pH 3.0 to 7.0 containing 0.1 M NaCl). The flow rate was adjusted to 25.0 ml/hour with a fraction volume of 4.0 ml. The fractions containing enzyme activity (fraction 1-31) were pooled and dialyzed. This dialyzed enzyme sample was further purified on Sephadex G-100 column (2.5 x 90 cm). The column was equilibrated with 0.1 M citrate-phosphate buffer pH 3. Enzyme sample was eluted with same buffer, adjusting flow rate 30 ml/hour and fraction volume was 4 ml/tube. Two distinct peaks were obtained.

Determination of Invertase activity: Invertase activity was measured as described earlier (8,15).

Table 1: Purification of invertase from Achras sapota fruits.

Purification Steps	Total protein mg	Total activity Units	Specific activity units / mg protein	Recovery
Crude	232	1675	7.22	100.00
Dialyzed	162	1275	7.80	76.12
DEAE Sephadex A - 50 Chromatography	140	1168	8.5	69.73
Sephadex G - 100 Chromatography				
I	19.40	286.0	14.71	17.10
II	84.17	874.6	10.39	52.01

A unit of invertase is defined as the amount of enzyme which catalyzes the liberation of one microgram of reducing sugars per 30 minutes under the conditions of assay.

Disc gel electrophoresis: The homogeneity of the purified enzyme was confirmed by polyacrylamide disc gel electrophoresis using the method of Davis (16), utilizing 10% polyacrylamide containing 1% SDS. 50 ml of enzyme sample was loaded on gel and a constant current supply of 4 mA/rod (13 x 0.6 cm) was applied for four hours. After electrophoresis run, gels were stained with commossie brilliant blue R-250 and destained with acetic acid-methanol water (7.5:5:87.5 v/v) till the appearance of blue band against clear background. The molecular weight of the purified Invertases was estimated by SDS-polyacrylamide gel using a series of protein with known molecular weight as standard.

Determination of protein: The absorbance at 280 nm was measured to monitor the protein during chromatographic separation. The protein content was measured by the method of Lowry *et al.* (17), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Figure 1 shows the elution pattern of Invertase when passed through Sephadex DEAE A-50 using citrate phosphate pH (3.0-7.0) gradient. However, further elution pattern of Invertase on Sephadex G-100 is shown in Figure 2. It is evident that two distinct peaks of Invertase activities were obtained and these were named as Invertase I and II. The steps of purification procedure and percentage recovery of enzyme activities at each step are presented in Table 1. Invertase I and II of *Achras sapota* fruit were found to be homogenous on SDS Polyacrylamide disc gel electrophoresis as shown in Figure 3. Invertase I and II shows a specific activity of 14.77 and 10.39 Units/ml protein with recovery of 17.10% and 52.21%. Molecular weights of Invertase I and II were estimated nearly to 42000 and 39000 by SDS-Polyacrylamide disc gel electrophoresis. The molecular weight of Invertase from different plant

Table 2: Effect of chemicals (5mM) on the activity of the Invertase I and II from Achras sapota fruits.

Chemicals	% Relative activity* Invertase I	% Activation / (Inhibition)	% Relative activity* Invertase II	% Activation / (Inhibition)
Control	100.00	-	100.00	-
Co^{2+}	283.33	183.33	156.66	56.66
Ascorbic acid	100.00	-	190.00	90.00
Cysteine	93.33	(6.67)	120.00	20.00
Mercaptoethanol	91.66	(8.34)	110.00	10.00
Ca^{2+}	86.66	(13.14)	288.88	188.88
Zn^{2+}	86.66	(13.34)	83.33	(16.67)
Mn^{2+}	166.66	66.66	79.44	(20.56)
Ba^{2+}	83.33	(16.67)	82.22	(17.78)
Ag^{+}	0.00	-	43.33	(56.67)
EDTA	0.00	-	9.50	(90.50)
O-phenanthroline	0.00	-	10.00	(90.00)

* Expressed as % of the activity with no addition of chemicals.

sources have been found to be different from each other and range in between 11000 to 300000 (7,18,19).

The activity of the enzyme was measured at a pH range of 4 to 8 of 0.1 M citrate-phosphate buffer. The optimum activities of *Achras sapota* fruit invertase I and II were found around 3.5 and 6.5 as shown in Figure 4. Subsequent experiments were run at pH 3.5 and 6.5. The optimum pH (6.5) of Achras sapota fruit Invertase II is slightly higher but Invertase I (3.5) is lower than *Oxystelma esculantum* fruit 5.5 (8) and *Ricinus communis* leaves 5.5 (19). The reaction for Invertase I and II activities of *Achras sapota* fruit was proceeded in temperature range of 15 to 40°C as shown in Figure 5.

The maximum hydrolysis of sucrose by purified enzyme I and II occurred at temperature 20°C and then declined. Thermal stability of *Achras sapota* fruit Invertase I and II was examined in temperature range of 15 to 70°C. The enzymes were incubated at desired temperature for 10 minutes and remaining activities were determined by standard assay method. It is concluded from the results shown in Figure 6 that the activities of invertase I and II are fairly stable up to 30 and 40°C but they completely lose

Figure 1: DEAE Sephadex A-50 chromatography of Achras sapota fruit invertase.

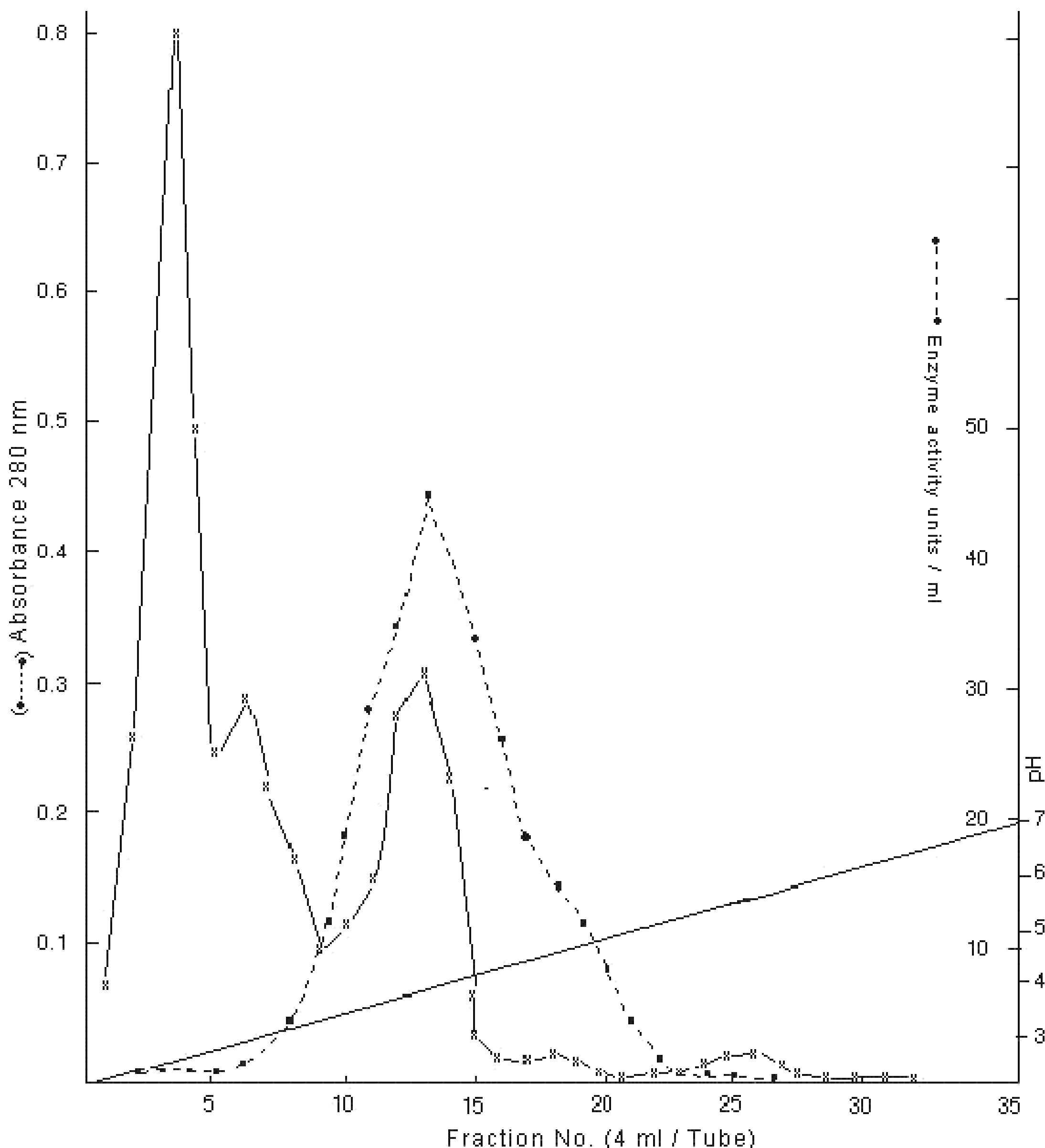
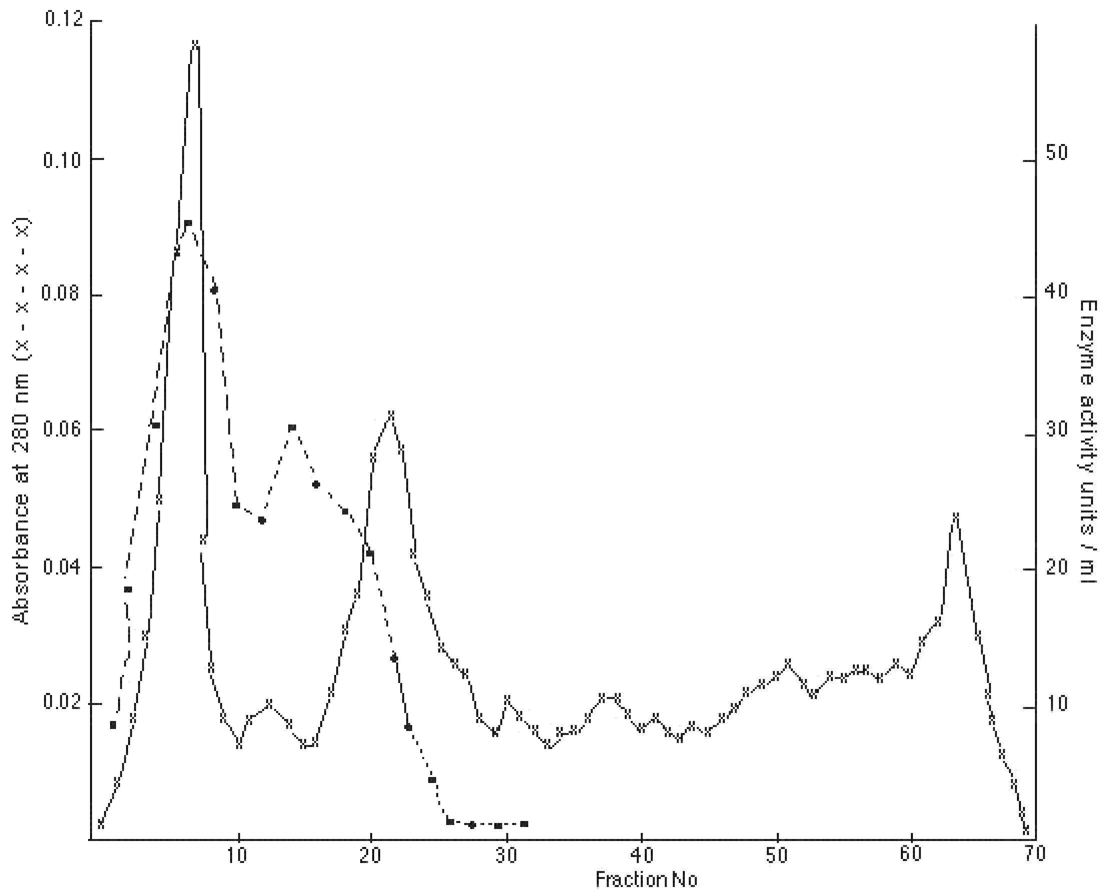


Figure 2: Sephadex G-100 column chromatography of Achras sapota seeds invertase.



their activities at 45 and 70°C respectively.

The effects of various metal ions and reagents on the activities of *Achras sapota* fruit Invertases I and II was studied and results are presented in Table 2. The Invertase I activity was highly increased by the addition of Co^{2+} and Mn^{2+} but the activity was slightly inhibited in presence of Zn^{2+}, Ca^{2+}, Ba^{2+}, cysteine and mercaptoethanol. However, invertase II activity was strongly stimulated in the presence of Ca^{2+}, Co^{2+} and ascorbic acid but cysteine and mercaptoethanol show slight activation effect whereas other bivalent cations such as Zn^{2+}, Mn^{2+}, and Ba^{2+} were also found slightly inhibitor. The activity of *Achras sapota* fruit Invertase II was inhibited to the extent of 90% but complete inhibition was observed with Invertase I by EDTA and ophenanthroline due to chelation with metal ions. The inhibition with EDTA reversed by the addition of Co^{2+} (Invertase I) and Ca^{2+} (Invertase II). This result indicates that may be invertase I and II are metalloenzyme (20,21). The activation of Invertase II with cysteine and mercaptoethanol and inhibition with heavy metal ion may suggests

Figure 3: Electrophoresis of the crude and purified invertase I and II of *Achras sapota* fruit.

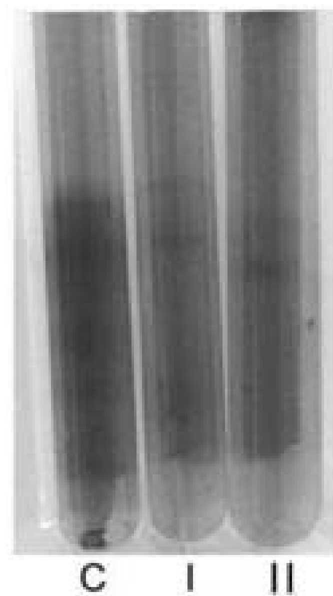
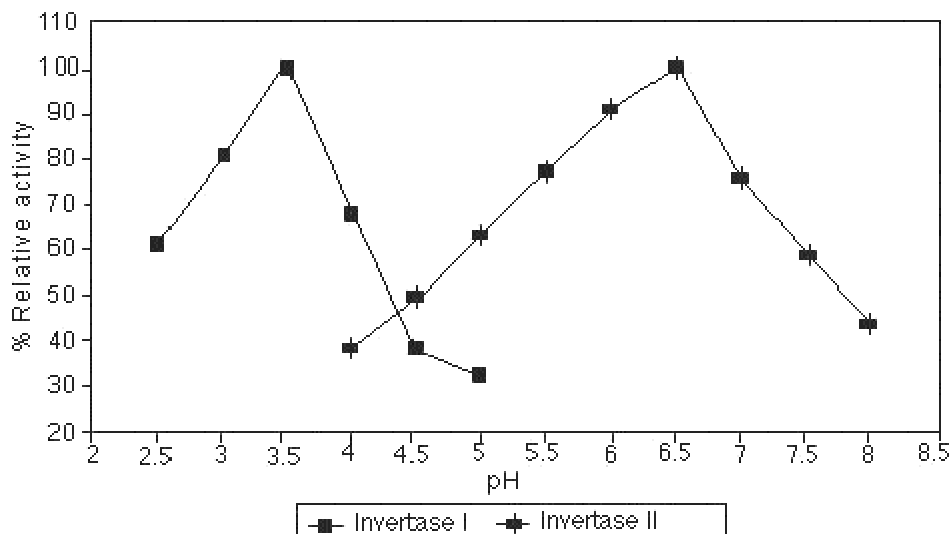


Figure 4: Effect of pH on Achras sapota fruit invertase I and II activities.



the presence of - SH group at active site. From this study, notion is that one or more active sites may have to be present in Invertase II enzyme of *Achras sapota* fruit.

The enzyme attacks sucrose, raffinose and stachyose but it does not hydrolyse inulin, cellobiose and maltose. In consequence this enzyme system is close to that of the *Carica papaya* fruit whose invertase activity shows similar behavior on these substrates (Figure 7) (22).

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Figure 5: Effect of temperature on Achras sapota fruit invertase I and II.

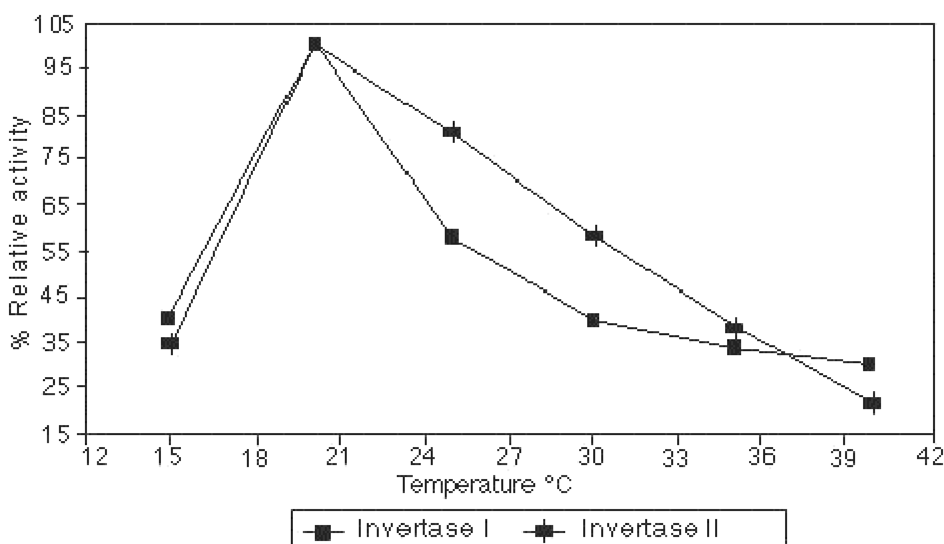


Figure 6: Effect of heat stability on Achras sapota fruit invertase I and II.

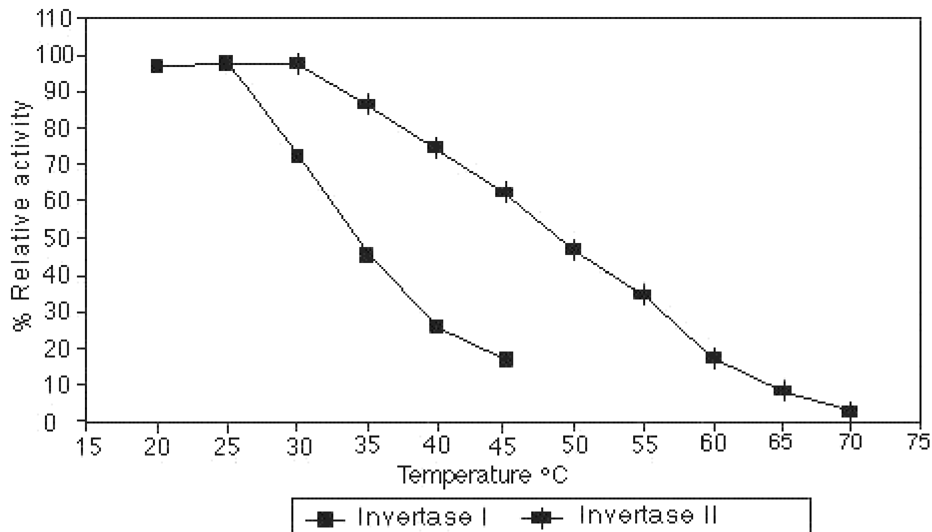
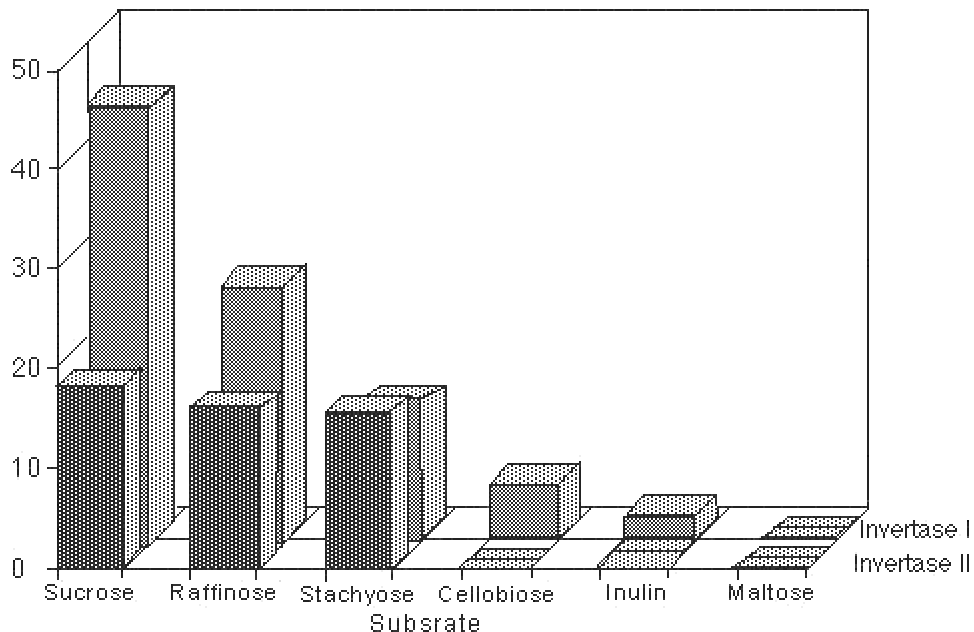


Figure 7: Effect of substrate on Achras sapota fruit invertase I and II.



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