Pak. J. Bot., 42(4): 2531-2540, 2010.

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ACID INVERTASE FROM *SACCHARUM OFFICINARUM* L.

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Abstract

An acid invertase was isolated and partially purified from mature sugarcane (var HSF 240) stem juice by a combination of Ammonium sulphate, DEAE-cellulose and gel filtration. The purified acid invertase had a specific activity of 17.05 $Umg^{-}1$. Invertase was characterized for various parameters. The pH and temperature optima of the enzyme were 3.0 and 45°C respectively. The K_m value and energy of activation (*E*a) of the enzyme was 5mM and 21.37 $kJmol^{-1}$, respectively. Irreversible thermal inactivation of the enzyme was studied at different temperatures that followed the first order kinetics. Different kinetic and thermodynamic parameters were also investigated. A slight increase in the activity of acid invertase was observed with Ca^{+2}, Mn^{+2} and Mg^{+2} ions while Cd^{2+}, Pb^{2+} and Hg^{2+} ions inhibited the activity.

Introduction

Sucrose is one of the predominant initial photosynthetic products and serves as the major form of carbohydrate translocation in higher plants. It is synthesized in the leaf tissue, from where it is transported to the heterotrophic parts of the plant. Invertase (β-D-fructofuranosidase, EC 3.2.1.26) cleaves the sucrose into D-glucose and D-fructose, the main forms of carbon and energy supply in plant metabolism. Invertases include a variety of forms, which can be categorized in terms of solubility, pH optima, isoelectric point (pI) and subcellular localization (Sturm & Chrispeels, 1990). Two types of invertases have been identified in higher plants, the soluble forms and cell-wall bound enzymes. Soluble forms are classified into acid and neutral/alkaline invertases depending upon their pH optima (Copeland, 1990). Soluble acid invertases are involved in sucrose metabolism and storage in the vacuole of young plant organs (Yelle *et al*., 1991; Lin & Sung, 1993). Alkaline invertases may be present exclusively in the cytoplasm of mature tissues and may regulate hexose and sucrose levels in the cytoplasm (Masuda *et al*., 1988). The rapid multiplication of early, mid and late maturing sugarcane clones have been investigated under different concentrations of commercial sugar and plant growth regulators (Khan *et al*., 2006).

To date there are few reports regarding the isolation, purification and characterization of soluble acid invertases. In storage organs, soluble acid invertase was purified from taproot of beet (Leigh *et al*., 1979), kernel of corn (Doehlert & Felker, 1987), shoot of Jerusalem airtichoke (Goupil *et al*., 1988), potato tuber (Burch *et al*., 1992), peach fruit (Moriguchi *et al*., 1991), mango fruit (Rahman *et al*., 2001), Japanese pear fruit (Hashizume *et al*., 2003), apple fruit (Pan *et al*., 2005) and suspension-cultured bamboo cells (Liu *et al*., 2006). In the present study, we report on the isolation, partial purification, kinetic and thermal characterization of a soluble acid invertase from sugarcane (*Saccharum officinarum* L.) variety HSF 240.

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Materials and Methods

All the chemicals used were of analytical grade and mainly purchased from Sigma Chemical Co., USA. Sugarcane (*Saccharum officinarum* L.) variety HSF 240 was collected from Ayub Agricultural Research Institute (AARI), Faisalabad Pakistan.

Isolation and purification of invertase: Unless stated otherwise, all steps were carried out at or near 4°C. Mature stems of about 9 months old, sugarcane were harvested from a plantation in AARI during spring season. Stems were excised with rootstalk and leaves were removed. Stems were bundled together and stored at 4°C till juice extraction. A stainless crusher was used to extract juice. The juice was then filtered through a four layer muslin cloth and chilled immediately at 4°C. The juice was further clarified by centrifugation at 8,000 rpm for 15-20 min., at 4°C. The clear supernatant/juice obtained was used as crude enzyme extract.

After centrifugation proteins in the supernatant/juice were fractioned by the addition of solid Ammonium sulphate. Precipitates were cut at 10 % intervals from 20% to 80% of salt saturation. The bulk of the invertase was precipitated between 40% to 80% saturation. The precipitate was collected by centrifugation at 10,000 rpm for 15 min. The precipitate was dispersed in 50 mM phosphate buffer (pH 7.0) and dialyzed extensively against distilled water to remove the salts. The dialyzed sample was then applied on DEAE- cellulose column (2.4 x 26 cm) equilibrated with Tris/HCl buffer (pH 7.5). The enzyme was eluted with a linear gradient of 0.1-0.5 M NaCl. The active fractions (2 mL) were collected and pooled together. Then sample was applied to Sephadex G-100 column (2.4 x 26 cm) previously equilibrated with phosphate buffer (pH 6.5) and eluted with the same buffer (Bhatti *et al*., 2007). The activity containing fractions were pooled together and assessed for kinetic and thermal studies.

Invertase assay: The activity of the enzyme was determined using sucrose as substrate. Appropriate amount (100µl) of the enzyme solution was mixed with buffered (acetate buffer pH 5.0) 50 mM aqueous sucrose solution (w/v). The reaction was performed at 40 °C for 40 min. The reaction was quenched by placing tubes in boiling water bath for 5 min., and then immediately cooled in ice. The released glucose was determined using glucose kit (Biocon, Germany) spectrophotometricallly at 500 nm (Bhatti *et al*., 2006).One unit of invertase activity was defined as the amount of enzyme required to liberate 1 µmole of glucose equivalent $ml^{-1}min^{-1}$ at pH 5.0 and 40°C.

Protein determination: Protein concentration was determined by the method of Bradford (1976) with BSA (Bovine Serum Albumin) as standard.

Effect of pH and temperature: The effect of pH on invertase activity was determined by assaying the enzyme as mentioned before with the difference that the activity was determined at different pH ranging from 2–9 at 40°C as described earlier (Bhatti *et al*., 2006; Amin *et al*., 2008). Optimum temperature of the invertase activity was determined by measuring the actively at different temperatures (25-70 °C) following the procedure as described by Amin *et al*., (2008).

Effect of substrate concentration: The effect of substrate concentration was determined by measuring the activity of sugarcane invertase in the reaction mixtures containing

variable amounts of sucrose [4.0–15.0 mM)] at pH 5.0.The data were plotted to determine the values of kinetic constants (V_{max} & K_m).

Thermal denaturation studies: Thermal denaturation studies of sugarcane invertase were carried out by incubating the enzyme in 50 mM Sodium acetate buffer (pH 5.0) at different temperatures (50- 66°C) in the absence of substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to assay (Violet & Meunier, 1989). The residual activity was measured and expressed as a % of the initial activity. From a semi-logarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated.

The temperature dependence of k_d was analyzed from the Arrhenius plot; the activation energy for thermal denaturation (*Ea*) was obtained from the slope of the plot. Enthalpy of inactivation (ΔH^*) was calculated according to the equation:

$$\Delta H^* = Ea - RT \qquad \text{(Eq. 1)}$$

where R = universal gas constant = 8.314 JK^{-1} mol^{-1} and T is absolute temperature.

The free energy of activation (ΔG^*) for thermal denaturation at different temperatures were obtained from the equation:

$$\Delta G^* = - RT \ln (k_d h/k T) \qquad \text{(Eq. 2)}$$

where h is the Planck constant and k is Boltzmann constant.

The entropy of activation (ΔS^*) for thermal denaturation was calculated from Equation 3:

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \qquad \text{(Eq.3)}$$

Effect of metal ions: The effect of metal ions on the activity of sugarcane acid invertase was examined by incubating the enzyme in the presence of particular metal ion solution (1m*M*) at 40°C for 40 minutes. The residual activity (%) was determined by the standard method (Bhatti *et al*., 2006).

Results and Discussion

Acid invertase isolated from sugarcane was successively purified by Ammonium sulphate, DEAE-cellulose column chromatography and gel filtration through Sephadex G-100. The results of various purification steps are summarized in Table 1. The specific activity of crude acid invertase was 2.86 U mg^{-1}. The onset of acid invertase precipitation occurred at 40%, while complete precipitation was observed at 80% of Ammonium sulphate at 0°C. The % age recovery of acid invertase was 76 after Ammonium sulphate fractionation. Acid invertase isolated from apple fruit was found to be precipitated at 90 % Ammonium sulphate saturation with 86.87% age recovery (Pan *et al*., 2005). Similarly soluble invertases were precipitated from suspension-cultured bamboo (*Bambusa edulis*) cells with 20-60% Ammonium sulphate (Liu *et al*., 2006).

Table 1.Partial purification of acid invertase from sugarcane.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	560	196	2.86	1.00	100
$(NH_4)_2SO_4$ fractionation	426	106	4.02	1.40	76
DEAE-cellulose chromatography	136	19	7.16	2.50	24.28
Sephadex G-100 chromatography	29	1.7	17.05	5.96	5.18

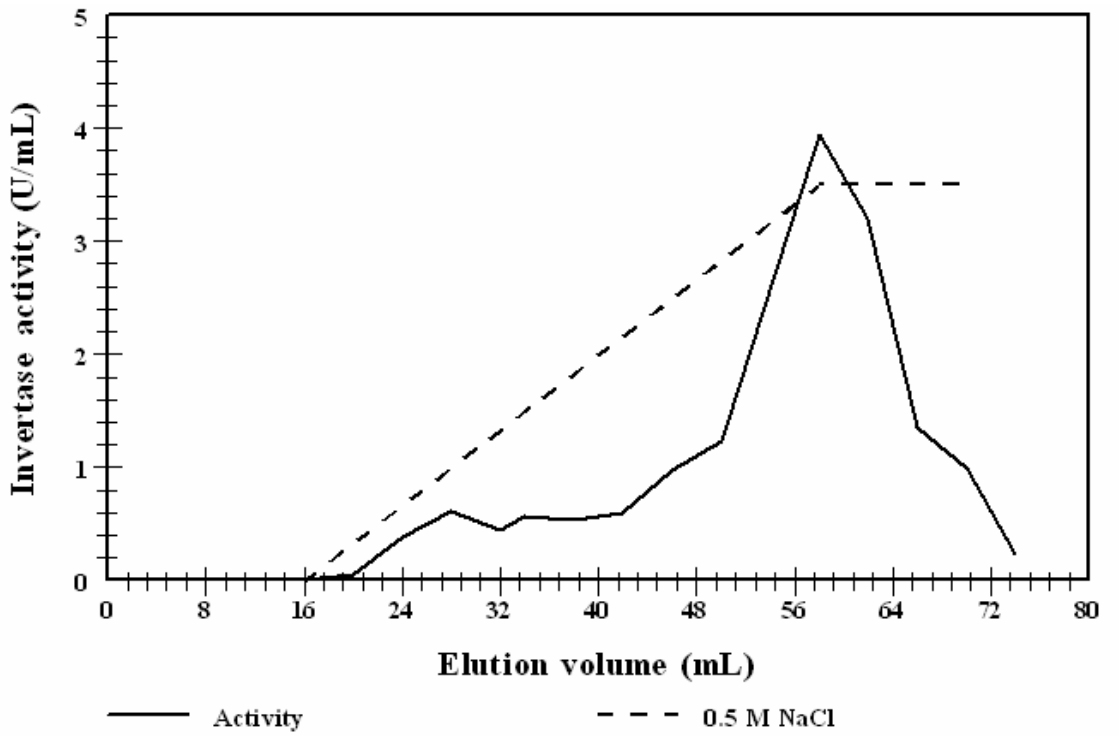

Fig. 1. Ion-exchange chromatogram of acid invertase purification. The enzyme was eluted with increasing concentration of 0.5M NaCl solution.

Major contaminating proteins were removed by passing the partially purified invertase through DEAE-cellulose column by gradient elution with varying concentration of 0.5 M NaCl solution (Fig. 1). The fractions with maximum activity were collected and pooled together. Acid invertase from DEAE-cellulose column was further purified by passing through gel filtration column containing Sephadex G-100. The % age recovery of acid invertase after gel filtration was 5.18 with specific activity 17.05 U mg^{-1}. A soluble acid invertase was purified from *Oryza sativa* (Isla *et al*., 1995) using Ammonium sulphate, gel filtration and Brushite chromatography with specific activity of 12.8 U mg^{-1} and 20% yield. Mahbubur-Rahman *et al*., (2004) purified invertase from sugarcane variety Isd-28 by successive ion-exchange chromatography on DEAE-cellulose and CM-cellulose having specific activity 50.79 U mg^{-1}. Similarly two soluble acid invertase isozymes (AIV I and AIV II) were isolated and purified from Japanese pear fruit by a combination of Ammonium sulphate, DEAE-Sephacel, ConA-Sepharose with % yield of 1.58 and 5.00 (Hashizume *et al*., 2003).

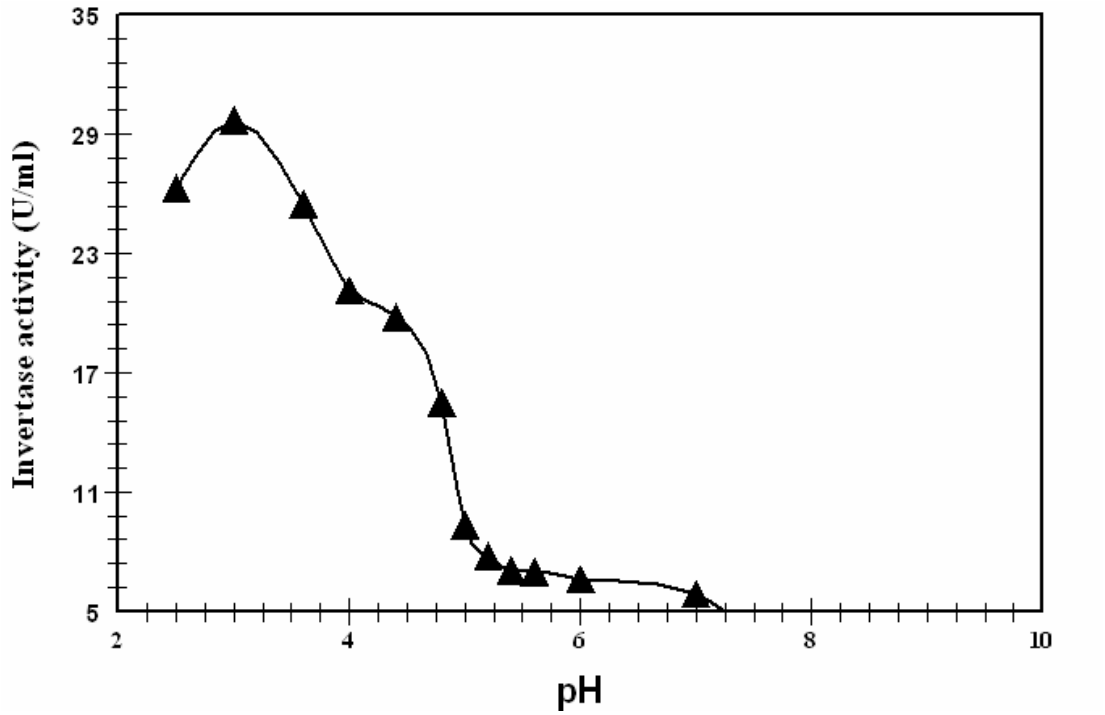

Fig. 2. Effect of pH on the activity of acid invertase. The activity was determined at different pH ranging from 2 – 9 at 40°C.

The partially purified acid invertase showed maximum activity (29.7 U/ml) at pH 3.0 (Fig. 2). Increase in pH beyond optimum caused a rapid inactivation of the enzyme with almost 99% loss in activity observed at pH 8.0. These observations indicate that the purified enzyme was relatively stable in the acidic pH region. Vorster & Botha (1998) reported that the optimum pH of sugarcane neutral invertase was 7.2. Ikram-ul-Haq & Ali (2007) isolated an invertase from *Saccharomyces cerevisiae* in a batch culture and reported that the enzyme exhibited maximum activity at pH 7.8. Similar results have also been reported by Mahbubur-Rahman *et al*., (2004). An optimum pH of 4.5 was also noted for Japanese pear fruit invertase (Hashizume *et al*., 2003). The change in pH affects the ionization of essential active site amino acid residues, which are involved in substrate binding and catalysis i.e., breakdown of substrate into products. Some ionizable residues may be located on the periphery of the active site, commonly known as non-essential residues. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity.

Figure 3 shows the effect of temperature on the catalytic activity of sugarcane acid invertase. The activity of the enzyme increased gradually with temperature and the maximum activity (23.51 U/ml) was observed at 45°C. Further, the activity gradually decreased with the rise of temperature and there was a drastic drop in activity above 65°C (Fig. 3). Arrhenius plot in the temperature range from 25°C to optimum appears linear and activation energy (Ea) was found to be 21.37 $kJmol^{-1}$. It is obvious from the Arrhenius plot (Fig. 4) that the enzyme had a single conformation up to transition temperature. Mahbubur-Rahman *et al*., (2004) reported that the optimum temperature of sugarcane invertase was 60°C. Similar results were also reported for Cherry fruit invertase (Krishan & Pueppke, 1990).

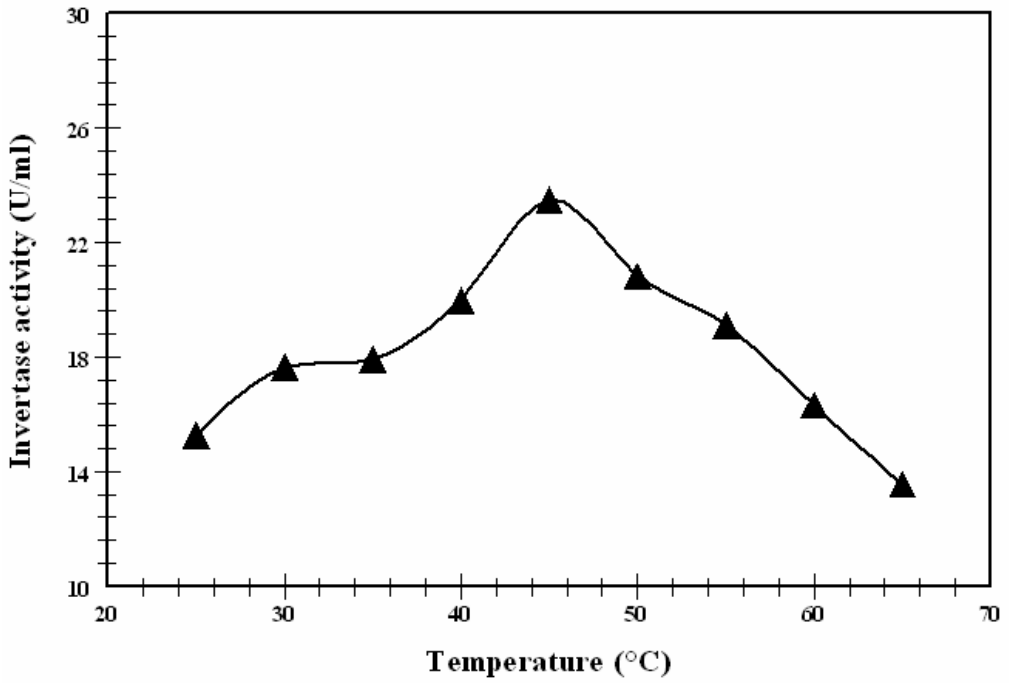

Fig. 3. Effect of temperature on the activity of acid invertase. The enzyme activity was determined at different temperatures (25-70 °C).

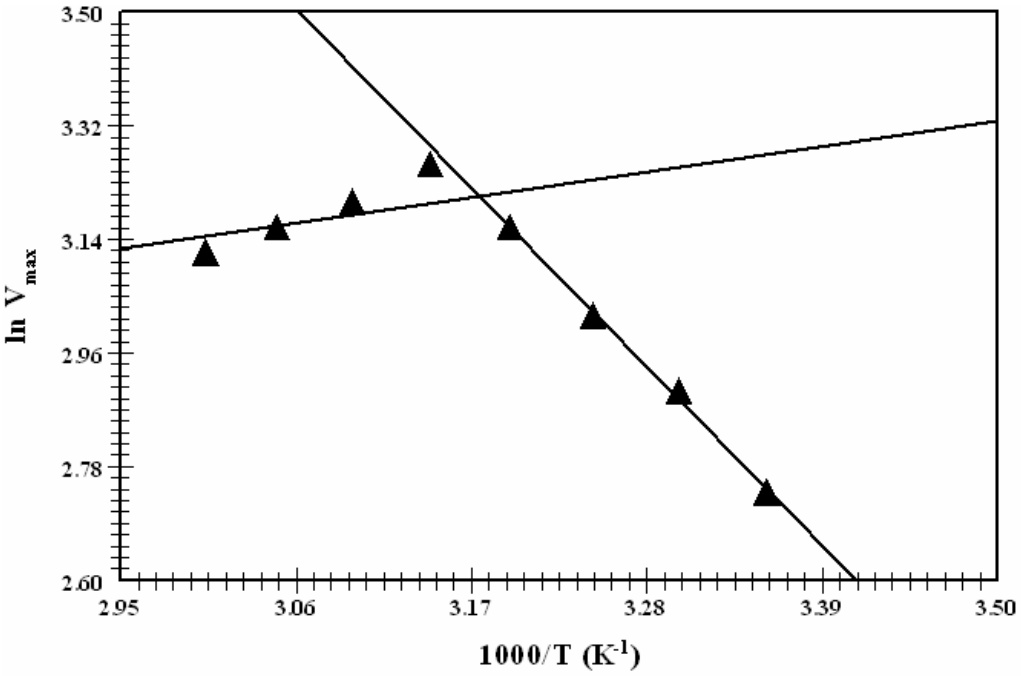

Fig. 4. Arrhenius plot for determination of activation energy (Ea) for sucrose hydrolysis. The enzyme activity was determined at different temperatures (25-70 °C). Then a plot of ln enzyme activity v/s 1/T was drawn to determine Ea value.

Sucrose hydrolysis reaction of sugarcane acid invertase nearly conformed to Michaelis-Menten kinetics. Kinetic parameters were determined from the Lineweaer-Burk plot as shown in Fig. 5. The Michaelis constant (K_m) and maximum velocity (V_{max}) were 5mM, and 5.26 µmol/min, respectively. Vorster & Botha (1998) reported that the K_m value of sugarcane invertase was 9.8±0.37mM, while Hatch *et al*., (1963) and Mahbubur-Rahman *et al*., (2004) reported that the K_m value of sugarcane invertase was 8 mM.

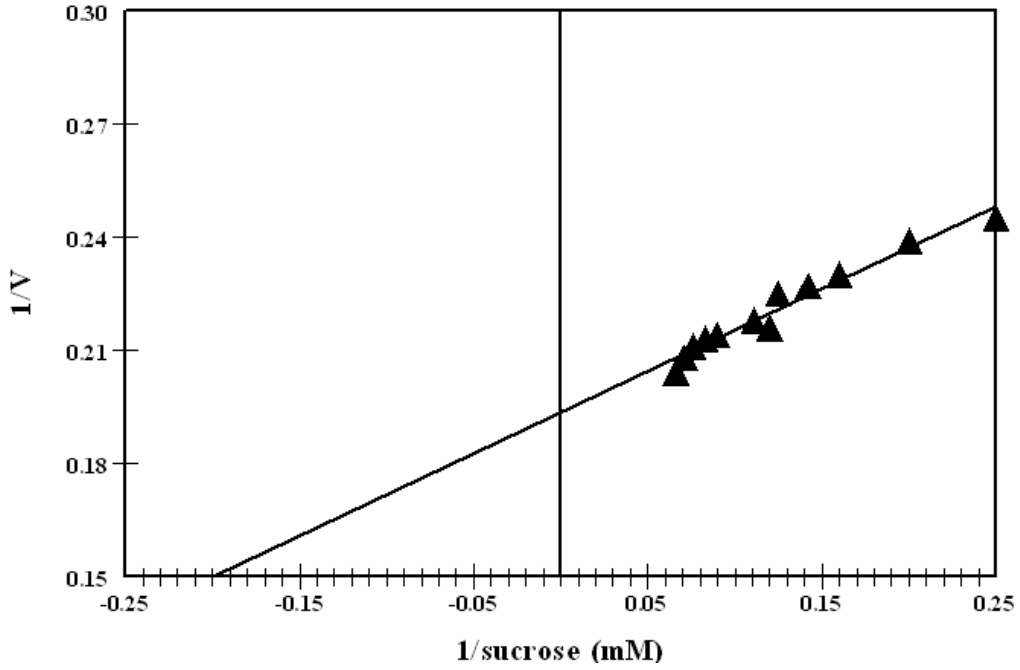

Fig. 5. Double reciprocal plot for sucrose hydrolysis by sugarcane acid invertase. The enzyme activity in the reaction mixtures containing variable amounts of sucrose [4.0 – 15.0 mM)] at pH 5.0 was determined.

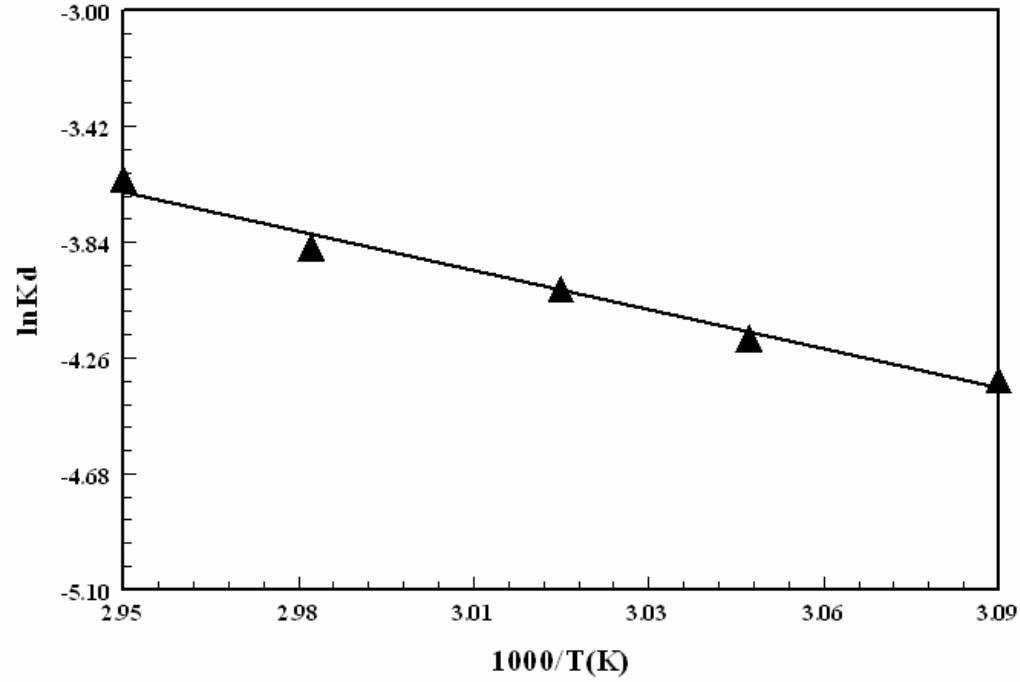

Fig. 6. Arrhenius plots for the determination of energy of activation for thermal inactivation of acid invertase Thermal denaturation studies of sugarcane invertase were carried out by incubating the enzyme in 50 mM sodium acetate buffer (pH 5.0) at different temperatures (50- 66 °C) in the absence of substrate.

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates, while thermophilicity is the capability of enzymes to work at elevated temperatures in the presence of substrate (Georis *et al*., 2000). The results regarding the thermal inactivation of sugarcane acid invertase are shown in Table 2 and Fig. 6. The thermal inactivation of enzymes is accompanied by the disruption of non-

covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (ΔH^*) (Daniel, 1996). The opening up of enzyme structure is accompanied by an increase in disorder or entropy of activation (ΔS^*) (Vieille & Zeikus, 1996) but contrary to this we found that the acid invertase from sugarcane has negative ΔS^* and positive ΔH^* between the temperature range of 50 to 66°C (Table 2). The denaturation of many proteins like chicken egg albumin and lactoglobulin at low temperatures and in the presence of urea (Nelson, 1962) show negative ΔS^* because water ordering increases in the vicinity of non-polar amino acids which are exposed during unfolding. This ordering of water around hydrophobic residues is disrupted at higher temperatures; therefore, this could not be the reason for negative ΔS^* in case of sugarcane acid invertase. The thermal denaturation of β-amylase from *Fusarium solani* at 61°C gave ΔS^* of -288 $Jmol^{-1}$ K^{-1} (Amin *et al*., 2008). Similarly thermal denaturation of invertase from *Fusarium* sp. exhibited ΔS^* of -68.04 $Jmol^{-1}$ K^{-1} at 65°C (Shaeen *et al*., 2008). However, in case of α-amylase (apo-enzyme) from *Bacillus licheniformis* thermal denaturation even at higher temperature of 80°C gave ΔS^* of -150 $Jmol^{-1}$ K^{-1} and ΔH^* of 50 k J mol^{-1} whereas surprisingly the same enzyme in the presence of Ca^{2+} ions gave positive ΔS^* (Violet & Meunier, 1989). At low temperatures, some enzymes such as chymotrypsinogin show cold denaturation phenomenon which means that below a certain critical temperature, called the temperature of maximum stability (T_{max}), both ΔS^* and ΔH^* have negative values. The negative entropy of activation (ΔS^*) observed for sugarcane acid invertase suggested that there was negligible disorderness. The value of ΔH^* decreased with increase in the temperature indicating that the conformation of the enzyme was altered. Moreover a high value (105.55 $kJmol^{-1}$) for free energy of thermal denaturation (ΔG^*) at 64°C indicated that the sugarcane acid invertase exhibited a resistance against thermal unfolding at higher temperatures.

Enzymes could be made more thermostable by either stabilizing the native form by putting non-covalent bonds including hydrogen bonds, salt bridges and hydrophobic interactions or by decreasing the entropy of unfolding (Daniel, 1996) Other possible reasons for stabilization of reactive proteins have been reviewed by Matthews (1993). Immobilization also causes a drop in the values of both ΔH^* and ΔS^*. Further decrease in these variables makes the enzyme more thermostable.

The effect of different metal ions on the activity of sugarcane acid invertase is shown in Table 3. A slight increase in the activity of acid invertase was observed with Ca^{+2}, Mn^{+2} and Mg^{+2} ions while Cd^{2+}, Pb^{2+} and Hg^{2+} ions inhibited acid invertase activity as compared to enzyme activity in the absence of such ions (control). Nakanishi *et al*., (1991) reported that effect of 0.01 M of KCl,, NaCl, $MgCl_2$ and $CaCl_2$ on the activity of both wine and grape juice invertase were negligible. Voster & Botha (1998) reported similar effects of Hg^{2+} on the activity of sugarcane neutral invertase. It was also reported that 0.005 M $FeCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$ and $AlCl_3$ reduced the activity of invertase approximately 80, 73, 32, 45 and 22% respectively (Mahbubur-Rahman, 2004).

Acknowledgement

We are thankful to Director AARI for providing the sugarcane variety *Saccharum officinarum* L. HSF 240.

Table 2. Kinetic and thermodynamic parameters for irreversible thermal denaturation of sugarcane acid invertase.

Temp.(K)	$K_d(min^{-1})$	$t_{1/2}(min^{-1})$	$\Delta H^*(kJmol^{-1})$	$\Delta G^*(kJ/mol)$	$\Delta S^*(Jmol^{-1}K^{-1})$
323	0.0125	55.45	39.55	102.08	-193.59
327	0.015	46.21	39.51	102.88	-193.78
331	0.018	38.51	39.48	103.67	-193.94
335	0.020	34.66	39.44	104.66	-194.67
339	0.023	30.14	39.41	105.55	-195.103

Ea = 42.23 k $Jmol^{-1}$(calculated from Fig. 6), $t_{1/2}$ (half-life) = $0.693/k_d$, $\Delta H^* = E_a$ (42.23 kJ mol^{-1}) $- RT$, $\Delta G^* = -RT \ln (k_d h/k.T)$, $\Delta S^* = (\Delta H^* - \Delta G^*)/T$

Table 3. Effect of metal ions on sugarcane acid invertase activity.

Salt concentration (1 mM)	Residual activity of acid invertase (%)
Control (H_2O)	100
K^+	112
Na^+	126
Mn^{2+}	156
Pb^{2+}	32
Hg^{2+}	2
Mg^{2+}	126
Cd^{+2}	54
Ca^{2+}	146

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(Received for publication 15 March 2009)