JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Studies on Kinetics and Thermostability of a Novel Acid Invertase from *Fusarium solani*

HAQ NAWAZ BHATTI, MUHAMMAD ASGHER,* A. ABBAS, RAKHSHANDA NAWAZ, AND MUNIR A. SHEIKH

Department of Chemistry, University of Agriculture, Faisalabad, Pakistan, 38040

The present investigation deals with purification and thermal characterization of an acid invertase produced by *Fusarium solani* in submerged culture. The maximum enzyme activity (9.90 U mL^{-1}) was achieved after 96 h of cultivation at pH 5.0 and 30 °C in a basal medium containing molasses (2%) as the carbon and energy source supplemented with 1% peptone. Invertase was purified by ammonium sulfate fractionation and column chromatography on DEAE-cellulose and Sephadex G-200. The purified enzyme was proven to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular mass of the enzyme was 65 kDa. The optimum pH and temperature for activity were 2.6 and 50 °C, respectively. The K_m value for sucrose was 3.57 mM with an activation energy of 4.056 kJ mol^{-1}. Enthalpies of activation (ΔH^*) were decreased while entropies (ΔS^*) of activation increased at higher temperatures. The effects of α-chymotrypsin and 4 M urea were tetraphasic with periodic gain and loss of enzyme activity. A possible explanation for the thermal inactivation of invertase at higher temperatures is also discussed.

KEYWORDS: ***β*-D-Fructofuranosidase; *Fusarium solani*; purification; thermodynamics; thermal stability**

INTRODUCTION

Invertase (*β*-D-fructofuranosidase, EC 3.2.1.26) is one of the most widely used industrial enzymes that catalyzes the hydrolysis of sucrose into glucose and fructose by recognizing the fructose moiety of sucrose. It is extensively used in confectioneries, fermentation of cane molasses into ethanol, in calf feed preparation, and also in the manufacture of inverted sugars as food for honeybees (*1*, *2*). Invertase has been widely studied especially in yeasts *Saccharomyces cerevisiae* and *Schwannionmyces occidentalus* (*3*−*5*) and in filamentous fungi such as *Aureobasidium* sp. ATCC 20524 (*6*) and *Aspergillus niger* (*7*).

Invertase is mainly used in the food (confectionery) industry where fructose is preferred over sucrose because it is sweeter and does not crystallize as easily. For health and taste reasons, its use in the food industry requires that invertase be highly purified. The hydrolyzed sugar mixture obtained by invertase has the advantage of being colorless in contrast to the colored products obtained by acid hydrolysis (*8*).

The filamentous fungus *Fusarium solani* produces a large amount of amyloglucosidase (*9*); however, as far as we know, it has not been studied as a possible producer of invertase. To exploit new industrial potentials of invertase, it is necessary to investigate new microbial strains and to understand the structure−stability relationship of this important enzyme. In this paper, we describe the production, purification, and thermal characterization of an invertase from a locally isolated *F. solani* strain in order to identify its economic advantages in industrial processes such as syrup production.

EXPERIMENTAL PROCEDURES

All of the chemicals used were of analytical grade and mainly purchased from Sigma (United States) unless otherwise mentioned.

Organism. A pure culture of *F. solani* was obtained from the National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture Faisalabad. It was propagated on potato dextrose agar slants.

Inoculum Preparation. Inocula were prepared by transferring spores from 5 to 6 days old slant cultures into 500 mL Erlenmeyer flasks containing 150 mL of sterile Vogel's medium. The composition of the inoculum medium was (g/L) as follows: glucose, 20.0; trisodium citrate, 2.5; KH_2PO_4, 5.0; NH_4NO_3, 2.0; $(NH_4)_2SO_4$, 4.0; $MgSO_4 \cdot 7H_2O$, 0.2; peptone, 2.0; trace element solution, 10 mL; and vitamin solution, 5.0 mL. The trace elements solution had the following composition (g/L): $CuSO_4$, 0.08; H_2MoO_4, 0.05; $MnSO_4 \cdot 4H_2O$, 0.07; $ZnSO_4 \cdot 7H_2O$, 0.043; and $Fe_2(SO_4)_3$, 0.05. The vitamin solution contained (g/L) the following: biotin,0.2; folic acid, 0.2; thiamine−HCl, 0.5; riboflavin, 0.5; pyridoxin HCl, 1.0; cyanocobalamine, 0.1; nicotinic acid; 0.5; Ca−pantothenate; 0.5; *p*-aminobenzoic acid, 0.5; and thioctic acid, 0.5. The pH of the medium was adjusted to 5.0 using 1 M HCl/1 M NaOH. The flasks were incubated on a rotary shaker at 150 rpm at 30 °C for 48 h to get a homogeneous spore suspension (10^6−10^7 spores mL^{-1}). The spore suspension was used as inoculum in the growth media for the production of invertase.

Invertase Production. Invertase was produced under liquid state growth conditions by cultivating the fungus in 250 mL Erlenmeyer

* To whom correspondence should be addressed. Tel: +92(041)-9200161-70/3309. Fax: +92-(041)-9200764. E-mail: mabajwapk@yahoo.com.

10.1021/jf053194g CCC: $33.50
Published on Web 06/06/2006

flasks containing 50 mL of the basal medium (BM). The composition of the BM was (g/L) as follows: molasses, 20.0; trisodium citrate, 2.5; KH_2PO_4, 5.0; NH_4NO_3, 2.0; $(NH_4)_2SO_4$, 4.0; $MgSO_4 \cdot 7H_2O$, 0.2; peptone, 2.0; microelement solution, 10 mL; vitamin solution, 5.0 mL; and initial pH, 5. The conditions were optimized for enhanced production of the enzyme by the one-variable-at-a-time approach.

Protein Determination. The protein concentration was determined by the method of Bradford (*10*) with bovine serum albumin (BSA) as the standard.

Invertase Assay. The invertase activity was determined using sucrose as the substrate. An appropriate amount (100 μL) of the enzyme solution was mixed with buffered (MES buffer, pH 5.0) 50 mM aqueous sucrose solution (w/v) and shaken in a water bath at 40 °C for 40 min. The reaction was quenched by placing tubes in a boiling water bath for 5 min and then immediately cooling on ice. The released glucose was determined using a glucose kit (Biocon, Germany) spectrophotometrically at 500 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent mL^{-1} min^{-1} at pH 5.0 and 40 °C.

Purification of Invertase. The crude extract containing the enzyme was filtered through Whatman no. 1 filter paper. Ammonium sulfate was added to make a 45% (w/v) saturation of the culture filtrate (450 mL), and the solution was left overnight at 4 °C. After centrifugation at 10000*g* for 15 min, the pellet of the precipitated proteins was discarded whereas the supernatant was treated with 40% (w/v) ammonium sulfate to give a final concentration of 85% (w/v).The solution was again kept overnight and centrifuged as described above. This time, the supernatant was discarded whereas the pellet containing invertase was dialyzed to remove salts. The dialyzed sample was applied on a DEAE-cellulose column equilibrated with Tris/HCl buffer pH 7.5. The linear gradient of NaCl (0−0.5 M) in 20 mM Tris/HCl, pH 7.5, was used as elution buffer. Three milliliter size fractions were collected.The active fractions were pooled and dialyzed against distilled water. The sample from DEAE-cellulose column was then applied to Sephadex G-200 column previously equilibrated with acetate buffer, pH 5.0, and eluted with the same buffer as described previously (*9*). Two milliliter size fractions were collected, and the total protein and enzyme activity were determined.

Subunit Molecular Mass. The purity of the purified enzyme and its subunit molecular mass were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) using the Laemmli system (*11*) on 10% polyacrylamide gel. The standard proteins were β-galactosidase (116 kDa), BSA (66.2 kDa), ovulbumin (45 kDa), lactate dehydrogenase (35 kDa), RE Bsp981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa) (middle molecular mass calibration kit, Fermentas).The gel containing different molecular mass markers was stained with Coomassie blue G-250 solution.

Effect of pH. 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 values ranging from 2 to 7 at 40 °C as described earlier (*9*, *12*).

Effect of Temperature and Activation Energy. Invertase was assayed at different temperatures ranging from 30 to 70 °C at pH 5.0 as described before. The activation energy for substrate hydrolysis was determined from the Arrhenius plot as described earlier (*13*, *14*).

Determination of Kinetic Constants. Invertase from *F. solani* was assayed in the reaction mixtures containing variable amounts of sucrose (2.0−5.0 mM) at pH 5.0. The data were plotted to determine the values of kinetic constants (V_{max} and K_m).

Kinetics of Thermal Inactivation. Kinetics of thermal inactivation of invertase was studied by incubating the enzyme in 50 mM sodium acetate buffer (pH 5.0) at different temperatures (50−70 °C) in the absence of substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to assay as described above. The residual activity was measured and expressed as a % of the initial activity. From a semilogarithmic plot of residual activity vs time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half-lives were estimated.

Activation Energy Calculations. The thermal stability of the invertase was determined by inactivation rate constant (k_d) as a function of temperature in the range 50−70 °C. The temperature dependence of k_d was analyzed from the Arrhenius plot; the activation energy (E_a) was obtained from the slope of the plot as described earlier (*15*). The activation enthalpy (ΔH^*) was calculated according to the equation

$$\Delta H^* = E_a - RT \quad (1)$$

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

The values for free energy of inactivation (ΔG^*) at different temperatures were obtained from the equation

$$\Delta G^* = -RT \ln(k_d h/kT) \quad (2)$$

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

The activation entropy (ΔS^*) was calculated from eq 3.

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (3)$$

Table 1. Purification of Invertase[a] from *F. solani*

treatment	total units	total protein (mg)	specific activity (U mg^{-1})	purification factor	% recovery
crude	4500	93.9	47.92	1.00	100
$(NH_4)_2SO_4$ precipitation (45–85%)	3890	61.2	63.56	1.33	86.44
anion exchange chromatography	1230	15.6	78.85	1.64	27.33
gel filtration	420	0.58	245.56	3.11	9.33

[a] All values were after dialysis against distilled water.

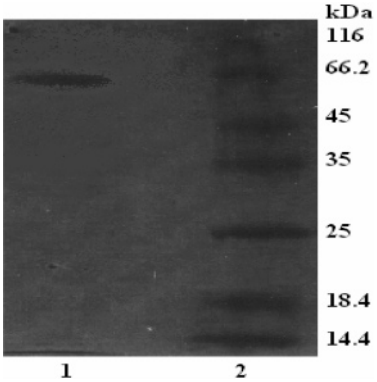

Figure 1. Ten percent SDS–PAGE of *F. solani* invertase. Lane 1, invertase; and lane 2, standard protein markers.

Effect of Protease. The effect of protease on the activity of invertase was determined by incubating the enzyme at 30 °C in 10 mM Tris/HCl buffer, pH 7, containing α-chymotrypsin (0.5 mg mL^{-1}) according to Rashid and Siddiqui (*14*). Different time course aliquots were withdrawn and immediately assayed for enzyme activity.

Effect of Urea. The effect of urea on the activity of invertase was determined by incubating the enzyme at 30 °C in 10 mM Tris/HCl buffer, pH 7, containing 4 M urea as described earlier (*14*). Aliquots withdrawn at different time intervals were immediately assayed at 40 °C for glucoamylase activity.

Effect of Metal Ions. The effect of metal ions on the activity of invertase was examined by incubating the enzyme in the presence of metal ion solution (1 mM) at 40 °C for 40 min. The residual activity (%) was determined by the standard method.

RESULTS AND DISCUSSION

All of the experiments and analyses were run in triplicate, and data values have been given as means ± standard errors (SE). The ±SE values have been presented as error bars in the figures.

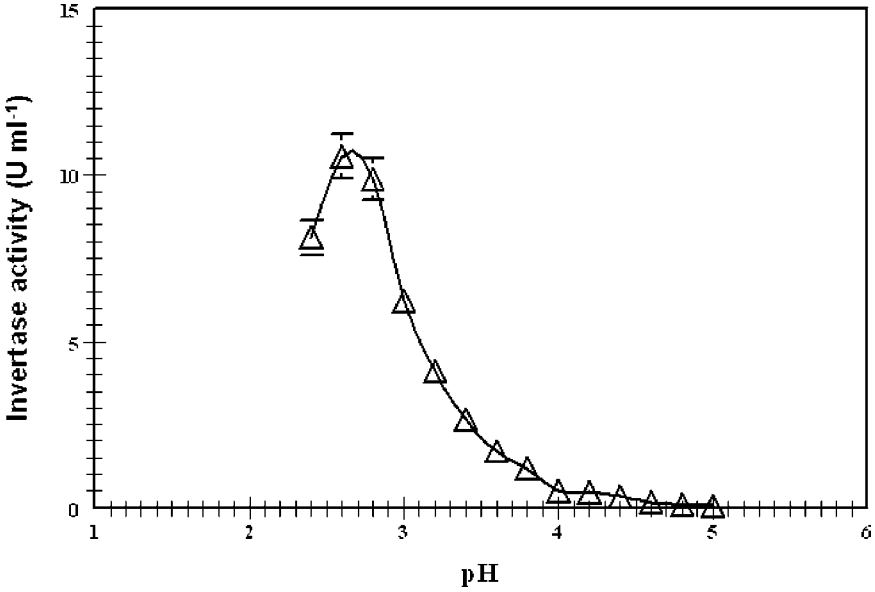

Figure 2. Effect of pH on *F. solani* invertase activity.

Production and Purification of Invertase. The maximum invertase activity (9.90 U mL^{-1}) was achieved after 96 h at pH 5.0 and 30 °C using molasses (2%) as the carbon source in submerged fermentation. Crude invertase was purified by ammonium sulfate precipitation, anion exchange chromatography, and gel filtration. The purification results are shown in **Table 1**. The crude extract contained 93.9 mg of total protein having a specific activity of 47.92 U mg^{-1}. Ammonium sulfate precipitation resulted in a 1.33-fold increase in specific activity of the enzyme with a recovery of 84.6%. The partially purified enzyme was then applied to anion exchange chromatography and gel filtration chromatography with % recoveries of 27.33 and 9.33, respectively. The elution profile from the G-100 column showed a single peak with symmetrical distribution of activity. The invertase yield obtained by this purification procedure is low; nevertheless, our goal was to purify and characterize the isolated enzyme.

Enzyme Purity and Apparent Molecular Mass. The invertase activity of the eluted protein was confirmed by testing the enzyme activity of the portions of the gel. The final enzyme preparation was homogeneous, as estimated by SDS−PAGE (**Figure 1**), and was purified 3.11-fold from the culture supernatant (**Table 1**). The apparent molecular mass of invertase as determined from the calibration curve of molecular mass protein standards run on SDS−PAGE was 65 kDa. Rubio et al. (*16*) purified an invertase from *Rhodotorula glutinis* by ammonium sulfate, anion exchange chromatography, and gel filtration, and the estimated molecular mass was found to be 47 kDa by SDS−PAGE. A molar mass of 50 kDa has been reported for an intracellular invertase from *A. niger* IAM 2544 (*17*).

Effect of pH. The results on effect of pH on invertase activity are presented in **Figure 2**. The pH for maximum sucrose hydrolysis was determined as 2.6. An increase in pH beyond optimum caused a rapid inactivation of the enzyme with 100% loss in activity observed at pH 5.0. A pH optimum from 3.85 to 5.5 has previously been reported for invertases isolated from different yeasts and fungi (*16*, *18*−*21*). 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 nonessential residues. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity.

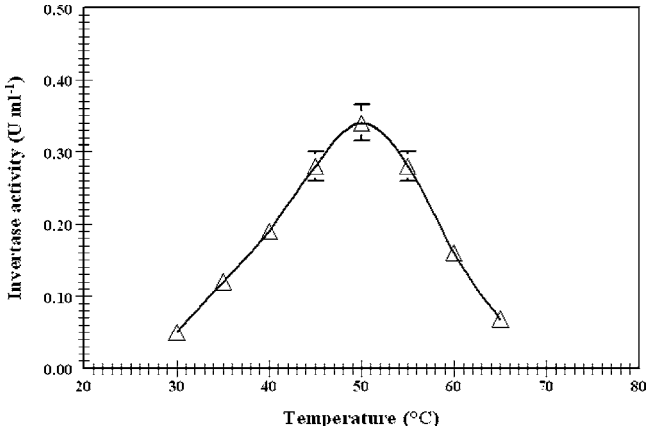

Figure 3. Effect of temperature on *F. solani* invertase activity.

Effect of Temperature on the Catalytic Activity. Invertase was assayed at different temperatures, i.e. 30, 35, 40, 45, 50, 55, 60, and 65 °C, at pH 5.0. **Figure 3** shows the effect of temperature on the catalytic activity of invertase isolated from *F. solani.* In the bell-shaped curve, the maximum activity of the enzyme was at 50 °C. An Arrhenius plot in the temperature range from 30 °C to the optimum appears linear, and the activation energy (E_a) was found to be 4.06 kJ mol^{-1}. It is obvious from the Arrhenius plot (**Figure 4**) that the enzyme had a single conformation up to the transition temperature. The value of E_a for invertase isolated from *F. solani* was approximately 1.52- and 6.71-fold less than that of isolated from baker's yeast (*21*) and a strain of *R. glutinis* (*16*), respectively. This indicates that less amount of energy is required to form the activated complex with sucrose in the case of invertase obtained from *F. solani* as compared to other reported invertases.

Kinetic Constants. Kinetic parameters of the enzymatic reaction were estimated by the direct linear method of the Lineweaver−Burk plot of the initial sucrose hydrolysis rates from the experimental data. The Michaelis constants K_m and V_{max} for invertase estimated from the Lineweaver−Burk plot were 3.57 mM and 0.33 μmol min^{-1} (**Figure 5**). The value of apparent K_m for invertase isolated from *F. solani* was ap-

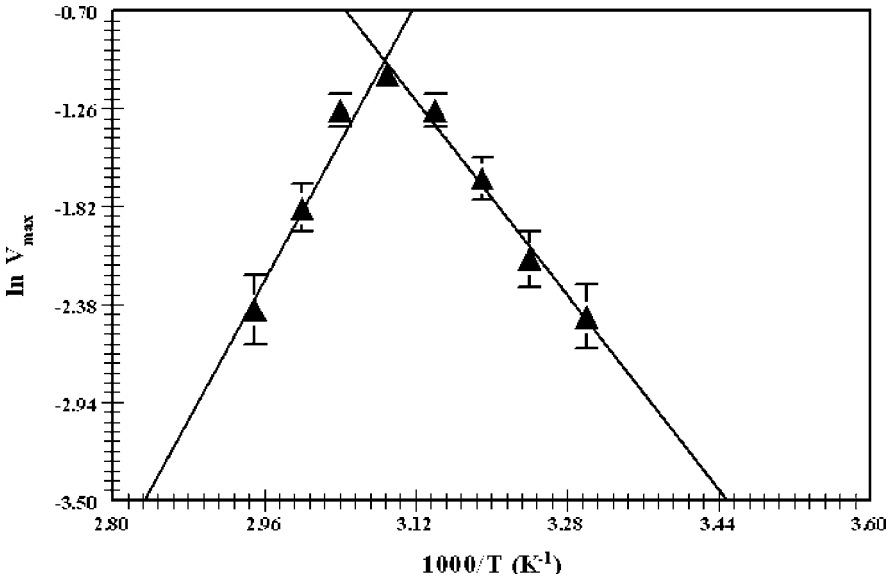

Figure 4. Arrhenius plot for activation energy of sucrose hydrolysis by *F. solani* invertase.

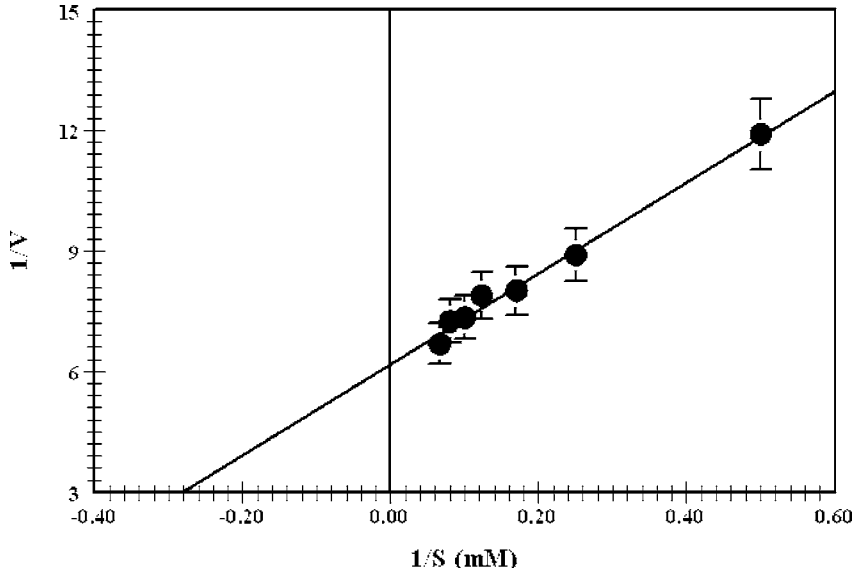

Figure 5. Double reciprocal plots to determine the kinetic constants for sucrose hydrolysis by *F. solani* invertase at 40 °C.

Table 2. Kinetic and Thermodynamic Parameters for Thermal Inactivation of Invertase from *F. solani*[a]

	min^{-1}		kJ mol^{-1}		
temp (K)	k_d	$t_{1/2}$	ΔH^*	ΔG^*	ΔS^* (J mol^{-1} K^{-1})
323	0.053	13.07	1.379	98.28	–300.00
328	0.151	4.58	1.338	96.90	–291.34
333	0.219	3.16	1.29	97.39	–297.52
338	0.286	2.42	1.25	98.15	–286.68
343	0.337	2.05	1.21	99.17	–285.59

[a] E_a = 4.06 kJ mol^{-1} (calculated from **Figure 7**). k_d values (first-order rate constant of denaturation) are determined from **Figure 6**. $t_{1/2}$ (half-life) = $0.693/k_d$. $\Delta H^* = E_a$ (4.06 kJ mol^{-1}) – RT. $\Delta G^* = -RT \ln (k_{cat}h/k \cdot T)$. $\Delta S^* = (\Delta H^* - \Delta G^*)/T$.

proximately 3.08-fold less than that of isolated from *Candida utiliz* (*19*). A comparison with the results obtained by Akgol et al. (*21*) showed that the K_m value of *F. solani* invertase was 6.72-fold lower than that obtained from baker's yeast. This indicates that the invertase isolated from *F. solani* has a higher

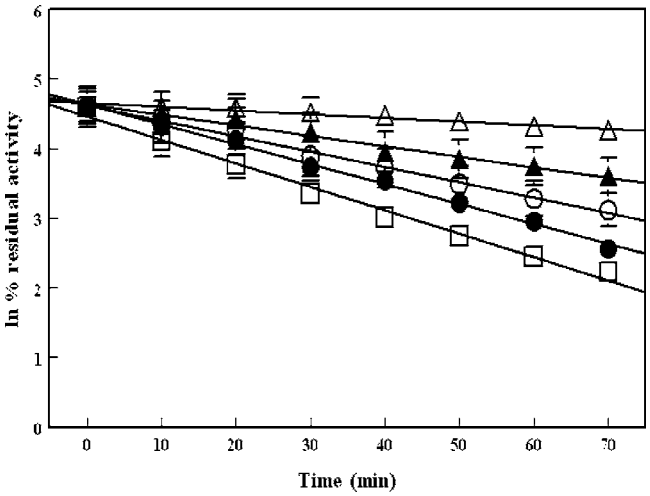

Figure 6. First-order plots of the effect of thermal denaturation of *F. solani* invertase. Samples were incubated at 50 (△), 55 (▲), 60 (○), 65 (●), and 70 °C (□) in 50 mM sodium acetate buffer, pH 5.0.

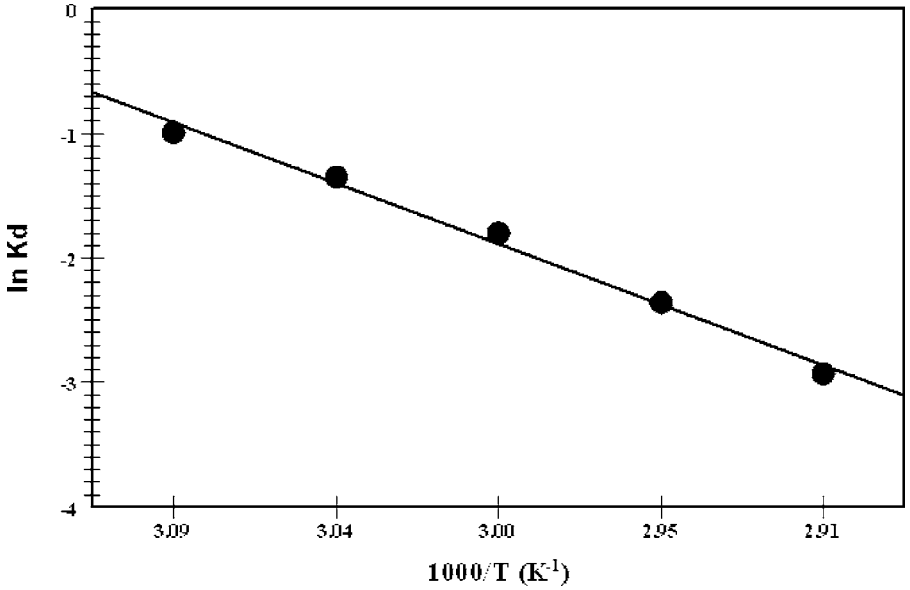

Figure 7. Arrhenius plots for the determination of energy of activation for thermal inactivation of *F. solani* invertase.

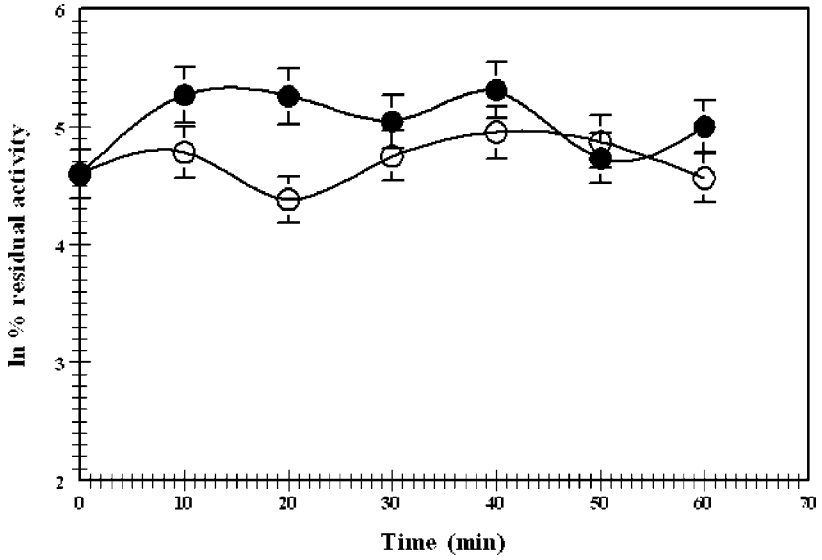

Figure 8. Effect of α-chymotrypsin (○) and 4 M urea (●) on *F. solani* invertase activity.

affinity for the sucrose. Moreover, the V_{max} value of the enzyme is 3.44-fold higher than that reported from a strain of *R. glutinis* (*16*), indicating that the invertase possesses a high catalytic activity.

Kinetics of Thermal Inactivation. 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 (*22*). The results regarding the thermal inactivation of invertase are shown in **Table 2** and **Figures 6** and **7**. The thermal denaturation of enzymes is accompanied by the disruption of noncovalent linkages, including hydrophobic interactions, with a concomitant increase in the enthalpy of activation (ΔH^*) (*23*). The opening up of enzyme structure is accompanied by an increase in disorder or entropy of activation (ΔS^*) (*24*), but contrary to this, we found that the invertase from *F. solani* has negative ΔS^* and positive ΔH^* between the temperature range of 50 and 70 °C (**Table 2**). The denaturation of many proteins such as chicken egg albumin (*25*) and lactoglobulin at low temperatures and in the presence of urea (*26*) shows a negative ΔS^* because water ordering increases in the vicinity of nonpolar amino acids, which are exposed during unfolding (*27*). 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 invertase. The unfolding of hemoglobin in acid at 45 °C also shows negative ΔS^* of -155 J mol^{-1} K^{-1} (*25*), whereas the thermal denaturation of CMCas from *A. niger* at 65 °C gave ΔS^* of -209 J mol^{-1} K^{-1} (*13*). In case of α-amylase (apo-enzyme) from *Bacillus licheniformis*, thermal denaturation even at a higher temperature of 80 °C gave ΔS^* of -150 J mol^{-1} K^{-1}, ΔG^* of kJ mol^{-1}, and ΔH^* of 50 kJ mol^{-1}, whereas surprisingly the same enzyme in the presence of Ca^{2+} ions gave positive ΔS^* (*28*). Apart from *A. niger* CMCase, this is the only other enzyme with negative ΔS^* at such a high temperature. At low temperatures, some enzymes such as chymotrypsinogin show a 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 invertase suggested that there was negligible disorderness as that of β-glucosidase from *A. wentii* (*29*). The value of ΔH^* decreased with an increase in the

Table 3. Effect of Metal Ions on Invertase Activity

compound	concn (mM)	relative activity (%)	compound	concn (mM)	relative activity (%)
control (H_2O)	1	100	$Pb(NO_3)$	1	99
KCl	1	120	$HgCl_2$	1	100
NaCl	1	160	$MgSO_4$	1	205
$CuSO_4$	1	220	$CoCl_2$	1	169
$BaCl_2$	1	150			

temperature indicating that the conformation of the enzyme was altered. Moreover, a high value (99.17 kJ mol^{-1}) for free energy of thermal denaturation (ΔG^*) at 70 °C indicated that the invertase exhibited a resistance against thermal unfolding at higher temperatures.

Enzymes could be made more thermostable by either stabilizing the native form by putting noncovalent bonds including hydrogen bonds, salt bridges, and hydrophobic interactions or by decreasing the entropy of unfolding (*23*). Other possible reasons for stabilization of reactive proteins have been reviewed by Matthews (*30*). Immobilization also causes a drop in the values of both ΔH^* and ΔS^*. A further decrease in these variables makes the enzyme more thermostable (*31*).

Effect of Protease and Urea. The time course studies of the effect of protease (α-chymotrypsin) on invertase activity revealed a complex pattern showing periodic activation and inhibition (**Figure 8**). The results indicate that different conformational forms of invertase are generated as a result of successive proteolytic nicking. Rashid and Siddiqui (*14*) also reported a periodic trend of the effect of proteases on the activity of β-glucosidase from *A. niger*. For the same reason, urea treatment also showed periodic gain and loss in activity of invertase. Therefore, invertase from *F. solani* could be used in a hydrophobic environment.

Effect of Metals on Invertase Activity. As shown in **Table 3**, Pb^{2+} and Hg^{2+} ions did not inhibit the enzyme activity at a concentration of 1 mM. The results revealed that Cu^{2+} and Mg^{2+} ions stimulated invertase activity about 2-fold while Na^+, Ba^{2+}, and Co^{2+} increased it about 1.5-fold as compared to enzyme activity in the absence of such ions (control). The effect of metal ions could be important in the use of raw materials with a high salt content. In contrast, these metal ions inhibited invertase activity in other microorganisms such as *A. japonicusi* (*6*). Hg^{2+} does not affect invertase activity, which suggests that tryptophan and cysteine are not essential for its activity.

In conclusion, we have shown that the invertase isolated from a culture filtrate of *F. solani* has a high sucrose affinity and could be used in food industries. Moreover, it has been found that apart from α-amylase from *B. licheniformis* (*28*) and CMCas from *A. niger* (*13*), invertase also has a transition state of negative entropy at high temperatures.

LITERATURE CITED

(1) Chen, J.; Saxton, J.; Hemming, F. W.; Puberty, J. F. Purification and partial characterization of high and low molecular weight form (S- and F-form) of invertase secreted by *Aspergillus nidulans*. *Biochem. Biophys. Acta*. **1996**, *1296*, 207−218.

(2) Sanchez, M. P.; Huidobro, J. F.; Mato, I.; Munigategui, S.; Sancho, M. T. Evolution of invertase activity in honey over two years. *J. Agric. Food Chem*. **2001**, *49*, 416−422.

(3) Fontana, A.; Ghomm, C.; Guiraud, J.; Navarro, J. Continuous alcoholic fermentation of sucrose using using flocculating yeast. The limits of invertase activity. *Biotechnol. Lett*. **1992**, *14*, 505−510.

(4) Costaglioli, P.; Meilhoc, F.; Janatova, I.; Klein, R.; Mason, J. Secretion of invertase from *Schwannionmyces occidentalus*. *Biotechnol. Lett*. **1997**, *19*, 632−627.

(5) Kiran, S.; Ali, S.; Haq, I. Time course study for yeast invertase production by submerged fermentation. *J. Biol. Sci*. **2003**, *3*, 984−988.

(6) Hayashi, S.; Nonoguchi, M.; Shimokawa, Y.; Takasaki, Y.; Imada, K. Effect of deglycosylation on the properties of β-frucofuranosidase P-1 from *Aureobasidium sp*. ATCC 20524. *J. Ind. Microbiol*. **1992**, *9*, 251−255.

(7) Romero-Gomez, S.; Auger, C.; Viniegra-Gonzalez, G. Invertase production by *Aspergillus niger* in submerged and solid-state fermentation. *Biotechnol. Lett.* **2000**, *22*, 1255−1258.

(8) Bayramoglu, M. Y.; Senel, S.; Alaeddinoglu, N. G.; Patir, S.; Arica, M. Y. Covalent immobilization of invertase on to a reactive film composed of 2-hydroxyethyl methacrylate and glycidyl methacrylate: Properties and applications in continuous flow system. *Biochem. Eng. J*. **2003**, *14*, 117−126.

(9) Bhatti, H. N.; Huma, F.; Nawaz, R.; Sheikh, M. A.; Rashid, M. H.; Khalid, A. M. Partial purification and properties of amyloglucosidase from *Fusarium solani*. *Int. J. Biol. Biotechnol.* **2005**, *2*, 577−582.

(10) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(11) Laemmli, U. K. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(12) Stamford, T. L.; Stamford, N. P.; Coelho, L. C.; Araujo, I. M. Production and characterization of a thermostable glucoamylase from Streptosporangium sp. Endophyte of maize leaves. *Bioresour. Technol.* **2002**, *83*, 105−109.

(13) Siddiqui, K. S.; Azhar, M. J.; Rashid, M. H.; Rajoka, M. I. Stability and identification of active site residues of carboxymethylcellulase from *Aspergillus niger* and *Cellulomonas biazotea*. *Folia Microbiol.* **1997**, *42*, 312−318.

(14) Rashid, M. H.; Siddiqui, K. S. Thermodynamic and kinetic study of stability of the native and chemically modified β-glucosidases from *Aspergillus niger*. *Proc. Biochem.* **1998**, *33*, 109−115.

(15) Siddiqui, K. S.; Shamsi, A. M.; Anwar, M. A.; Rashid, M. H.; Rajoka, M. I. Partial and complete alteration of surface charges of carboxymethylcellulase by chemical modification: Thermo stabilization in water-miscible organic solvent. *Enzyme Microb. Technol*. **1999**, *24*, 599−608.

(16) Rubio, M. C.; Runco, R.; Navarro, A. R. Invertase from a strain of *Rhodotorula glutinis*. *Phytochemistry* **2002**, *61*, 605−609.

(17) Muramatsu, M.; Kakuki, T. Enzymatic synthesis of novel fructosyl and oligofructosyl trehaloses by Aspergillus sydowi β-fructofuranosidase. *Biosci., Biotechnol., Biochem.* **2005**, *59*, 208−212.

(18) Quiroga, E. N.; Vattunone, M. A.; Sampietro, A. R. Purification and characterization of invertase from *Pycnoporus sanguineus*. *Biochim. Biophys. Acta* **1995**, *1251*, 75−80.

(19) Chavez, F. P.; Rodriguez, L.; Diaz, J.; Delgado, J. M.; Cremata, J. A. Purification and characterization of an invertase from Candida utilities: Comparison with natural and recombinant yeast invertases. *J. Biotechnol.* **1997**, *53*, 67−74.

(20) Hocine, L. L.; Wang, Z.; Jiang, B.; Xu, S. Purification and partial characterization of fructosyltransferase and invertase from *Aspergillus niger* AS0023. *J. Biotechnol.* **2000**, *81*, 73−84.

(21) Akgol, S.; Kacar, Y.; Denizli, A.; Arica, M. Y. Hydrolysis of sucrose by invertase immobilized onto novel magnetic poly(vinyl alcohol) microspheres. *Food Chem.* **2001**, *74*, 281−288.

(22) Georis, J.; Esteves, F. L.; Brasseur, J. L.; Bougnet, V.; Devreese, J. L. B.; Giannotta, F.; Granier, B.; Frere, J. M. An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. *Protein Sci*. **2000**, *9*, 466−475.

(23) Daniel, R. M. The upper limits of enzyme thermal stability. *Enzyme Microb. Technol*. **1996**, *19*, 74−79.

(24) Vieille, C.; Zeikus, J. G. Thermozymes: Identifying molecular determinants of protein structural and functional stability. *TIBIOTECH* **1996**, *14*, 183−190.
(25) Eyring, H.; Stearn, A. E. The application of the theory of absolute reaction rates to protein. *Chem. Rev*. **1939**, *24*, 253−270.
(26) Nelson, C. A.; Hummel, J. P. Reversible denaturation of pancreatic ribonuclease by urea. *J. Biol. Chem*. **1962**, *237*, 1567−1574.
(27) Privalov, P. L.; Gill, S. J. Stability of protein structure and hydrophobic interaction. In *Advance Protein Chemistry*; Anfirses, C. B., Edsal, J. T., Richards, F. M., Eisenberg, D. S., Eds.; Academic Press: San Diego, 1988; Vol. 39, pp 191−234.
(28) Violet, M.; Meunier, J. C. Kinetic study of irreversible thermal denaturation of *Bacillus licheniformis* alpha-amylase. *Biochem. J.* **1989**, *263*, 665−670.
(29) Declerck, N.; Machius, M.; Joyet, P.; Weigand, G.; Hubber, R.; Gaollardin, C. Hyperthermostabilization of *Bacillus licheniformis* alpha-amylase and modulation of its stability over a 50 °C temperature range. *Protein Eng*. **2003**, *16*, 287−293.
(30) Matthews, B. W. Structural and genetic analysis of protein stability. *Annu. Rev. Biochem*. **1993**, *62*, 139−160.
(31) Saleem, M.; Rashid, M. H.; Jabbar, A.; Perveen, R.; Khalid, A. M.; Rajoka, M. I. Kinetic and thermodynamic properties of an immobilized endoglucanase from *Arachniotus citrinus*. *Proc. Biochem*. **2005**, *40*, 849−855.

Received for review December 21, 2005. Revised manuscript received April 24, 2006. Accepted May 3, 2006.

JF053194G