Thermal stability of α-amylase in aqueous cosolvent systems

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The activity and thermal stability of α-amylase were studied in the presence of different concentrations of trehalose, sorbitol, sucrose and glycerol. The optimum temperature of the enzyme was found to be 50 ± 2°C. Further increase in temperature resulted in irreversible thermal inactivation of the enzyme. In the presence of cosolvents, the rate of thermal inactivation was found to be significantly reduced. The apparent thermal denaturation temperature $(T_m)_{app}$ and activation energy (E_a) of α-amylase were found to be significantly increased in the presence of cosolvents in a concentration-dependent manner. In the presence of 40% trehalose, sorbitol, sucrose and glycerol, increments in the $(T_m)_{app}$ were 20°C, 14°C, 13°C and 9°C, respectively. The E_a of thermal denaturation of α-amylase in the presence of 20% (w/v) trehalose, sorbitol, sucrose and glycerol was found to be 126, 95, 90 and 43 kcal/mol compared with a control value of 40 kcal/mol. Intrinsic and 8-anilinonaphthalene-1-sulphonic acid (ANS) fluorescence studies indicated that thermal denaturation of the enzyme was accompanied by exposure of the hydrophobic cluster on the protein surface. Preferential interaction parameters indicated extensive hydration of the enzyme in the presence of cosolvents.

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1. Introduction

Enhancement of the structural stability of enzymes is of great importance for their application in several industrial processes. The mechanism of thermal denaturation depends on various physicochemical parameters of the solution in which the reaction is catalysed. Any process that enhances the structural stability and rate of reaction of enzymes has a favourable impact on their industrial application (Wasserman 1984; Timasheff 1998; Matsumoto *et al.* 1999; Sousa 2009). It has been shown that aqueous solutions of sugars and polyhydric alcohols protect the native conformation of various enzymes and proteins. Stabilization of various enzymes by sugars and polyols has been extensively described (Breccia *et al.* 1999; Bustos *et al.* 1999; Li *et al.* 2000; Saunders *et al.* 2000; Wendorf *et al.* 2004; Domenico *et al.* 2004; Ashie *et al.* 2008; Gangadhara *et al.* 2008; Sukenik and Harries 2009). Since many organisms synthesise certain polyhydric alcohols in response to environmental stress conditions, they have evolutionary significance (Bolen and Baskakov 2001; Bolen 2004). However, some cosolvents such as ethanol and polyethylene glycol are known to destabilize various enzymes and proteins (Gekko 1981; Hancock and Hsu 2000; McClement 2002; Cioci and Lavecchia 2004; Kamatari *et al.* 2008).

It has been shown that the presence of cosolvents in protein solution changes the physicochemical properties of the protein mainly by altering solvent/water structure (Xie and Timasheff 1997a, b, c; Rosgen and Hinz 2000; De Cordt *et al.* 2004a, b). The interactions between protein, solvent and cosolvent can be understood in terms of various thermodynamic and preferential interaction parameters

Keywords. α-Amylase; cosolvents; preferential interaction parameters; thermal stability

Abbreviations used: ANS, 8-anilinonaphthalene-1-sulphonic acid; BSA, bovine serum albumin; CNPG3, 2-chloro-p-nitrophenyl-α-D-maltotrioside; $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$, preferential interaction parameter; E_a, activation energy; F_u, fraction unfolded; TIM, triosephosphate isomerase; $(T_m)_{app}$, apparent thermal denaturation temperature

(Klibanov 1983). The surface of protein is heterogeneous in nature and can greatly influence solvent structure and the distribution of cosolvents in its vicinity (Tony *et al.* 1994). The degree of cosolvent-mediated protein stabilization under various denaturing conditions depends largely upon the nature of the cosolvent, its interaction with water molecules and nature of the protein itself (Arakawa and Timasheff 1982; Rajendran *et al.* 1995; Kaushik and Bhat 2003; Mukaiyama *et al.* 2007; Venkatesu *et al.* 2007). Preferential interaction parameters provide the evidence that cosolvents stabilize proteins/enzymes by their partial preferential exclusion from protein surfaces, but the detailed mechanisms of stabililization have not yet been understood (Arakawa and Timasheff 1982; Gupta *et al.* 2003). In this investigation, α-amylase, a well-studied molecule, was used as a model enzyme to study the mechanism of thermal stabilization in the aqueous systems of trehalose, sorbitol, sucrose and glycerol.

α-Amylase (α-D-1, 4-glucan glucanohydrolase; EC: 3.2.1.1) is a starch-degrading enzyme which catalyses the hydrolysis of α-D-1, 4-glycosidic bonds in starch, producing maltose and various oligosaccharides. Various α-amylases have been used in the starch liquefaction process for a long time and have tremendous potential in the food, textile, paper and pharmaceutical industries. Bacterial α-amylases, especially from the genus *Bacillus*, are widely used in various starch-based industries; the majority of industrial applications require their use at high temperatures (Nielsen and Borchert 2000; Pandey *et al.* 2000). α-Amylase is a metalloenzyme that requires at least one calcium ion per enzyme molecule for its activity and structural stability (Vihinen and Mantsala 1989). It belongs to family-13 and its whole structure is divided into three domains known as domains A, B and C. Domain A consists of a central $(\beta/\alpha)_8$ structure, also known as TIM (triosephosphate isomerase), a barrel with highly symmetrical folds of eight inner parallel β-strands surrounded by eight α-helices. Domain B is inserted between the third β-strand and third α-helix of the $(\beta/\alpha)_8$ structure. Domain C is constituted by the C-terminal end of the amino acid sequence with a Greek key motif (Farber and Petsko 1990; Nielsen and Borchert 2000).

2. Materials and methods

2.1 *Materials*

α-Amylase type II (A6380), trehalose, sorbitol, sucrose, glycerol, corn starch, maltose, dinitrosalisylic acid, 8-anilinonaphathalene-1-sulphonic acid (ANS) and $CaCl_2$ were procured from Sigma Chemicals Company, St Louis, MO, USA. 2-Chloro-p-nitrophenyl-α-D-maltotrioside (CNPG3) was procured from Pointe Scientific, Inc., Canton, MI, USA. All chemicals used were of analytical grade. The enzyme was dialysed against 0.02 M citrate buffer (pH 5.9) to remove any additives present, freeze-dried (–80ºC) and desiccated at 0°C for further use. This enzyme was found to be homogeneous on SDS-PAGE and used for all experiments. Quartz triple distilled water was used for the preparation of all the solutions and 2 mM of $CaCl_2$ was used throughout the experiment.

2.2 *Determination of protein concentration*

Protein concentration of α-amylase solution was determined by using the extinction coefficient of α-amylase ($E_{1\%}^{1cm}$) in aqueous medium. The absorbance of the enzyme solution was recorded at 280 nm in a Shimadzu UV-1601 UV-Visible spectrophotometer (Japan) and the concentration of α-amylase was calculated using an $E_{1\%}^{1cm}$ value of 14.46 (Rajendran *et al.* 1995).

$$A_{280} = \varepsilon \, c \, l, \quad (1)$$

where ε is the extinction coefficient, c is the concentration of protein in mg/ml and l is the path length in cm. Alternatively, the concentration of α-amylase solution was also estimated by the Lowry method (Lowry *et al.* 1951) using bovine serum albumin (BSA) as standard.

2.3 *Determination of α-amylase activity*

2.3.1 *Using starch as a substrate:* α-Amylase activity was measured by using the Bernfeld method (Bernfeld 1955) for estimation of reducing sugar. The enzyme solution was prepared in 0.02 M citrate buffer, pH 5.9, containing 2 mM of $CaCl_2$. The enzyme and starch solution was preincubated for 5 min at 37°C. The reaction mixture (2 ml), containing 1 ml of 1% starch solution and 1 ml of enzyme solution, was incubated for 5 min in a temperature-controlled shaking water bath at 37°C. The enzymatic reaction was terminated by the addition of 2 ml of 1% (w/v) alkaline dinitrosalicylic acid solution. The reaction mixture was subjected for 10 min to a boiling water bath for colour development. After cooling, it was diluted five times using triple distilled water, mixed thoroughly, and the absorbance recorded at 540 nm in a Shimadzu UV-1601 UV-Visible spectrophotometer (Japan). The activity of α-amylase was determined by using the standard plot of maltose. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1.0 μmol equivalence of maltose at pH 5.9 at 37°C in 5 min from 1 ml of 1% (w/v) corn starch solution.

2.3.2 *Using CNPG3 as substrate:* The α-amylase activity was also determined by using an artificial substrate, 2-chloro-p-nitrophenyl-α-D-maltotrioside (CNPG3). The enzyme hydrolyses CNPG3 to release 2-chloro-nitrophenol,

maltotriside and glucose (Dupuy *et al.* 1987; Foo and Bais 1998). The amount of 2-chloro-nitrophenol released reflects the degree of hydrolysis, which was monitored by spectrophotometry. Of the enzyme solution, 25 μl was added to 1 ml of CNPG3 solution and incubated at 37°C for 3 min, and absorbance was recorded at 405 nm in a Shimadzu UV-1601 UV-Visible spectrophotometer. Enzyme activity was determined using the following equation:

$$\text{Unit of } \alpha\text{-amylase} = \frac{\Delta\text{Abs} / \text{min x total assay volume}}{\text{milimolar absorptivity of CNPG3 x sample volume x } l} \quad (2)$$

where l is the path length of light and ΔAbs is the change in the absorbance at 405 nm. The specific activity of the enzyme was obtained by dividing the total activity with the protein concentration.

2.4 *Thermal inactivation of α-amylase*

Enzyme solutions were prepared in different concentrations of trehalose, sorbitol, sucrose and glycerol, and allowed to equilibrate for 12 h under cold conditions (8°C). Thermal inactivation of α-amylase was done by incubating the enzyme solution at 60°C in the presence of different concentrations of cosolvents. Samples were withdrawn after various incubation periods, placed on an ice bath and the residual specific activity was determined at 37°C. Enzyme without heat treatment was treated as the control and the percentage residual activity was calculated based on the original activity. The thermal inactivation of α-amylase was also carried out at different temperatures in the presence of 20% (w/v) of each cosolvent at temperatures ranging between 30 and 80°C. Enzyme solutions were incubated for 10 min at each temperature, cooled on an ice bath and the residual activity measured at 37°C. Enzyme solution in buffer was considered as the control.

2.5 *Thermal denaturation of α-amylase*

Thermal denaturation of α-amylase in the presence of different concentrations of each cosolvent was performed by recording the spectra in a Cary Varian 100-bio UV-Visible spectrophotometer (Mulgrave Victoria, Australia) at 287 nm using a path length of 1 cm and scan rate of 1°C/min in the temperature range of 30–90°C. Throughout the experiments, the concentration of protein solution was 8.6 x 10^{-6} M in 0.02 M citrate buffer, pH 5.9 containing 2 mM $CaCl_2$. The $(T_m)_{app}$ of α-amylase was obtained after normalizing the absorbance of the native and denatured states of the enzyme. The fraction unfolded (F_u) of enzyme were obtained at different temperatures and $(T_m)_{app}$ was determined using the following equations:

$$F_u = \frac{A_T - A_N}{A_D - A_N}, \quad (3)$$

where A_N and A_D are the absorbances of the native and denatured states of the protein and A_T is the absorbance of protein at temperature T (ºC). The standard enthalpy change (ΔH^o) and standard entropy change (ΔS^o) at $(T_m)_{app}$ were calculated using the equilibrium constant (K) of protein denaturation (Tanford 1968; Pace and Scoltz 1997; Atkin and Paula 2006).

$$K = F_u / 1 - F_u \quad (4)$$

$$\Delta G^o = -\text{RT ln } K, \quad (5)$$

At $(T_m)_{app}$ the native and denatured states of the protein remain in equilibrium, therefore K = 1 and ΔG^o = 0. As per the standard equation (Tanford 1968; Pace and Scoltz 1997):

$$\Delta S^o = \Delta H^o / T \quad (6)$$

$$\Delta H^o = -R\, d(\ln K) / d(1/T), \quad (7)$$

where R is the universal gas constant and T is the absolute temperature.

2.6 *Thermal denaturation kinetics of α-amylase*

The rate of thermal denaturation of α-amylase was estimated by recording the spectra at 287 nm using a path length of 1 cm in a Cary Varian 100-bio UV-Visible spectrophotometer (Mulgrave Victoria, Australia). The isothermal denaturation of α-amylase was carried out in the presence of 20% (w/v) solution of each cosolvent at temperatures of 60°C, 62°C, 64°C, 66°C and 68°C. The log of percentage residual native structure versus time of denaturation was plotted at each temperature and apparent rate constants (k) were obtained from the slope of the straight line. Using the Arrhenius equation (the lnk versus $1/T$) was plotted and the E_a was calculated from the slope using methods described elsewhere (Privalov 1979; Samborska *et al*. 2005; Atkin and Paula 2006).

2.7 *Fluorescence measurements*

Intrinsic and ANS fluorescence measurements of α-amylase were carried out in the presence of 20% (w/v) trehalose, sorbitol, sucrose and glycerol using a Shimadzu Spectrofluorophotometer model RF-5000. The enzyme solutions were preincubated in 20% (w/v) of each cosolvent and allowed to equilibrate in cold conditions for 12 h. Intrinsic fluorescence spectra of the enzyme in each

cosolvent were recorded after incubation at 60°C for 30 min. The excitation was set at 280 nm and emission spectra were recorded in the range of 300–400 nm. For measurement of the ANS fluorescence, the enzyme samples were prepared in 20% solution of each cosolvent, incubated at 60°C for 30 min and cooled to 25°C. Aliquots of ANS stock solution were added to the enzyme samples, mixed well and allowed to equilibrate in the dark for 20 min. Appropriate controls of the respective cosolvents were taken to eliminate the interference in fluorescence spectra of the enzyme. The molar ratio of protein and ANS was adjusted as 1:100. The excitation wavelength was set at 380 nm and emission spectra were recorded in the range of 400–600 nm (Lehrer 1971; Eftink and Ghiron 1982; Engelhard and Evan 1995).

2.8 *Measurements of partial specific volume and preferential interaction parameters*

The density of α-amylase in the presence of different concentrations of trehalose and sorbitol were measured using a precise densitometer (Anton Par DMA-4500/5000, Software: V5.009b, Austria). The density measurements were carried out at 20°C and partial specific volumes of the enzyme were calculated under both isomolal and isopotential conditions. The partial specific volumes and preferential interaction parameters were calculated using standard procedures described elsewhere (Lee and Timasheff 1974, 1981; Lee *et al.* 1979; Prakash and Timasheff 1981). The samples of α-amylase were dried in small glass vials over phosphorus pentoxide at 40°C for 36 h in a vacuum oven. After cooling to 20°C, 2 ml of 0.02 M citrate buffer, pH 5.9 or the respective cosolvent solutions were added, sealed by thermoplastic self-sealing film (Parafilm M) and allowed to stand for 4 h before the density measurements (isomolal) were taken. For the isopotential condition, protein samples were dialysed against the respective cosolvent solutions to achieve complete equilibrations for 36 h in cold (14°C) and brought to 20°C before density measurements. The notations of the components in the systems were set in accordance with Stochmayer (1950); buffer as component 1, protein as component 2 and cosolvents as component 3. The apparent partial specific volumes of α-amylase in isomolal (Φ_2) and isopotential (Φ'_2) conditions were measured as a function of protein concentrations and extrapolated to zero protein concentrations to obtain Φ^0_2 and Φ'^0_2. The isomolal and isopotential terms refer to the condition in which the molarity of the solvent component and their chemical potential, respectively, are kept identical in the protein solution and reference solvents. The partial specific volume of protein (Φ_2) is related to the density of protein and buffer as described elsewhere (Stockmayer 1950; Kielley and Harrington 1960; Inoue and Timasheff 1972).

$$\Phi_2 = 1/\rho_0\,[1-\{(\rho-\rho_0)/c\}], \tag{8}$$

where ρ and ρ_0 are the density of the protein solutions and corresponding buffer, respectively, c is the concentration of protein expressed in g/ml. The preferential interaction parameters of α-amylase were calculated from the following equation (Inoue and Timasheff 1972).

$$(\delta g_3/\delta g_2)_{T,\mu1,\mu3} = \rho_0(\Phi^0_2 - \Phi'^0_2)/(1-\rho_0\bar{v}_3) \tag{9}$$

$$(\delta g_1/\delta g_2)_{T,\mu1,\mu3} = -1/g_3\,(\delta g_3/\delta g_2)_{T,\mu1,\mu3} \tag{10}$$

$$(\delta m_3/\delta m_2)_{T,\mu1,\mu3} = (M_2/M_3)\,(\delta g_3/\delta g_2)_{T,\mu1,\mu3}, \tag{11}$$

where gi is the concentration of component i in gram per gram of buffer, T is the absolute temperature (Kelvin), μi is the chemical potential of component i and $\bar{v}_3$ is the partial specific volume of component 3. The preferential interaction and preferential exclusion parameters of the cosolvents, respectively, with the protein molecule are $(\delta g_3/\delta g_2)_{T,\mu1,\mu3}$ and $(\delta g_1/\delta g_2)_{T,\mu1,\mu3}$. $(\delta m_3/\delta m_2)_{T,\mu1,\mu3}$ is the preferential interaction parameter of the cosolvents with proteins in molar terms, whereas the *mi* and *Mi* are the molal concentration and molecular weight of component *i*, respectively.

3. Results

3.1 *Effect of cosolvents on thermal inactivation of α-amylase*

α-Amylase showed optimum activity at a temperature of 50 ± 2°C. Increasing the temperature of the reaction mixture above 55°C leads to irreversible thermal inactivation of the enzyme. Addition of cosolvents to the enzyme solution was shown to have a significant impact on thermal inactivation. The rate of thermal inactivation of α-amylase was found to be significantly reduced in the presence of cosolvents. Stabilization of the enzyme against thermal inactivation was highly dependent on the concentration of the cosolvents used. The stabilization effect of each cosolvent was found to be increased with increasing concentrations. As shown in figure 1, there was complete loss of α-amylase activity after 30 min of incubation at 60°C. More than 90% of enzyme activity was preserved even after 40 min of incubation at the same temperature in the presence of a 40% (w/v) concentration of each of the cosolvents. All the four cosolvents were shown to be effective in stabilizing α-amylase against thermal inactivation. More significant differences in the stabilizing capacity of the cosolvents could be obtained after incubating the enzyme at a still higher temperature, or after a longer period of incubation at the same temperature.

In order to determine the effect of cosolvents on thermal denaturation of the enzyme at higher temperatures, enzyme solutions were incubated for 10 min in the temperature range

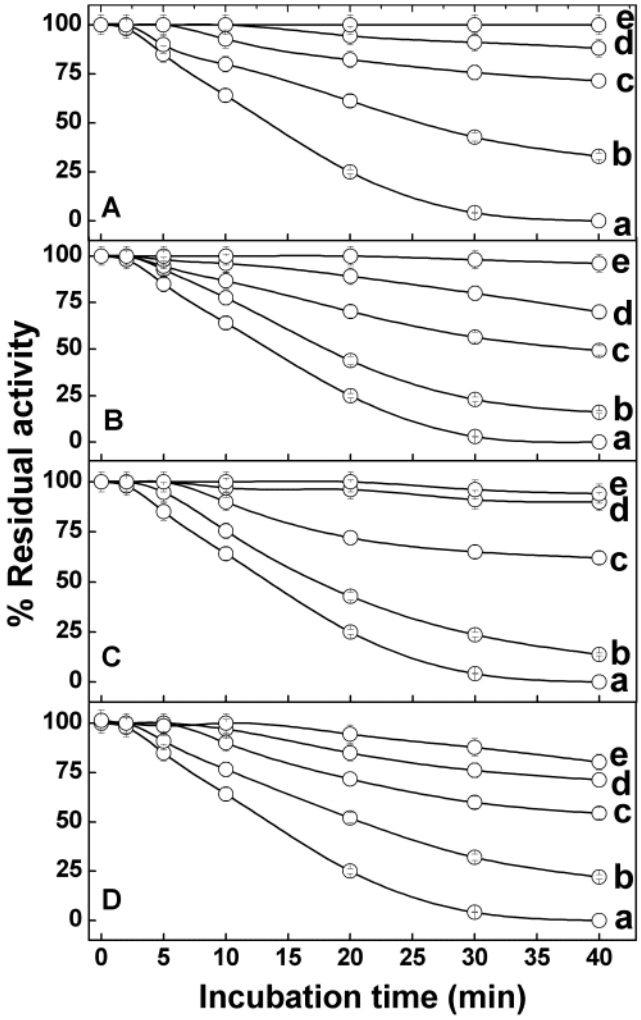

Figure 1. Thermal inactivation of *α*-amylase in presence of: **(A)** trehalose, **(B)** glycerol, **(C)** sucrose and **(D)** sorbitol. The enzyme was incubated at different concentrations of each cosolvent for 12 h under cold conditions (8°C) for equilibration. Thermal inactivation was carried out by subjecting the enzyme samples at 60°C in a temperature-controlled water bath and aliquots of enzyme were withdrawn after different incubation periods, cooled on an ice bath and residual activity was measured at 37°C. The percentage residual activities were calculated based on the original enzyme activity (without thermal treatment). The concentrations of each cosolvent were taken as: (a) 0.0, (b) 10%, (c) 20%, (d) 30% and (e) 40% (w/v).

of 30–80°C. There was complete thermal inactivation of *α*-amylase at 65°C. Addition of cosolvents has a significant effect on thermal inactivation of the enzyme as shown in figure 2. In the presence of 20% (w/v) trehalose, 45% activity was retained even at 80°C. The relative effectiveness of all the cosolvents used in stabilizing *α*-amylase against thermal inactivation varied and was found to be in the order of trehalose >glycerol >sucrose >sorbitol. The rate of

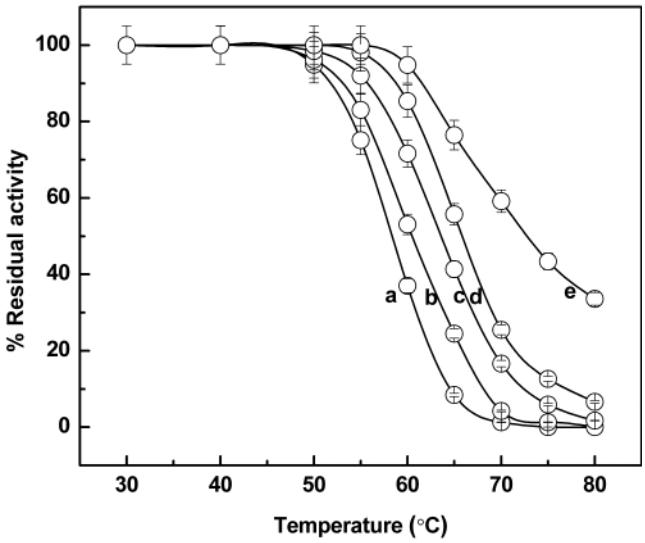

Figure 2. Thermal inactivation of *α*-amylase at different temperatures. The enzyme was incubated in 20% (w/v) of: (a) none, (b) glycerol, (c) sucrose, (d) sorbitol and (e) trehalose, for 12 h under cold conditions (8°C) for equilibration. Thermal inactivation of the enzyme was carried out at 30°C, 40°C, 50°C, 55°C, 60°C, 65°C, 70°C and 80°C. The enzyme samples were incubated at each temperature for 10 min, cooled on an ice bath and residual activity was measured at 37°C. The percentage residual activities were calculated based on the original enzyme activity (without thermal treatment).

thermal inactivation was dependent on the temperature and incubation period at a particular temperature.

3.2 *Effect of cosolvents on thermal unfolding of α-amylase*

Thermal denaturation of *α*-amylase was carried out in the presence of different concentrations of each cosolvent by recording the difference spectra of enzyme solution in the temperature range of 30–90°C. The $(T_m)_{app}$ of the enzyme in the presence of different concentrations of cosolvents was determined using equation 3. The $(T_m)_{app}$ of *α*-amylase was found to be 61 ± 1°C. The presence of cosolvents significantly altered the thermal denaturation profile of the enzyme. The thermal transition curve was found to have shifted to a higher temperature in the presence of each of the cosolvents (figure 3A). The $(T_m)_{app}$ of the enzyme was found to increase linearly with increasing concentrations of the cosolvents as shown in figure 3B. At equal concentrations, trehalose was found to be relatively more effective compared with the other cosolvents in stabilizing the enzyme against thermal denaturation. In the presence of 40% trehalose, the $(T_m)_{app}$ of *α*-amylase was found to be 81 ± 1°C, nearly 20°C higher than the control, whereas in the presence of the same concentration of glycerol, sucrose and sorbitol, the $(T_m)_{app}$

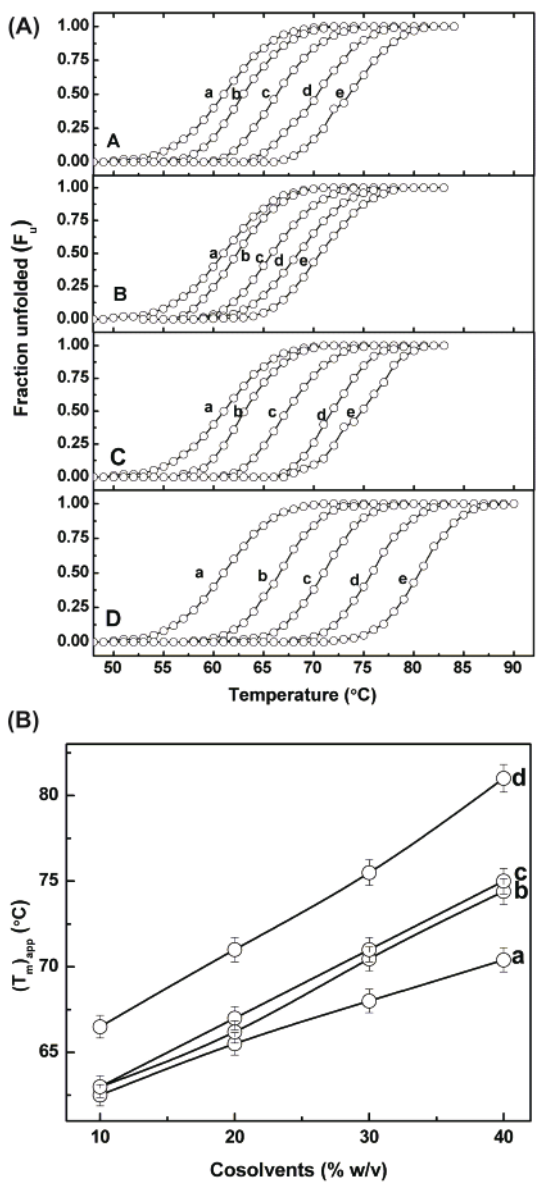

Figure 3. **(A)** Thermal denaturation profile of α-amylase in the presence of: (A) sucrose, (B) sorbitol, (C) glycerol and (D) trehalose. The concentration of each cosolvent was taken as: (a) 0.0, (b) 10%, (c) 20%, (d) 30% and (e) 40% (w/v). The enzyme samples were equilibrated under cold conditions with different concentrations of cosolvents for 12 h prior to thermal denaturation. The thermal denaturation of α-amylase in different concentrations of each coslvent was monitored by measuring the increase in absorbance at 287 nm in a UV-Visible spectrophotometer using a scan rate of 1°C/min. The absorbances of native and unfolded fractions of the enzyme at each temperature were normalized and fraction unfolded (F_u) were calculated using the equation given in Materials and methods. The $(T_m)_{app}$ was determined at the temperature where the F_u and K were found to be 0.5 and 1.0, respectively (equations 3 and 4). **(B)** Apparent T_m α-amylase in the presence of different concentrations of cosolvents: (a) sucrose, (b) sorbitol, (c) glycerol and (d) trehalose.

was 75 ± 1°C, 74 ± 1°C and 70 ± 1°C, respectively. The changes in enthalpy (ΔH^0) and entropy (ΔS^0) at $(T_m)_{app}$ in the presence of the cosolvents were obtained by using the van't Hoff equation (Tanford 1968; Pace and Scoltz 1997; Atkin and Paula 2006) and are summarized in table 1. A marginal difference was observed in ΔH^0 and entropy ΔS^0 in the presence and absence of cosolvents. It has been shown that the stabilization effect of polyols is dependent on the unfavourable free energy of transfer of amino acid side chains from the solvent to the solution of the cosolvent (Xie and Timasheff 1997; Gekko 1981; McClement 2002). This indicates that the presence of cosolvents in protein solution does not favour the denatured state and hence stabilizes the protein.

3.3 *Kinetics of thermal unfolding of α-amylase in the presence of cosolvents*

The kinetic study of thermal denaturation of α-amylase was performed in order to quantitate the E_a in the presence of cosolvents. The E_a of thermal denaturation of α-amylase was estimated by isothermal denaturation of the enzyme in the presence of each cosolvent at different temperatures. In the presence of 20% (w/v) trehalose, sorbitol, sucrose and glycerol, the E_a of thermal denaturation of α-amylase was found to be 126, 95, 90 and 43 kcal/mol, respectively, compared with the control value of 40 kcal/mol. The higher E_a value of thermal denaturation of α-amylase in cosolvent solutions shows that the protein is more stable in their presence rather than in their absence. The increasing slope of the Arrhenius plot in the presence of cosolvents indicates enhancement in the thermal stability of the enzyme (Privalov 1979). It is clear from figure 4 that the E_a of the thermal denaturation reaction is sensitive to changes in temperature and provides a more quantitative thermodynamic approach to monitor the thermal stabilization of proteins in different conditions.

3.4 *Effect of cosolvents on intrinsic and ANS fluorescence of α-amylase*

The effect of cosolvents on structural alteration of the enzyme at higher temperatures was determined by monitoring the changes in intrinsic and ANS fluorescence behaviours. The measurement of intrinsic fluorescence provides valuable information about the changes in the local conformations of the protein. Relative exposure of the hydrophobic residue was monitored by measuring the intrinsic fluorescence spectra and ANS binding. Thermal denaturation of the enzyme induces conformational changes, which might result in an increase or decrease in intrinsic fluorescence intensity. The fluorescence intensity of the enzyme was measured in

Table 1. Standard enthalpy change (ΔH^0) and standard entropy change (ΔS^0) at $(T_m)_{app}$, of α-amylase in the presence of different cosolvents

Cosolvents	Concentration (%w/v)	$(T_m)_{app}$ (°C) (± 1)	ΔH^0 at $(T_m)_{app}$ (kcal/mol) (± 20)	ΔS^0 at $(T_m)_{app}$ (cal/mol.*K*) (±50)
Buffer	--	61	94	280
Trehalose	10	66	110	325
	20	71	120	350
	30	75	121	347
	40	81	131	370
Glycerol	10	62	114	339
	20	65	100	296
	30	68	101	295
	40	70	107	312
Sucrose	10	63	108	320
	20	66	113	334
	30	71	105	305
	40	74	114	328
Sorbitol	10	63	124	370
	20	67	108	318
	30	71	123	357
	40	75	123	353

the presence of 20% solutions of each cosolvent. Appropriate controls were set for each respective cosolvent. The intrinsic fluorescence emission maximum (λ_{max}) of α-amylase was found to be 340 nm. After thermal treatment of the enzyme at 60°C for 30 min, the intrinsic fluorescence intensity was found to be significantly increased with a red shift of 4 nm in the λ_{max} (figure 5A). In the presence of different cosolvents, the fluorescence intensity of the enzyme was found to be lower than the control and no shift was observed in λ_{max}. These results indicate that the enzyme is relatively protected against thermal denaturation. ANS binding has been extensively used to probe the conformational changes that occur during protein denaturation (Engelhard and Evan 1995). It is a hydrophobic molecule that binds preferentially to the hydrophobic clusters on the protein surface. After heat treatment, the ANS fluorescence intensity of α-amylase was found to be significantly increased. In the presence of cosolvents, it was found to be relatively lower than that of the control (figure 5B). These observations suggest that at higher temperatures, protein unfolds and exposes the non-polar clusters to the solvent, which bind with ANS and increase the fluorescence intensity. In the presence of cosolvents, the enzyme showed relatively lower binding with ANS, which indicates the lower exposure of hydrophobic clusters than in their absence. These observations indicate that the presence of cosolvents prevents unfolding of the protein molecule.

3.5 *Preferential interaction parameters of α-amylase in the presence of cosolvents*

The preferential interaction parameters of α-amylase were determined in the presence of trehalose and sorbitol in isomolal and isopotential conditions at 20°C. The partial specific volume of α-amylase was calculated from density measurements and was found to be 0.721 ± 0.002 and 0.728 ± 0.003 ml/g in isomolal and isopotential conditions, respectively. The other preferential interaction parameters of the enzyme were calculated using equations 8, 9, 10 and 11 based on density measurements of the protein in different concentrations of trehalose and sorbitol. The values are summarized in table 2. As shown in figure 6, the preferential interaction parameter $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$ of α-amylase was found to be negative in the presence of both the cosolvents. This indicates the relatively low density of the cosolvents in the vicinity of the protein compared with the bulk solvent. A negative value of $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$ has also been reported in the presence of glycerol and sucrose (Rajendran *et al.* 1995). A negative value of $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$ indicates that the cosolvents are preferentially excluded from the immediate vicinity of the protein and the protein is relatively more hydrated compared with the situation in the absence of cosolvents. The maximum exclusion was observed in the presence of 10% and 40% sorbitol. In the presence

of trehalose, the preferential exclusion was found to be approximately similar at all the concentrations used. This might be due to structural propensities and differences in the molar concentrations of each cosolvent.

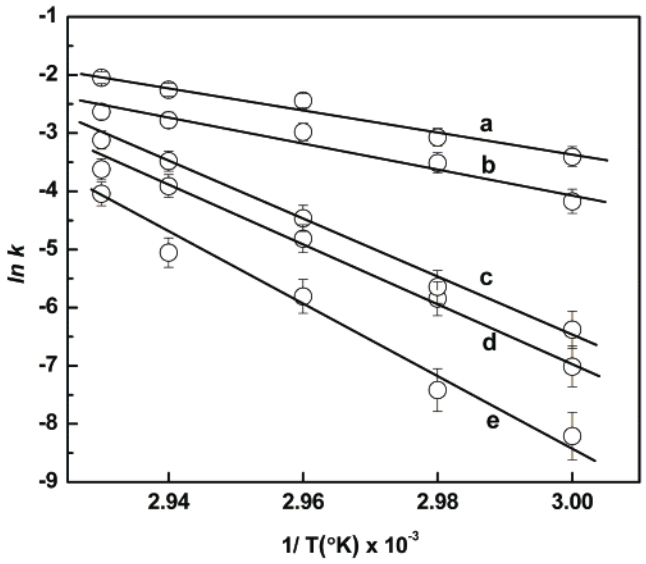

Figure 4. Arrhenius plot of the thermal denaturation of α-amylase in the presence of 20% concentration of different cosolvents: (a) control, (b) sucrose, (c) sorbitol (d) glycerol and (e) trehalose. Isothermal denaturation of α-amylase was monitored by measuring the increase in absorbance of the enzyme using a UV-Visible spectrophotometer with a scan rate of 1°C/min. The isothermal denaturation was carried out at 60°C, 62°C, 64°C, 66°C and 68°C till complete denaturation. The rate constant of the denaturation process at each temperature was obtained from the slope of the straight line of fraction unfolded vs incubation time. The E_a was calculated from the slope of Arrhenius plot according to the method given elsewhere (Tanford 1968; Pace and Scoltz 1997).

4. Discussion

It is evident from the above results that the thermal stability of α-amylase can be significantly improved by using cosolvents. The increase in $(T_m)_{app}$, E_a and reduction in the

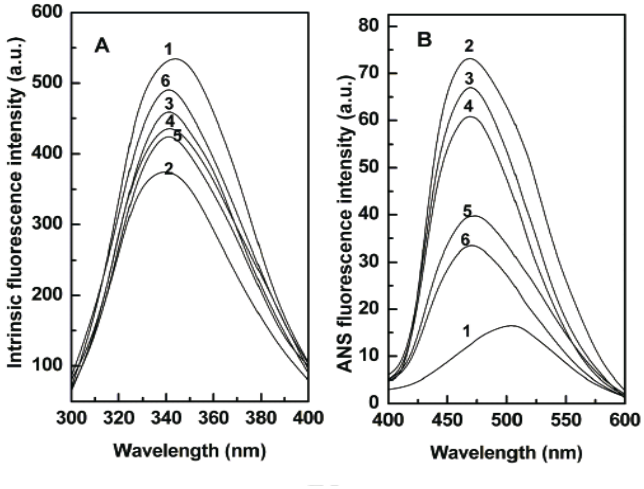

Figure 5. Fluorescence emission spectra of α-amylase were measured in the presence of 20% concentration of each cosolvent. The enzyme and cosolvent solutions were made in 20 mm citrate buffer containing 2 mM $CaCl_2$ pH 5.9. **(A)** Intrinsic fluorescence spectra were recorded after heat treatment of the enzyme at 60°C for 30 min in 20% (w/v) of each cosolvent. **(B)** 8-anilinonaphathalene-1-sulphonic acid(ANS) fluorescence spectra were recorded after heat treatment of the enzyme at 60°C for 30 min, and cooled followed by the addition of ANS stock solution. The ratio of ANS to enzyme was 100:1. ANS fluorescence spectra were recorded in the range of 400–600 nm after excitation at 380 nm. Appropriate controls of cosolvents were set to eliminate any interference. Curves 1 and 2 show α-amylase before and after heat treatment (60°C) in the absence of cosolvents, respectively. Curves 3, 4, 5 and 6 are the fluorescence spectra of α-amylase after heating in presence of 20% (w/v) trehalose, sorbitol, sucrose and glycerol, respectively.

Table 2. Partial specific volumes and preferential interaction parameters of α-amylase in the presence of cosolvents (sorbitol and trehalose) at 20ºC in 0.02 mM citrate buffer, pH 5.9

Cosolvents	Conc. (%w/v)	Φ_2^0	Φ'^0_2	g_3 (g/g)	m_3 (mol/ 1000 g of water)	$(\delta g_3/\delta g_2)_{T,\mu1,\mu3}$ (g/g)	$(\delta g_1/\delta g_2)_{T,\mu1,\mu3}$ (g/g)	$(\delta m_3/\delta m_2)_{T,\mu1,\mu3}$ (mol/mol)
Buffer	--	0.721 ± 0.002	0.728 ± 0.003	--	--	--	--	--
Sorbitol	10	0.724 ± 0.001	0.793 ± 0.001	0.107	0.58	–0.232 ± 0.015	2.169 ± 0.178	–61.91 ± 5.11
	20	0.724 ± 0.002	0.759 ± 0.003	0.229	1.26	–0.113 ± 0.012	0.493 ± 0.08	–30.15 ± 2.22
	30	0.727 ± 0.002	0.746 ± 0.002	0.372	2.04	–0.063 ± 0.004	0.170 ± 0.098	–16.87 ± 1.91
	40	0.701 ± 0.002	0.750 ± 0.001	0.541	2.97	–0.177 ± 0.05	0.327 ± 0.08	–47.22 ± 2.30
Trehalose	10	0.719 ± 0.001	0.761 ± 0.003	0.106	0.28	–0.122 ± 0.081	1.148 ± 0.011	–15.71 ± 2.50
	20	0.721 ± 0.003	0.747 ± 0.003	0.229	0.60	–0.077 ± 0.052	0.339 ± 0.055	–9.98 ± 1.20
	30	0.721 ± 0.002	0.762 ± 0.001	0.371	0.98	–0.129 ± 0.048	0.349 ± 0.075	–16.63 ± 1.66
	40	0.758 ± 0.003	0.788 ± 0.003	0.538	1.42	–0.100 ± 0.004	0.186 ± 0.096	–12.85 ± 0.85

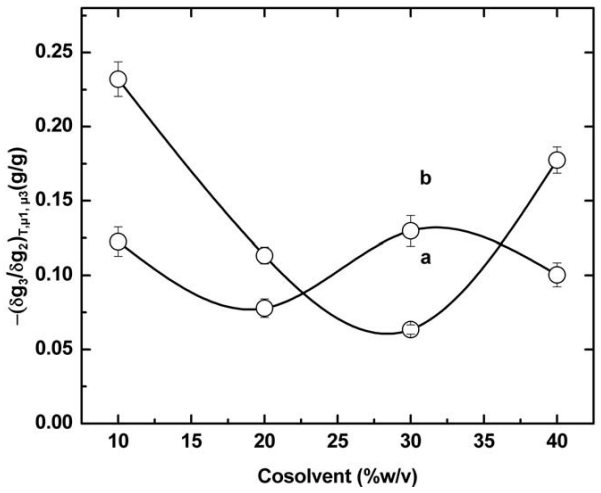

Figure 6. Preferential interaction of cosolvents (sorbitol and trehalose) with *α*-amylase at pH 5.9 and 20°C. The preferential interactions of *α*-amylase were obtained by measuring the protein density in different concentrations of (a) sorbitol and (b) trehalose, and $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$ values were calculated using the equations 8, 9, 10 and 11 described in Materials and methods.

rate of thermal inactivation of *α*-amylase in the presence of cosolvents indicate that the cosolvents have a protective and stabilizing effect against thermal denaturation/inactivation. The thermal denaturation of *α*-amylase is an irreversible process and accompanied by extensive aggregation. Since thermal aggregation of the protein may begin even before complete unfolding, the thermal denaturation temperature or melting temperature does not reflect the actual value as in the case of reversibly unfolded proteins. Therefore, it is preferable to refer to the thermodynamic parameters obtained from the thermal denaturation of *α*-amylase as apparent. The changes in the E_a of thermal denaturation of the enzyme in the presence of cosolvents were found to be increased. This indicates the relative stability of the protein in the presence of cosolvents (Timasheff 1993).

The preferential interaction parameters indicate that the enzyme molecules were preferentially hydrated in the presence of cosolvents. This could probably be due to the steric exclusion of cosolvents. Generally, the surface of the enzyme is dominated by hydrophilic groups with a small fraction of hydrophobic groups. The preferential hydration in the presence of cosolvents may partially internalize the surface hydrophobic residues in the protein interior and subsequently enhance the hydrophobic interactions (Sola-Penna and Meyer-Fernandez 1998). Thermal denaturation results in opening of the protein core and exposure of hydrophobic domains to aqueous medium. Additional hydration of protein resists the unfolding process by preventing the unfolding in order to minimize the unfavourable interactions of non-polar amino acid residues with water. Cosolvents are known to exert their stabilizing effect primarily by altering the water structure in the immediate vicinity of the protein (Lin and Timasheff 1996; Gekko and Timasheff 1981). Although the thermal stability of *α*-amylase was found to be significantly improved in the presence of all the cosolvents, trehalose was found to be a more effective stabilizer compared with sorbitol, sucrose and glycerol at equal concentrations (%w/v). The extraordinary stabilizing effect of trehalose may be due to several reasons. Usually trehalose occupies a volume that is at least 2.5 times larger than sucrose in aqueous solution. It has less dynamic conformations than any of the other sugars due to its anisotropic hydration adjacent to glycosidic oxygen (Sola-Penna and Meyer-Fernandez 1998; Choi *et al.* 2006). Therefore, it may substitute more water molecules in solution compared with other sugars. In a similar study with *α*-amylase from *Aspergillus oryzae*, sucrose was found to be more effective in providing thermal stability compared to trehalose (Samborska *et al.* 2006). Cosolvent-induced thermal stabilization varies among different cosolvents. They may interact in different ways with the same proteins from different sources, based on their structural and physicochemical properties (Gekko and Timasheff 1981; Lin and Timasheff 1996). Apart from the variations in the thermodynamic properties, the preferential interaction parameters and hydration of the enzyme play a major role in determining the thermal stability of the protein/enzyme. Based on the above thermodynamic parameters, it is clear that the thermal denaturation of the enzyme is reduced in the presence of cosolvents, and the nature of interactions between protein, solvent and cosolvents is such that it favours stability of the protein.

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