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ORIGINAL PAPER

Intrinsic halotolerance of the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis*

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Abstract The halotolerance of a cold adapted α-amylase from the psychrophilic bacterium *Pseudoalteromonas haloplanktis* (AHA) was investigated. AHA exhibited hydrolytic activity over a broad range of NaCl concentrations (0.01–4.5 M). AHA showed 28% increased activity in 0.5–2.0 M NaCl compared to that in 0.01 M NaCl. In contrast, the corresponding mesophilic (*Bacillus amyloliquefaciens*) and thermostable (*B. licheniformis*) α-amylases showed a 39 and 46% decrease in activity respectively. Even at 4.5 M NaCl, 80% of the initial activity was detected for AHA, whereas the mesophilic and thermostable enzymes were inactive. Besides an unaltered fluorescence emission and secondary structure, a 10°C positive shift in the temperature optimum, a stabilization factor of >5 for thermal inactivation and a ΔT_m of 8.3°C for the secondary structure melting were estimated in 2.7 M NaCl. The higher activation energy, half-life time and T_m indicated reduced conformational dynamics and increased rigidity in the presence of higher NaCl concentrations. A comparison with the sequences of other halophilic α-amylases revealed that AHA also contains higher proportion of small hydrophobic residues and acidic residues resulting in a higher negative surface potential. Thus, with some compromise in cold activity, psychrophilic adaptation has also manifested halotolerance to AHA that is comparable to the halophilic enzymes.

Keywords Acidic protein · *Pseudoalteromonas haloplanktis* α-amylase · Halophilic · Halotolerance · Psychrophilic · Stability

Abbreviations

AHA	*Pseudoalteromonas haloplanktis* α-amylase
PPA	Porcine pancreatic α-amylase
BAA	*Bacillus amyloliquefaciens* α-amylase
BLA	*Bacillus licheniformis* α-amylase

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This article is dedicated to Late Dr. P. V. Sundaram.

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Introduction

Selective pressure from the environment on the microorganisms results in the production of enzymes that are stable and functional in their respective habitats (Pflüger and Müller 2004). The enzymes adjust to the environmental limitations through adaptations at the molecular level. Examination of proteins isolated from organisms growing in naturally extreme environments of temperature, pH, pressure and salt conditions and comparison with mesophilic enzymes could provide valuable information regarding the differences in the molecular structure contributing to the differences in catalytic and conformational stability (Madigan and Marrs 1997; D'Amico et al. 2003; Venkatesh et al. 2005). Enzyme adaptation to function at multiple extremes could provide new impetus to the biotechnology research and development for the deployment of enzymes in application industries.

Pseudoalteromonas haloplanktis TAB23 is a psychrophilic Gram-negative bacterium collected along the Antarctic ice-shell close to the French station Dumont d'Urville where the water temperature remains stable between –2 and 2°C and the salinity is close to 0.5 M. It secretes a Cl^- and Ca^{2+}-dependent α-amylase (E.C. 3.2.1.1) which is a well characterized cold-adapted enzyme for low temperature catalysis of starch (Feller et al. 1992; D'Amico et al. 2003; Georlette et al. 2004). *Pseudoalteromonas haloplanktis* α-amylase (AHA) is characterized by low structural stability, lower activation free energy ($\Delta G^{\neq}$) for both heat inactivation and reversible unfolding, increased conformational plasticity or flexibility and lower activation energy (E_a) for the catalytic hydrolysis compared to its mesophilic homologues (D'Amico et al. 2003). The important cold adaptive features are the weakening of cohesion factors due to drastic decrease in the number of salt bridges and aromatic interactions, hydrophilic surface, loose packing of the core hydrophobic residues, decrease in proline and arginine contents and the stabilization of helices dipoles (Feller 2003). AHA and other psychrophilic enzymes adopt a flexible conformation owing to improved electrostatic potentials of the protein surface and therefore provide better accessibility of the active site to the substrate resulting in higher catalytic efficiency (Feller 2003; Smalås et al. 1994). All these features have made AHA a cold-active and thermolabile protein in comparison with the mesophilic α-amylases from porcine pancreas (PPA), *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA).

Interestingly, some of the cold-adaptive features such as enrichment of negative residues and small hydrophobic residues in their primary sequence, of AHA resemble the halophilic enzymes characterized till date. Proteins are called halophilic when they are fractionated from halophilic microorganisms requiring at least 2.5 M NaCl in the medium for optimum growth and generally they require multimolar concentrations of KCl or NaCl (>2.5 M) for proper folding, functioning and conformational stability (Mevarech et al. 2000; Madern et al. 2000; Danson and Hough 1997). On the other hand, halotolerant proteins are those that are not from such high saline sources but remain active over broad salinity ranging from low to very high salt concentration without any obligatory salt dependence (Madern et al. 2000; Premkumar et al. 2005). Halophilic proteins generally contain a large excess of acidic residues resulting in a higher negative surface potential (Madern et al. 2000 and 2004). The presence of excess acidic residues like the halophilic proteins (Aghajari et al. 1998; Aghajari et al. 2002) and the reduced inactivation rate in 0.5 M NaCl (Feller et al. 1992) observed for AHA indicated that this enzyme could be a candidate to test halotolerance. In view of exploring the salt tolerance of AHA, we examined the effect of high salinity on the tertiary and secondary structure, catalytic activity, substrate binding, thermal stability and compared it with that observed under low salt concentrations. Wherever needed, the salt tolerance of mesophilic BAA and BLA, though less acidic than AHA and other halophilic α-amylases was also compared.

Materials and methods

Chemicals

α-Amylase from the Antarctic psychrophile *P. haloplanktis* (AHA) was obtained and purified as described previously. The purified sample gave a single band in denaturing and native PAGE (Feller et al. 1992; D'Amico et al. 2001) *B. licheniformis* α-amylase (BLA) was purchased from Sigma Chemical Company in a purified form. *B. amyloliquefaciens* α-amylase (BAA) was supplied by Spic Science Foundation, Chennai, India. All other chemicals including soluble potato starch, KI and I_2 were of analytical grade either from E. Merck or SRL, India. Protein estimation was done according to the method of Lowry using BSA as standard (Lowry et al. 1951).

Activity assay

The activities of AHA (1.12 nM), BAA (0.5 nM) and BLA (0.5 nM) were assayed with 0.09 ml of 0.1 %

soluble starch at 25°C in the presence of 0.01–4.5 M NaCl at pH 7.0 in 0.02 M borate buffer containing 0.01 M $CaCl_2$ (buffer A) in a final assay volume of 0.9 ml. After 10 min incubation, the reaction was stopped by the addition of 0.1 ml of KI_3 reagent. The starch–iodine complex formation was read at 550 nm to estimate the extent of starch hydrolysis (Srimathi and Jayaraman 2005). pH-induced changes in the activity in the presence of 0.01 M and 2.7 M NaCl were checked using 0.02 M glycine–HCl (pH 2.0–3.5), sodium acetate–acetic acid (pH 4.0–5.5), MES–NaOH (pH 6.0–6.5), borate–boric acid (pH 7.0–9.0) and glycine–NaOH (pH 9.5–11.0). Suitable substrate blanks were used to correct the enzyme activity under different salt concentrations, temperature and pH.

Activation and inactivation

The effect of temperature (5–60°C) on the activity of AHA was checked by incubating AHA with starch at different temperatures for 10 min and estimating the amount of starch hydrolyzed. The activation energy (E_a) was calculated from the Arrhenius plot of –ln k versus $1/T$ using the equation $k = A \exp(-E_a/RT)$, where k is the activation rate constant, R the gas constant (8.314 J mol^{-1} K^{-1}), T the temperature in Kelvin and A the collision constant (D'Amico et al. 2003). The thermal inactivation was carried out by incubating the enzyme (0.1 μM) in the presence of either 0.01 M or 2.7 M NaCl in buffer A. Aliquots of 10 μl removed at regular time intervals were transferred to pre-cooled buffer A containing 0.01 M NaCl and the residual activity was determined by assaying with starch at 25°C in the same buffer. The half-life of inactivation ($t_{½}$) was calculated from the plot of log percentage residual activity versus time using the relationship $t_{½} = 0.693/k_i$ where k_i is the inactivation rate constant obtained from the slope of the curve. The enthalpy, free energy and entropy of activation were also calculated using appropriate equations (Sundaram and Srimathi 2004; D'Amico et al. 2003).

Fluorescence measurements

Protein fluorescence emission was measured in the presence of 0.01–4.5 M NaCl using a protein concentration of 0.1 μM in buffer A at 25°C in a 1 cm path length quartz cuvette. An excitation wavelength of 280 nm was used to record the emission spectrum between 300 and 450 nm with a spectral resolution of 1 nm. Both the excitation and emission slit widths were set at 10 nm. All the measurements were carried out on a Shimadzu Spectrofluorimeter RF1501 and the data were analyzed using the Personal fluorescence software.

Circular dichroism

The far UV CD spectra (190–250 nm) of the α-amylases were recorded in a Jasco Spectropolarimeter J715, equipped with a Peltier type temperature controller (Jasco PTC348) under constant nitrogen flux using a protein concentration of 0.67 μM in a 1 cm quartz cuvette. The final spectrum is an average of four scans (50 nm min^{-1} scan speed; 4 nm bandwidth; 0.2 nm resolution and 4 s response time). These measurements were carried out in the presence of 0.01–4.5 M NaCl at pH 7.0 (25°C) in 0.02 M borate buffer with 0.18 mM $CaCl_2$. The thermal unfolding experiments were performed using a heating rate of 50°C h^{-1} with a 0.5°C resolution. The fraction of unfolded protein (f_U) was estimated as given in Sundaram and Srimathi (2004). The melting temperature (T_m) was obtained from the sigmoidal fit of the temperature versus $\Theta_{222\ nm}$ using Sigma Plot curve fitting software.

Results and discussion

Hydrolytic activity

Pseudoalteromonas haloplanktis α-amylase, BAA and BLA exhibited maximum activity in the presence of 0.5 M NaCl (Fig. 1). The hydrolysis rates corresponding

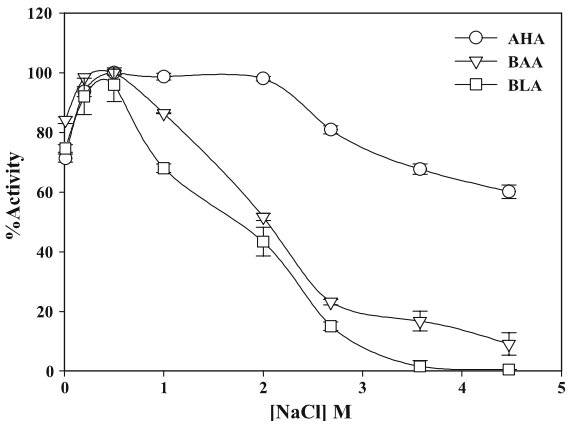

Fig. 1 Hydrolytic activity of *Pseudoalteromonas haloplanktis* α-amylase (*AHA*), *Bacillus amyloliquefaciens* α-amylase (*BAA*) and *B. licheniformis* α-amylase (*BLA*) in the presence of different concentrations of NaCl in buffer A (pH 7.0) at 25°C. Observed hydrolysis of starch in the presence of 0.5 M NaCl has been referenced to 100% activity

to the maximum activity are 4.9, 3.5 and 1.6 μg min^{-1} respectively for AHA, BAA and BLA. Upon increasing the salt concentration (>0.5 M), both BLA and BAA showed steady decrease in their hydrolytic activity, with almost negligible or no activity at 4.5 M NaCl. But their activity could be retrieved by dilution to lower salt concentration (data not shown). On the other hand, no significant change in the hydrolysis was observed for AHA in the presence of 0.5–2.0 M NaCl. Further increase in salt concentration, resulted in slight decrease in the hydrolytic activity. At the highest salt concentration studied (4.5 M), AHA retained about 80% of the activity seen at 0.01 M NaCl (Fig. 1). This 20% decrease in activity could however be retrieved by simple dilution with buffer A containing 0.01 M NaCl (data not shown). The loss of activity seen for BAA and BLA could thus be due to the loss of catalytic water and altered substrate binding site conformation. This trend suggests that the psychrophilic α-amylase is highly halotolerant and as speculated by Gerday et al. (2000) could function even at lower water activity whereas its mesophilic and thermophilic analogs are sensitive to changes in the salt concentration. Therefore, detailed analysis of the effect of NaCl on the stability and activity of the psychrophilic enzyme was undertaken.

Effect of salt on Michaelis constants

By definition, the halophilic enzymes are functional above 2.5 M NaCl (Madern et al. 2000). We have chosen a moderate NaCl concentration of 2.7 M for the characterization of halotolerance of AHA so that there is considerable activity to check the catalytic parameters and inactivation constants. The effect of NaCl on the steady-state kinetic constants was analyzed in the presence of 0.01 M and 2.7 M NaCl by assaying with varying concentrations of starch (5–120 mg l^{-1}) at 25°C in buffer A. Michaelis–Mentens parameters for the enzyme kinetics were estimated to be 601 ± 15 mg l^{-1} (K_m) and 14.9 ± 0.65 s^{-1} (k_{cat}) in the presence of 0.01 M NaCl. However, when the salt concentration was increased to 2.7 M NaCl K_m decreased to 436 ± 12 mg l^{-1} without altering the k_{cat} (14.15 ± 0.68 s^{-1}). This indicates that the presence of salt increases the apparent binding constant of the enzyme-substrate complex without affecting the catalytic turn over significantly. This could be due to the changes in the enzyme electrostatic potential upon binding to salt. However, the catalytic efficiency (k_{cat}/K_m), increased from 0.025 s^{-1} mg^{-1} l in 0.01 M NaCl to 0.032 s^{-1} mg^{-1} l in 2.7 M NaCl and remained the same even at 4.5 M NaCl (0.035 s^{-1} mg^{-1} l).

Temperature dependence of activity

The effect of temperature on the hydrolytic activity of AHA was studied. In the presence of 0.01 M NaCl the enzyme exhibited a temperature optimum of 25°C (Fig. 2a). In the presence of 2.7 M NaCl, the hydrolytic activity of the enzyme was lower at temperatures below 20°C (~20% decrease at 10 and 15°C) though a 2.5-fold decrease in activity was observed at 5°C compared to that in 0.01 M NaCl (Fig. 2a). Interestingly at temperatures greater than 20°C, the presence of NaCl enhances the hydrolytic activity, indicating that AHA is a better catalyst in the presence of 2.7 M NaCl than at low NaCl (Fig. 2a). Increased thermal stability of AHA could be evidenced from the shift in T_{opt} to 35°C in 2.7 M NaCl compared to 25°C in 0.01 M NaCl (Fig. 2a,) which is comparable to that obtained for the mesophilic α-amylase from *B. amyloliquefaciens* (Srimathi and Jayaraman 2005). This

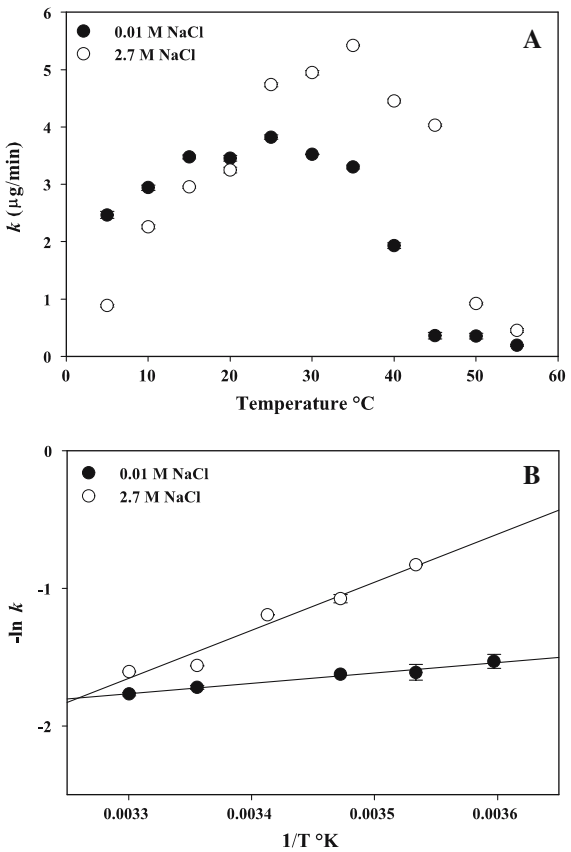

Fig. 2 **a** Effect of temperature on the hydrolytic activity of AHA in the presence of low (0.01 M) and high (2.7 M) concentration of NaCl and **b** Arrhenius plot for temperature dependence of activity. *k* is the rate of starch hydrolysis at different temperatures. The temperature optimum shifted from 25°C in 0.01 M NaCl to 35°C in 2.7 M NaCl while the Arrhenius activation energy increased from 6.3 ± 0.6 to 29.1 ± 2.2 kJ mol^{-1}

indicates that increase in salinity results in decrease in cold activity at 5°C but enhances the optimum temperature for activity.

The Arrhenius plot for thermal activation showed that the activation energy (E_a) is 6.3 ± 0.6 and 29.1 ± 2.2 kJ mol^{-1}, respectively, at low and high salinity (Fig. 2b). The enthalpy of activation was also found to increase. Generally, psychrophilic enzymes encounter a reduced activation barrier owing to their flexible conformation (D'Amico et al. 2003; Georlette et al. 2004). The higher Arrhenius activation energy (ΔE_a = 22.8 kJ mol^{-1}) and enthalpy of activation ($\Delta\Delta H^{\#}$ = 22.8 kJ mol^{-1}) support the fact that AHA acquires higher conformational rigidity in the presence of high salt concentrations indicating that more energy is needed to acquire the conformational dynamics essential for optimal activity (Fig. 2a, b). The suppression of cold activity, enhanced temperature optimum and activation barrier at 2.7 M NaCl suggests that it is possible that the shielding of the protein surface charges play an important role in determining the psychrophily and halotolerance. Ion binding makes the protein conformation more compact. This partially disables the flexible conformation required to perform the catalytic steps at low temperatures. Thus, the presence of excess acidic amino acid residues seems to be one of the cold adaptive features involved in salt tolerance.

Thermal inactivation and catalytic stability

Thermal inactivation of AHA is reversible upon gradual cooling (data not shown). However, in the present study, the sudden drop in temperature upon mixing the heat-inactivated sample with a pre-cooled assay buffer prevented proper protein folding required for complete reversibility. This enabled the measurement of residual activity to determine the half-life periods. Higher resistance of AHA to thermal inactivation in 0.5 M NaCl has been reported earlier (Feller et al. 1992). To validate the higher salt tolerance and for comparison with other halophilic α-amylases, we have examined the thermal inactivation of AHA at 2.7 M NaCl in the present study. As expected, the enzyme had higher resistance to thermal inactivation in 2.7 M NaCl compared to that observed in 0.01 M NaCl at all the temperatures studied (Fig. 3). The half-lives of inactivation in 0.01 M NaCl were 83 ± 5, 15 ± 1 and 3 ± 0.4 min, respectively, at 40, 45 and 50°C. The corresponding values in 2.7 M NaCl were 478 ± 19, 166 ± 11 and 20 ± 0.8 min. Compared to that in 0.01 M NaCl, the half-life periods were 5.8-, 11.1- and 6.7-fold higher, respectively, at 40, 45 and 50°C in the presence

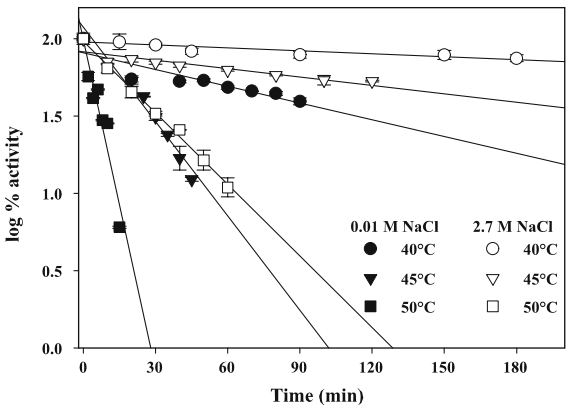

Fig. 3 Thermal inactivation kinetics of AHA in the presence of low and high NaCl concentrations. The enzyme (0.02 μM) was incubated at different temperatures in the presence of either 0.01 M or 2.7 M NaCl at 25°C in buffer A, and the residual activity of both the samples after definite time intervals was assayed in buffer A containing 0.01 M NaCl (pH 7.0) irrespective of the NaCl concentration present during inactivation. The starch hydrolysis rates corresponding to the initial activity are 3.8 and 4.8 μg min^{-1} at 0.01 and 2.7 M NaCl

of 2.7 M NaCl. Also, the activation energy for inactivation (E_{ai}) calculated using the inactivation rate constant at various temperatures showed that an E_{ai} of 166 kJ mol^{-1} in 0.01 M NaCl increased to 268 kJ mol^{-1} in the presence of 2.7 M NaCl indicating enhanced stability. The positive shift of the inactivation temperature and higher $t_{½}$ concurrent with the increase in T_{opt} is a clear indication of improved catalytic stability of AHA in the presence of higher concentrations of NaCl. But the pH dependence of activity at 25°C was similar both at low and high salinity with an optimum pH of 6.5 indicating the unchanged ionization states of the catalytic residues (data not shown).

Tertiary structure

The tryptophan fluorescence of AHA was recorded in the presence of different concentrations of NaCl at 25°C in buffer A (Fig. 4). AHA exhibits an emission λ_{max} of 348 nm (λ_{ex} = 280 nm) in its native state (0.01 M NaCl, buffer A). This value is higher compared to the fluorescence emission maximum of 336 nm observed for the mesophilic (BAA) and thermostable (BLA) α-amylases (Fig. 4). Such red shift has been attributed to the higher exposure of the hydrophobic residues in the case of these psychrophilic enzymes (D'Amico et al. 2003). AHA retained its gross tertiary structure even in 4.5 M NaCl as evidenced from the absence of any apparent change in the λ_{max} (Fig. 4). The decrease in the intensity in 4.5 M NaCl could possibly be due to the quenching effects of the

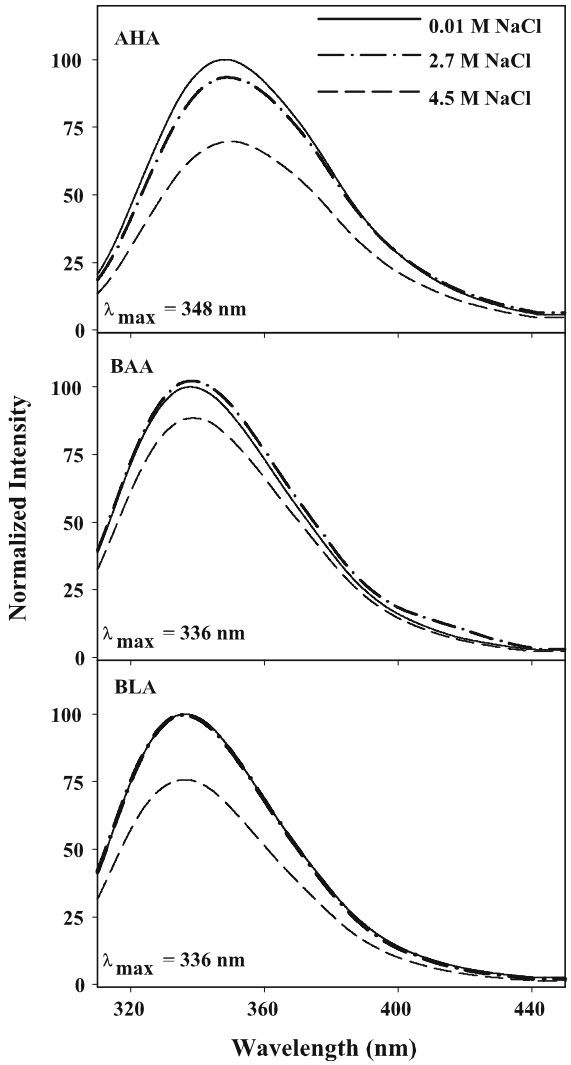

Fig. 4 Tryptophan fluorescence of AHA, BAA and BLA in the presence of 0.01, 2.7 and 4.5 M NaCl in buffer A (pH 7.0) at 25°C. The fluorescence spectra are normalized with respect to 0.01 M NaCl

salt. This suggests that the salt tolerance of AHA is different from that observed for the halophilic enzymes that show either partially or completely unfolded tertiary structure at lower salt concentrations (Wright et al. 2002; Madern and Zaccai 2004). The intact protein tertiary structure and higher stability to thermal inactivation under high NaCl concentrations could be attributed to the surface acidic residues playing a major role in the organization of specific solvation shell described for halophilic proteins (Madern et al. 2004).

Secondary structure

Presence of high salt concentration did not induce any detectable change in the secondary structure of AHA measured at 25°C (Fig. 5, inset a). Nucleoside diphosphate kinase from the halophilic archaeon *Halobacterium salinarum* (*Hs*NDk) capable of functioning over a wide range of NaCl concentration, has been reported to show only 19% α-helix in 0.2 M NaCl compared to 29% in 3.8 M NaCl (Ishibashi et al. 2002). A far UV CD spectrum corresponding to a partially unfolded conformation has also been reported for the halophilic dihydrofolate reductase from *Haloferax volcanii* (Wright et al. 2002). However, no such change in the secondary structure was observed for AHA as it showed unchanged far UV CD spectra even at 4.5 M NaCl suggesting its extreme salt tolerance (Fig. 5, inset a). Also, no significant change in the CD or fluorescence spectra was observed between 0 and 50 mM NaCl (unpublished results of previous work) though a slight increase in stability was observed as shown by DSC experiments (Feller et al. 1999). Thus, the unaltered secondary and tertiary structure explains the ability of the enzyme to retain the overall fold and hence the catalytic activity even at very high salt concentration.

Thermal unfolding

Thermal unfolding of AHA was carried out at increasing concentrations of NaCl with a constant $CaCl_2$ of 0.18 mM in 0.02 M borate buffer at pH 7.0. At the protein concentration (0.67 μM) used for unfolding, no salting-out or aggregation was observed even in the presence of 4.5 M NaCl (measured from the absorbance at 350 nm, data not shown). But the unique reversible thermal unfolding of AHA in low salt conditions reported earlier (Feller et al. 1999) could not be achieved in the presence of higher salt concentrations (0.5–4.5 M NaCl). This could be due to the prevention of the electrostatic interactions required to fold back to the native conformation under high salt concentration. But the protein refolded to the native state upon cooling and dilution to 0.01 M NaCl (data not shown). The thermal unfolding curves of AHA in the presence of 0.01, 2.7 and 4.5 M NaCl at pH 7.0 (Fig. 5) and the changes in the T_m values as a function of salt concentration (Fig. 5, inset b) show that the T_m is minimum (42.4°C) at the lowest NaCl concentration (0.01 M) used. At 0.5 M, the T_m improved to 48.1°C. Further increase in NaCl up to 2.0 M did not alter the T_m significantly (Fig. 5, inset b). But at 2.7 M NaCl where most of the characterization is done, the T_m was at its maximum of 50.7°C. Above 2.7 M and up to 4.5 M the T_m remained constant at 50°C (Fig. 5, inset b). It is striking to note that the most stable state corresponding to 2.7 M

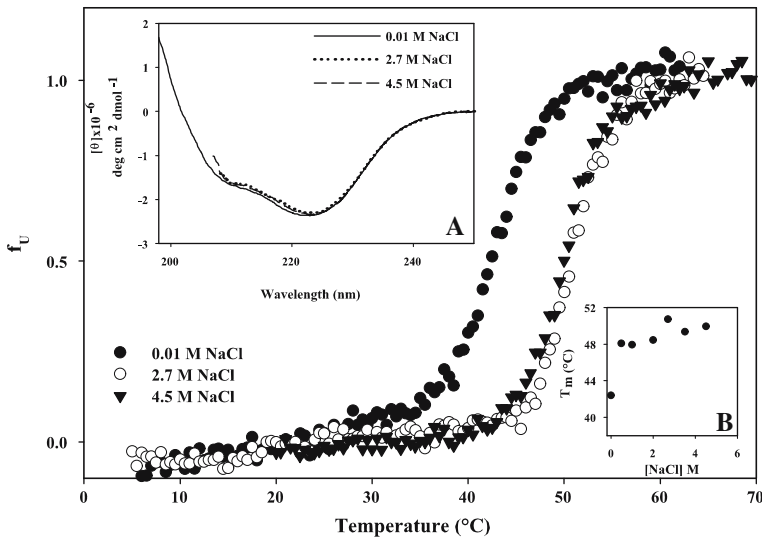

Fig. 5 Thermal unfolding of AHA in the presence of low (0.01 M) and high (2.7 and 4.5 M) concentration of NaCl monitored from the changes in Θ_{222} using circular dichroism. The corresponding melting temperatures (T_m) are 42.4, 50.7 and 50.1°C. The far UV CD spectrum of AHA in buffer A at 25°C in the presence of low and high salt concentrations is given as *inset a*. Variation in T_m values of AHA as a function of salt concentration is given as *inset b*. A protein concentration of 0.67 μM in 0.02 M borate buffer containing 0.18 mM $CaCl_2$ at pH 7.0 was used for all the CD measurements

NaCl shows ~15% lesser activity than the maximum activity (0.5–2.0 M NaCl) but ~12% higher activity compared to that in 0.01 M NaCl. Since AHA binds one chloride ion for allosteric activation like all chloride-dependent amylases, the use of 0.01 M NaCl as the lowest salt concentration allows removing the effects of allosteric activation from the true halotolerance. It has to be noted that in the presence of 0.5–3.0 M NaCl, AHA shows simultaneous increase in both stability and activity compared to that in the presence of 0.01 M NaCl. The ΔT_m of 8.3°C reported at higher salinity in the present work is significant especially for a non-halophilic enzyme of psychrophilic origin exhibiting the lower limit of thermodynamic stability. Even at 4.5 M NaCl, the highest salt concentration used, 80% activity was retained with respect to that in 0.01 M NaCl besides a higher T_m (Fig. 5, inset b).

Salts can promote protein stability (a) by preferential hydration or binding (Timasheff 1998) or due to Hofmeister effect (Record et al. 1998), (b) by shielding of any repulsion between the similarly charged amino acid residues on the surface and (c) by specifically binding to ion binding sites in the protein. One or more of the above factors might be the reason for the higher T_m, T_{opt} and $t_{½}$ under high salinity. Also, improved melting temperature in the presence of higher salt concentrations make the psychrophilic AHA slightly mesophilic.

Comparison of the salt tolerance of AHA, BAA and BLA

The α-amylases AHA, BAA and BLA are fairly acidic with a net charge of –21, –17 and –12, respectively (Table 1). The higher content of acidic residues and lower content of basic residues in AHA results in 4.7% excess acidic residues in AHA compared to a 3.5 and 2.4% excess in BAA and BLA, respectively (Table 1). This results in a higher net negative charge for AHA also indicated by a theoretical p*I* of 4.82 that is fairly acidic compared to BAA or BLA (Table 1). The analysis of the three-dimensional structures of AHA (Aghajari et al. 2002) indicates that 65% of the solvent exposed acidic amino acids in AHA are not involved in any electrostatic interactions in the native state. On the other hand, more than 60% of the negatively charged residues are neutralized by the formation of salt bridges in BLA (Machius et al. 1998). Effectively, the negative surface potential is greater in AHA than BLA, thus facilitating the ion pair formation in AHA to a greater extent under saline conditions. The surface potential representation of the three α-amylases generated with GRASP supported this fact with the order of negative surface potential being AHA > BAA > BLA (data not shown). Thus, the salt tolerance decreases with increasing thermal stability with the most stable BLA being the least halotolerant enzyme.

Table 1 Comparison of the halophilic and halotolerant α-amylases

Parameters	AHA	BAA	BLA	AmyA	AmyH^{bacterial}	AmyH^{archaeal}	HMA	NAA
Domain of origin	Bacteria	Bacteria	Bacteria	Bacteria	Bacteria	Archaea	Archaea	Archaea
Source	*Pseudoalteromonas haloplanktis*	*Bacillus amyloliquefaciens*	*Bacillus licheniformis*	*Halothermothrix orenii*	*Halomonas meridiana*	*Haloarcula hispanica*	*Haloferax mediterranei*	*Natronococcus amylolyticus*
Special feature	Psychrophilic	Mesophilic	Mesophilic, thermostable	Thermophilic, moderately halophilic	Moderately halophilic	Halophilic	Halophilic	Haloalkaliphilic
p*I*[a]	4.82 (5.5)	5.31	6.05	5.30	4.51	4.20	4.18	4.02
Acidic residues (Asp/Glu) (%)	10.4	13.9	12.8	14.5	13.0	16.6	15.8	2.4
Basic residues (Arg/Lys) (%)	5.7	10.4	10.4	10.8	4.8	6.1	5.7	5.5
Excess acidic residues (%)	4.7	3.5	2.4	3.7	8.2	10.5	10.1	18.5
Net charge	–21	–17	–12	–18	–36	–41	–70	–87
Large hydrophobic residues[b] (%)	22.5	24.5	25.1	30.1	22.4	23.5	22.7	20.9
Small hydrophobic residues[c] (%)	16.1	13.2	13.8	10.8	18.5	16.5	14.1	17.4
Ratio of c/b[d]	0.72	0.54	0.55	0.36	0.83	0.70	0.62	0.83
NaCl (M) for optimum activity	0.5	0.5	0.5	0.85	1.7	4.0	3.0	2.5
Activity at NaCl >4.0 M (%)	57	9	0	90	NA	100	100	20

References: AmyA (Sivakumar et al. 2006); AmyH^{bacterial} (Coronado et al. 2000); AmyH^{archaeal} (Hutcheon et al. 2005); HMA (Perez-Pomares et al. 2003); NAA (Kobayashi et al. 1994)

[a] The p*I* given are theoretical values. For AHA, the estimated p*I* is given in parenthesis

[b] Phe, Trp, Tyr, Leu, Ile and Met

[c] Ala and Val. The amino acid composition of all the enzymes were obtained from the SwissProt database available at http://www.expasy.org

[d] Ratio of small hydrophobic amino acid residues to large hydrophobic amino acid residues

AHA and other halophilic/halotolerant α-amylases

Most halophilic and halotolerant enzymes reported till date are characterized by the presence of higher percentage of negatively charged residues either distributed throughout the protein surface or found concentrated in a localized region of the enzyme (Madern et al. 2000). In either case, ion pair formation in the presence of salt alleviates the charge repulsion and therefore increases the stability of the whole enzyme or a part of it, such that the essential dynamics of the enzyme required for its optimal functioning is preserved. In halophilic proteins, a negative surface potential generated by clustering of acidic residues allow the formation of protective hydrated ion network which prevents aggregation by reducing the salting out effect under high salinity and keep the protein functional and stable (Danson and Hough 1997; Madern et al. 2004). The surplus acidic residues in these proteins could promote the tight binding of the hydration shell to the protein due to the strong hydration of the carboxylate groups of glutamate and aspartate (Madern et al. 2000, 2004; Dym et al. 1994; Mevarech et al. 2000).

The details of the α-amylases chosen for comparison, namely AmyA from *Halothermothrix orenii* (Sivakumar et al. 2006), AmyH^{bacterial} from *Halomonas meridiana* (Coronado et al. 2000), AmyH^{archaeal} from *Haloarcula hispanica* (Hutcheon et al. 2005), HMA from *H. mediterranei* (Perez-Pomares et al. 2003) and NAA from *Natronococcus amylolyticus* (Kobayashi et al. 1994) are given in Table 1. The amino acid sequences were imported from SwissProt database. With respect to activity, maximum activity was observed at 0.5–2.0 M NaCl for AHA that is comparable to the archeal, halophilic α-amylases NAA and HMA, less than that observed for AmyH^{archaeal} (4.0 M) but certainly higher than that seen for the moderately halophilic bacterial AmyA and AmyH^{bacterial}. This suggests that AHA indeed behaves like a halophilic enzyme. This is also supported by the fact that at NaCl >4.0 M AHA retained 57% activity compared to 20% in NAA though AmyA and HMA retained 90 and 100% activity, respectively. Though NAA has the structural features that are more typical of halophilic proteins, compared to HMA and AHA it is less active in NaCl >4.0 M (Table 1). However, these are only the relative activities and the specific activities could however be different. Partially, the reasons for the difference in the degree of salt tolerance could be the ability of AHA and HMA to retain the crucial catalytic water even in the presence of competing salt (reduction in water activity) besides exhibiting the required conformational dynamics and flexibility to perform the catalytic steps. Reduction of enzyme activity of AHA at very high salinities might be due to this competition as proposed for NAA (Kobayashi et al. 1994) besides the altered flexibility of the active site region. These observations show that halophilic enzymes and hence AHA could function under low water activity conditions that exist when organic solvents are used as reaction media.

It was found that all these α-amylases are acidic which is a characteristic feature of many halophilic enzymes (Premkumar et al. 2005; Madern et al. 2004). AHA shows a negative charge of –21 that is higher than the moderately halophilic AmyA (Table 1). The proportion of acidic amino acids (Asp and Glu) in NAA, HMA, AmyH^{archaeal} and AmyH^{bacterial} is 18.5, 10.1, 10.5 and 8.2% higher than the basic residues (Arg and Lys), respectively, compared to 4.7% in AHA (Table 1). Also, halophilic proteins are enriched in smaller hydrophobic residues, particularly Ala whereas the mesophilic counter parts have higher content of large hydrophobic residues (Madern and Zaccai 2004). The ratio of small hydrophobic residues to large hydrophobic residues is in the order NAA = AmyH^{bacterial} > AHA > AmyH^{archaeal} > HMA > BLA = BAA > AmyA. Though AHA is less acidic than the extremely halophilic HMA, NAA and AmyH^{archaeal}, the proportion of smaller hydrophobic residues is comparable. This indicates that generally halophilic and halotolerant enzymes resist salinity by enriching their sequence with acidic and/or smaller hydrophobic residues. However, the structural basis of halophily in proteins is far from being firmly established as not many crystal data are available. The crystal structure of the poly-extremophilic enzyme AmyA, that is both halophilic and thermophilic, solved recently points the lack of acidic surface or higher proportion of smaller hydrophobic residues. This enzyme has both positive and negative residues evenly distributed on the surface (Sivakumar et al. 2006). This could be a compromise to be thermophilic and hence AmyA which is less acidic than AHA and other halophilic amylases might have factors other than the negative surface to adapt to high salinity. Interestingly the higher proportion of acidic and small hydrophobic residues is a cold-adaptive feature of AHA and several other psychrophilic enzymes (Aghajari et al. 1998; Georlette et al. 2004; D'Amico et al. 2003; Feller 2003; Smalås et al. 1994). In AHA, we find that the same structural factors mentioned above are possibly involved in both cold- and salt-adaptations. The comparable salt-tolerance of AmyA and AHA that are thermophilic and psychrophilic, respectively, and the differences in the contributing structural factors once again suggests that halophilic adaptations are poorly understood.

The multiple sequence alignment of AHA, BAA, BLA and AmyH^{bacterial} is given as supplementary data (Fig. 1, electronic supplement data). AHA showed strong similarity with the moderately halophilic AmyH^{bacterial} from *Halomonas meridiana* as indicated by the 52% sequence identity, 18% similarity and 12% weak similarity. Only 18.6% residues are different between AHA and AmyH^{bacterial}. It was found that out of 29 Asp and 26 Glu in AmyH^{bacterial}, 17 Asp and 12 Glu are conserved in AHA. However, AHA contains 13 Lys and 13 Arg compared to 5 and 16 in AmyH^{bacterial}. Thus, the main difference is increased Glu and decreased Lys in AmyH^{bacterial} that makes AHA less acidic than AmyH^{bacterial} even though AHA shows better salt tolerance (Table 1). Incidentally, the presence of 8 Cys that is unique in AHA (not seen in BAA or BLA) is intact in AmyH^{bacterial} also. The four disulfide bonds were found to be important in preserving the active site from ionic interactions and for the catalytic stability of AHA (Siddiqui et al. 2005). Thus, the unusual salt tolerance of the psychrophilic AHA is well supported by its halophilic-like structural features.

AHA and non-amylolytic halophilic/halotolerant enzymes

Like AHA, carbonic anhydrase I (*d*CAI) from the halophilic green algae *Dunaliella salina* found in Dead Sea (Premkumar et al. 2005) and the nucleoside diphosphate kinases *Hs*NDPk (Ishibashi et al. 2002) and *Nm*NDPk (Polosina et al. 2002), respectively, from the halophilic archaeon *Halobacterium salinarum* and the haloalkaliphilic archaeon *Natrialba magadii* are exceptions and classified as halotolerant owing to their ability to function over a wide range of NaCl concentrations. However, *d*CAI exhibited an unaltered catalytic efficiency only in 0–0.5 M NaCl compared to the tolerance of up to 4.5 M NaCl by AHA suggesting the better halotolerant adaptation of AHA. Both AHA and *d*CAI contain more acidic residues (10.4 and 21.4%, respectively) resulting in a net negative charge on the protein surface similar to that seen in halophilic proteins (Premkumar et al. 2005). Halotolerant malate dehydrogenase from the extremely halophilic bacterium *Salinibacter ruber* (*Sr*MalDH) showed only 20% activity at 3.5 M KCl (Madern et al. 2004) in contrast to 68% activity retained by the non-halophilic AHA at 3.5 M NaCl. Interestingly, AHA contains only 4.7% excess acidic residues compared to the 6.7% excess acidic residues in *Sr*MalDH. Thus, it could be seen that the salt tolerance of the psychrophilic α-amylase is comparable and/or superior to that observed for halophilic and halotolerant enzymes.

Conclusions

The above results suggest that the native state of the psychrophilic α-amylase AHA, is better stabilized in the presence of molar concentrations of NaCl than under low salt conditions. The generally observed lower activation energy for the catalysis by the flexible psychrophilic enzyme increased under high salt condition indicating a less flexible conformation with a surprisingly higher activity. Thus, the halotolerance of AHA seems to be a consequence of the selective pressure to be psychrophilic. This salt resistance has been acquired with the existing primary sequence-dependent psychrophilic adaptations. Though the definition of halophiles (Madern et al. 2000) excludes AHA from being called a halophilic protein, it has superior resistance to salinity like halophilic enzymes besides its well-established psychrophily. Hence, AHA could be classified as a polyextremophilic protein. Enhanced negative surface potential and enrichment of acidic and small hydrophobic residues are the psychrophilic properties, also the so far described characteristics of many halophilic proteins, seemingly important for the halotolerant behavior of AHA. We presume that these features could also be found in other psychrophilic enzymes. Most of the psychrophilic proteins, owing to the presence of excess acidic residues, show acidic p*I* like the halophilic proteins. It will not be inappropriate to propose that some of the acidic psychrophilic enzymes could be halotolerant though further investigation is necessary. It is possible that such enzymes could, in fact, have the required molecular adaptations to be called as polyextremophilic enzymes. Enzymes with multiple specificities and wide range of functional characteristics in various environmental and laboratory conditions could become the future target for research. Therefore, more investigations aimed at revealing the multi-environment adaptations could have potential industrial advantages.

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References

Aghajari N, Feller G, Gerday C, Haser R (1998) Structures of the psychrophilic *Alteromonas haloplanctis* α-amylase give insights into cold adaptation at a molecular level. Structure 6:1503–1516

Aghajari N, Feller G, Gerday C, Haser R (2002) Structural basis of α-amylase activation by chloride. Protein Sci 11:1435–1441

Coronado M, Vargas C, Hofemeister J, Ventosa A, Nieto JJ (2000) Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridiana*. FEMS Microbiol Lett 183:67–71

D'Amico S, Gerday C, Feller G (2001) Structural determinants of cold adaptation and stability in a large protein. J Biol Chem 276:25791–25796

D'Amico S, Marx J-C, Gerday C, Feller G (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278:7891–7896

Danson MJ, Hough DW (1997) The structural basis of protein halophilicity. Comp Biochem Physiol 117A:307–312

Dym O, Mevarech M, Sussman JL (1994) Structural features that stabilize halophilic malate dehydrogenase from an archaebacterium. Science 267:1344–1346

Feller G (2003) Molecular adaptations to cold in psychrophilic enzymes. Cell Mol Life Sci 60:648–662

Feller G, D'Amico S, Gerday C (1999) Thermodynamic stability of a cold-active α-amylase from the Antarctic bacterium *Alteromonas haloplanktis*. Biochemistry 38:4613–4619

Feller G, Lonhienne T, Deroanne C, Libioulle C, Beeumen JV, Gerday C (1992) Purification, characterization, and nucleotide sequence of the thermolabile α-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis A23*. J Biol Chem 267:5217–5221

Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx J-C, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42

Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000) Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol 18:103–107

Hutcheon GW, Vasisht N, Bolhuis A (2005) Characterisation of a highly stable α-amylase from the halophilic archaeon *Haloarcula hispanica*. Extremophiles 6:487–495

Ishibashi M, Arakawa T, Philo JS, Sakashita K, Yonezawa Y, Tokunaga H, Tokunaga M (2002) Secondary and quaternary structural transition of the halophilic archaeon nucleotide diphosphate kinase under high- and low-salt conditions. FEMS Microbiol Lett 216:235–241

Kobayashi T, Kanai H, Aono R, Horikoshi K, Kudo T (1994) Cloning, expression, and nucleotide sequence of the α-amylase gene from the haloalkaliphilic archaeon *Natronococcus* sp strain Ah-36. J Bacteriol 176:5131–5134

Lowry OH, Rosebrough NJ, Farr AL, Randall R (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275

Machius M, Declerck N, Huber R, Wiegand G (1998) Activation of *Bacillus licheniformis* α-amylase through a disorder → order transition of the substrate-binding site mediated by a calcium–sodium–calcium metal triad. Structure 6:281–292

Madern D, Camacho M, Rodriguez-Arnedo A, Bonete MJ, Zaccai G (2004) Salt-dependent studies of NADP-dependent isocitrate dehydrogenase from the halophilic archaeon *Haloferax volcanii*. Extremophiles 8:377–384

Madern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzyme. Extremophiles 4:91–98

Madern D, Zaccai G (2004) Molecular adaptation: the malate dehydrogenase from the extreme halophilic bacterium *Salinibacter ruber* behaves like a non-halophilic protein. Biochimie 86:295–303

Madigan MT, Marrs BL (1997) Extremophiles. Sci Am 276:82–87

Mevarech M, Frolow F, Gloss LM (2000) Halophilic enzymes: proteins with a grain of salt. Biophys Chem 86:155–164

Perez-Pomares F, Bautista V, Ferrer J, Pire C, Marhuenda-Egea FC, Bonete MJ (2003) α-Amylase activity from the halophilic archaeon *Haloferax mediterranei*. Extremophiles 7:299–306

Pflüger K, Müller V (2004) Transport of compatible solutes in extremophiles. J Bioenerg Biomembr 36:17–24

Polosina YY, Zamyatkin DF, Kostyukova AS, Filimonov VV, Fedorov OV (2002) Stability of *Natrialba magadii* NDP-Kinase: comparisons with other halophilic proteins. Extremophiles 6:135–142

Premkumar L, Greenblatt HM, Bageshwar UK, Savchenko T, Gokhman I, Sussman JL, Zamir A (2005) Three-dimensional structure of a halotolerant algal carbonic anhydrase predicts halotolerance of a mammalian homolog. Proc Natl Acad Sci USA 102:7493–7498

Record MTJ, Zhang W, Anderson CF (1998) Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: a practical guide to recognizing and interpreting polyelectric effects, Hofmeister effects and osmotic effects of salts. Adv Protein Chem 51:281–353

Siddiqui KS, Poljak A, Guilhaus M, Feller G, D'Amico S, Gerday C, Cavicchioli R (2005) Role of disulfide bridges in the activity and stability of a cold-active α-amylase. J Bacteriol 187:6206–6212

Sivakumar N, Li N, Tang JW, Patel BK, Swaminathan K (2006) Crystal structure of AmyA lacks acidic surface and provide insights into protein stability at poly-extreme condition. FEBS Lett 580:2646–2652

Smalås AO, Heimstad ES, Hordvik A, Willassen NP, Male R (1994) Cold adaptation of enzymes: structural comparison between salmon and bovine trypsins. Proteins 20:149–166

Srimathi S, Jayaraman G (2005) Effect of glycosylation on the catalytic and conformational stability of homologous α-amylases. Protein J 24:79–88

Sundaram PV, Srimathi S (2004) Analysis of catalytic and structural stability of native and covalently modified enzymes. In: Svendsen A (ed) Enzyme functionality: design, engineering and screening. Marcel Dekker, New York, pp 632–661

Timasheff SN (1998) Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. Adv Protein Chem 51:355–432

Venkatesh R, Srimathi S, Yamuna A, Jayaraman G (2005) Enhanced catalytic and conformational stability of Atlantic cod trypsin upon neoglycosylation. Biochim Biophys Acta 1722:113–115

Wright DB, Banks DD, Lohman JR, Hilsenbeck JL, Gloss LM (2002) The effect of salts on the activity and stability of *Escherichia coli* and *Haloferax volcanii* dihydrofolate reductases. J Mol Biol 323:327–344