http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (18): 3154-3157, 2007
ISSN 1028-8880
© 2007 Asian Network for Scientific Information

Denaturation of Bacillus Amyloliquefaciens α-amylase with Urea

[1]B. Shareghi, [1]M. Arabi and [2]M. Zargham
[1]Department of Biology, Faculty of Basic Sciences, Shahrekord University, Iran
[2]Isfahan University of Medical Sciences, Iran

Abstract: The urea induced denaturation of the *Bacillus amyoliqefaciens* α-amylase (E.C. 3.2.1.1) was studied by absorption measurements in the near ultra-violet region and specific activity measurements. Spectral measurements were made at pH 6.9 and over the temperature range 20-80°C. It has been observed that urea induced a cooperative transition. In the absence of denaturant, the Gibs energy changes were in the range of 8-15 kcal mol^{-1}. α-amylase lost 80% of its activity in the concentrated solution of urea. α-amylase was more thermostable than other mesophilic enzymes.

Key words: Denaturation, *Bacillus amyloliquefaciens*, α-amylase, urea, thermostable

INTRODUCTION

α-amylases (E.C. 3.2.1.1) are the enzyme hydrolyzing the interal α-1,4- glucosidic linkage in starch. Most α-amylase derived from animal, plants and microorganisms keep their unique enzymatic properties only in normal physiological condition (Janecek *et al.*, 1997). Nevertheless, there are some microbial α-amylase of thermophiles which exhibit exceptional stability (Vihinen *et al.*, 1994).

Protein denaturation is a highly cooperative process, which, for small globular proteins, maybe approximated by a two-state model (Pace, 1990a). Denaturation studies are capable of yielding information about the native state in terms of its cooperativity, intrinsic stability and the nature of forces required to maintain its tertiary structure (Chen *et al.*, 1999; Blaber and Culajay, 1999; Ahmad and Bigelow, 1986). Small organic molecules in aqueous solution can have profound effects on protein stability, structure and function. The use of these solutions to stabilize or destabilize proteins, depending on the co-solvent, is commonplace. In fact, protein studies are conducted almost exclusively in complex solutions. Chemical denaturation, with an agent such as urea, is one of the primary ways to assess protein stability, the effects of mutations on stability and protein unfolding. Despite its widespread use, the molecular basis for urea's ability to denature proteins remain unknown. Urea may exert its effect directly by binding to the protein, or indirectly, by altering the solvent environment (Timasheff, 2002a; Tobi *et al.*, 2003; Anderson *et al.*, 2002; Felitsky and Record, 2003; Schellman, 2002; Timasheff, 2002b; Dill and Shortle, 1991).

We report, here, the results of the denaturation of *B. amyloliqefaciems* α-amylase at pH 6.9 and different temperatures. It has been observed that urea induce cooperative denaturation. The product of denaturation has been characterized by determining thermodynamic parameters of unfolding as well as the midpoint of the thermal unfolding curves (T_m).

MATERIALS AND METHODS

α-amylase was purchased from Sigma Chemical Company. Urea was obtained from Merck Co. Sodium phosphate 0.02 M pH 6.9 was used as buffer.

Determination of α-amylase stability: Denaturation curve of α-amylase (0.5 mg mL^{-1}) from 20 to 80°C at 280 nm were made in a pharmacia Biotech-4000 UV-Visible spectrophotometer having cell holder whose temperature was regulated by an external thermostat, at a heating rate 1 K min^{-1}.

Effect of urea on α-amylase stability: To asses the effect of urea on enzyme stability, samples were made in sodium phosphate buffer 0.02 M, pH 6.9 and urea over the concentration range (1-8 M). Denaturation curve were made at 280 nm at the desired temperature.

Assay of α-amylase activity: The activity of -amylase was measured in a 1 mL reaction mixture using soluble potato starch as substrate in 20 mM sodium phosphate buffer pH 6.9. The concentration of reducing sugars obtained from

Corresponding Author: B. Shareghi, Department of Biology, Faculty of Basic Sciences, Shahrekord University, P.O. Box 115, Shahrekord-88186, Iran Tel: 00 98 381 4424419 Fax: 00 98 381 4424419

catalyzed reaction was measured by the dinitrosalicylic acid method according to Bernfeld (1995). Specific activity of enzyme (SPA) was calculated using:

$$\text{Unit mg}^{-1} = \frac{\mu\text{mole of maltose}}{\text{mg enzyme in reaction mixture} \times 3}$$

Effect of urea on α-amylase activity: The activity of α-amylase was measured at 62°C in 20 mM sodium phosphate buffer pH 6.9 and urea concentration over the 1-8 M range.

RESULTS

Figure 1 depicts the profiles of α-amylase denaturation at various urea concentration. The fraction of the denatured protein, F_d, was calculated by assuming a two-state process and using the relation,

$$F_d = \frac{Y_{obs} - Y_N}{Y_D - Y_N} \quad (1)$$

Where, Y_n and Y_D are respectively the optical properties of the native and denatured molecules under the conditions in which Y_{obs} has been determined.

It is obvious that increasing the concentration of urea, decreases the thermal stability of α-amylase. The difference in free energy between the folded and unfolded conformation, can then be calculated using the equations:

$$\Delta G^\circ_D = -RT\ln\left[F_d/(1 - F_d)\right] = -RT\ln\left[(Y_N - Y_{obs})/(Y_{obs} - Y_D)\right] \quad (2)$$

Where R is the gas constant and T is the absolute temperature. The free energy of denaturation, ΔG°_D, as a function of temperature for α-amylase in the presence and absence of urea is shown in Fig. 2. These results can be used to determine T_m at which $\Delta G^\circ_D = 0$, the entropy change at T_m, (ΔS°_m) and the enthalpy change at T_m, (ΔH°_m), (Pace, 1990b).

ΔS°_m can be calculated from the slope of denaturation curves at T_m. ΔH°_m was calculated using:

$$\Delta H^\circ_m = T_m \Delta S^\circ_m \quad (3)$$

It is obvious that on increasing the concentration of urea to α-amylase, the curves shift to lower temperatures (Fig. 2). Thermodynamic data of T_m, ΔH°_m and ΔS°_m at various concentration of urea are given in Table 1. Melting temperature (T_m) for native α-amylase is 355 K. This enzyme is more thermostable than other mesophilic

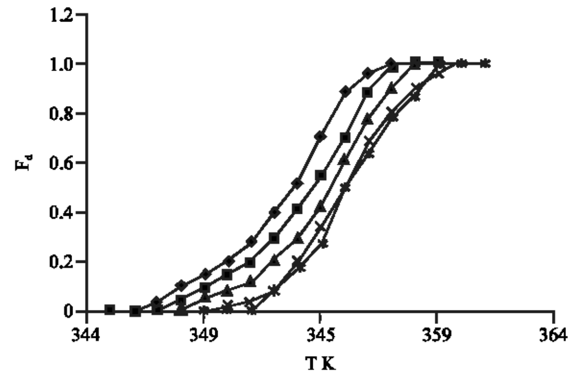

Fig. 1: Denaturation curves of α-amylase at various urea concentrations [urea] 0 M, [urea], 3 M [urea], 8 M [urea] and 1 M [urea] 6 M

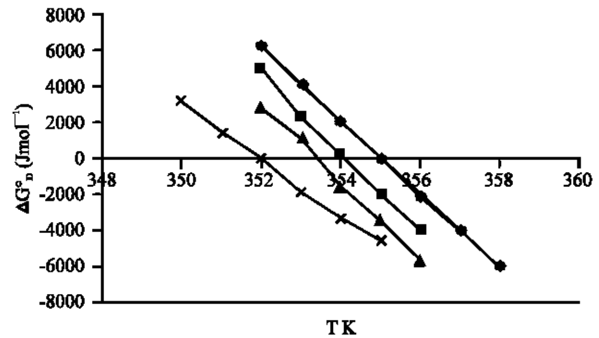

Fig. 2: ΔG°_D curves of α-amylase at various urea concentrations. [urea] 0 M, [urea] 3 M [urea], 8 M [urea] and 1 M [urea] 6 M

Table 1: Thermodynamic data of T_m, ΔS°_m, ΔH°_m at various urea concentration

Urea M	T_m K	ΔS°_m Jmol^{-1} K^{-1}	ΔH°_m kJmol^{-1}
0	355	2000	710
1	355	2000	710
3	354.2	1833	649
6	353.4	1714	605
8	352	1500	528

enzyme. Data from Fig. 1 can by used to plot the urea unfolding curves which is shown in Fig. 3, where the free energy of denaturation, ΔG°_D, is plotted against urea concentration. ΔG°_D was found to vary linearly with urea concentration according to the equation:

$$\Delta G^\circ_D = \Delta G^\circ_D(H_2O) - m\left[\text{urea}\right] \quad (4)$$

Where, ΔG°_D (H_2O) is the value of ΔG°_D in absence of urea and m is a measure of the dependence of ΔG°_D on urea concentration. The value of ΔG°_D (H_2O) at temperatures of 353 K and 354 K are 5 and 2.6 kJmol^{-1}

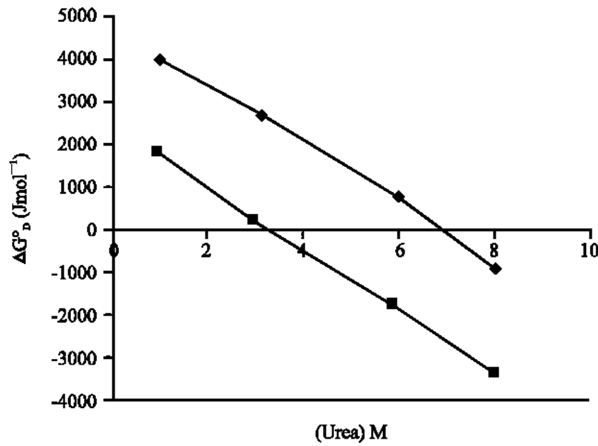

Fig. 3: ΔG°_{D} curves of α-amylase at various urea concentrations. —◆— T 353 K and —■— T 354 K

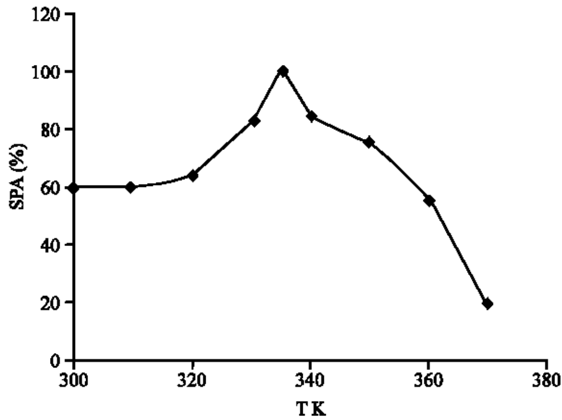

Fig. 4: Specific activity curve of α-amylase at various temperatures

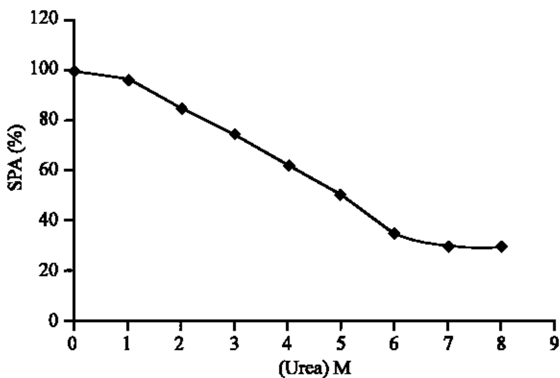

Fig. 5: Specific activity curve of α-amylase at various urea concentrations at 335 K

respectively, corresponding to decreasing stability of α-amylase. Plotting -amylase activity over the 300-370 K temperature range shows an activity maximum at 335 K (Fig. 4). In the presence of urea the enzyme loosed 80% of its initial activity (Fig. 5). Urea induced denaturation of α-amylase at temperatures more than 345 K, thus at 335 K urea has no denaturation effect on α-amylase structure. As amide reagents can inhibit α-amylase activity (Wetlaufer *et al.*, 1964), the decreasing of α-amylase activity at 335 K resulted from inhibition by urea.

DISCUSSION

Melting temperature (T_m) for native -amylase is 355 K. This enzyme is more thermostable than other mesophilic enzymes. α-amylase from *B. amyloliquefaciens* has 33% hydrophibic amino acids (Yamazaki *et al.*, 1983). The evidence indicate that the enhanced stability of enzyme is the result of a variety of stabilizing effects including hydrophobic interaction, ionic and hydrogen binding (Fontana, 1988; Tanford, 1980; Fitter and Heberle, 2000). It seems that hydrophobicity play an important role for α-amylase thermostability. Numerous example of thermal stability of α-amylase from *B-amylolquefaciens* have been found in previous papers. Suzuki *et al.* (1989) demonstrated that the deletion of Arg^{176} and Gly^{177} from the enzyme seemed to enhance its thermal stability. They suggested that this enhancement might have been due to improved internal packing, since these deletions lead to an increase in hydrophobicity. Rajendran *et al.* (1995) reported that some organic solvents, dimethyl sulfoxide (DMSO) and poly (ethylene glycol) increased the stability of α-amylase involving hydrophobic interaction with the hydrophobic regions in the protein. Therefore the internal hydrophobic packing in the core of the hydrophobic residues, which is exposed to the hydrated surface upon the heating, accelerated the unfolding of the enzyme. Chemical denaturation with urea is a general way to assess the stability of α-amylase and other proteins. It is obvious that increasing the concentration of urea, decreases ΔH°_{m} and ΔS°_{m} and T_m. ΔH°_{m} and ΔS°_{m} can be shown using:

$$\Delta H^{\circ}_{m} = H^{\circ}_{m}(D) - H^{\circ}_{m}(N) \tag{5}$$

$$\Delta S^{\circ}_{m} = S^{\circ}_{m}(D) - S^{\circ}_{m}(N) \tag{6}$$

As the denatured state of proteins is random coil, thus decreasing ΔS°_{m} and ΔH°_{m} in the presence of urea, implying unfolding of the native conformation of enzyme. Many studies have sought to explain the action of denaturants such as urea (Timasheff, 2002a; Tobi *et al.*, 2003; Anderson *et al.*, 2002; Felitsky and Record, 2003;

Schellman, 2002; Timasheff, 2002b; Dill and Shortle 1991; Sijpkes and Gill, 1993; Nandi and Robinson, 1984; Cheek and Lilley, 1988; Nozaki and Tanford, 1963). Present results are in good agreement with those reported by other groups. It is generally believed that one of two mechanism responsible for the ability of urea to destabilize the native conformation of globular proteins. In the first model urea interacts with the backbone peptide groups through the formation of hydrogen bonds, whereas in the second model aqueous urea dissolves the non-polar side chain buried in the interior of a native globular protein by decreasing the hydrophobic effect. By using a wide variety of model compound and lysozyme it was shown that protein denaturation is brought about through urea's interaction with polar groups as well as through hydration effects of non-polar group.

Briefly, we have observed that α-amylase from *B-amyloliquefaciens* is more thermostable than other mesophilic enzymes. It seems that hydrophobicity play an important role for α-amylase stability. Urea induced a cooperative transition at temperatures more than 345 K. At lower temperatures, urea acts as an inhibitor for α-amylase and has no effect on the enzyme stability.

REFERENCES

Ahmad, F. and C.C. Bigelow, 1986. Estimation of stability of globular proteins. Biopolymers, 25: 1623-1633.

Anderson, C.F., D.J. Felitsky, J. Hong and M.T. Record, 2002. Generalized derivation of an exact relationship linking different coefficient that characterize thermodynamic effects of preferential interactions. Biophys. Chem., 101: 497-511.

Bernfeld, P., 1955. Methods in Enzymology; Colovick, S.P. and N.O. Kaplan, (Eds.), Vol. 1, Academic Press, New York.

Blaber, S.I. and J.F. Culajay, 1999. Reversible thermal denaturation of human FGF-1 induced by low concentration of Guanidine hydrochloride. Biophys. J., 74: 470-477.

Cheek, P.J. and T.H. Lilley, 1988. Interaction between amides and urea in aqueous solution. J. Chem. Soc. Faraday Trans., 84: 1927-1932.

Chen, H.M., T.J. Dimagno, W. Wang and S.I. Chan, 1999. The effect of E75 of *Staphylococcal nuclease* on enzyme activity, protein stability and protein folding. Eur. J. Biochem., 261: 599-609.

Dill, K.A. and B. Shortle, 1991. Denatured states of proteins. Annu. Rev. Biochem., 60: 795-825.

Felitsky, D.J. and M.T. Record, 2003. Thermal and urea-induced unfolding of the marginally stable lac repressor DNA-binding domain: A model system for analysis of solute effects on protein processes. Biochemistry, 42: 2202-2217.

Fitter, J. and J. Heberle, 2000. Structural equilibrium fluctuations in mesophilic and thermophilic α-amylase. Biophysics J., 79: 1629-1636.

Fontana, A., 1988. Structure and stability of thermophilic enzymes. Biophys. Chem., 29: 181-193.

Janecek, S., B. Svensson and B. Henrissat, 1997. Domain evolution in the α-amylase family. J. Mol. Evol., 45: 322-331.

Nandi, P.K. and D.R. Robinson, 1984. Effect of urea and guanidine hydrochloride on peptide and non-polar groups. Biochemistry, 23: 6661-6663.

Nozaki, Y. and C. Tanford, 1963. The solubility of aminoacids and related compound in aqueous urea solutions. J. Biol. Chem., 238: 4071-4081.

Pace, C.N., 1990a. Measuring and increasing protein stability. TIB. THCK., 8: 63-68.

Pace, C.N., 1990b. Conformational stability of globular proteins. Trend Biochem. Sci., 15: 14-17.

Rajendran, S., C. Radha and V. Prakash, 1995. Mechanism of solvent-induced thermal stabilization of α-amylase from *Bacillus amyloliquefaciens*. Int. J. Peptide Protein Res., 45: 122-128.

Schellman, J.A., 2002. Fifty years of solvent denaturation. Biophys. Chem., 96: 91-101.

Sijpkes, A.H. and S.C. Gill, 1993. Urea-diketopiperazine interactions: A model for urea induced denaturation of proteins. Biophys. Chem., 46: 171-177.

Suzuki, F., N. Ito, T. Yuuki, H. Yamagata and S. Udaka, 1989. Improving thermostability of Raw-starch digesting amylase from a *Cytophaga* sp. by site-directed mutagenesis. J. Biol. Chem., 264: 18933-18938.

Tanford, C., 1980. The Hydrophobic Effect; Wiley, New York.

Timasheff, S.N., 2002a. Thermodynamic binding and site occupancy in the light of the Schellman exchange concept. Biophys. Chem., 101-102: 99-111.

Timasheff, S.N., 2002b. Protein-solvent preferential interactions, protein hydration and the modulation of biochemical reactions by solvent components. Proc. Natl. Acad. Sci., 99: 9721-9726.

Tobi, D., R. Elber and D. Thirumalai, 2003. Water and protein: A love-hate relationship. Biopolymers, 68: 359-369.

Vihinen, M., T. Peltonen and A. Liti, 1994. C-terminal truncations of a thermostable *Bacillus stearothermophilus* α-amylase. Protein Eng., 7: 1255-1259.

Wetlaufer, D.B., S.K. Malik and L. Stoller, 1964. Non-polar group participation in the denaturation of proteins by urea and guanidinium salts. Model compound studies. J. Am. Chem. Soc., 86: 508-514.

Yamazaki, H.J., 1983. Change in the properties and molecular weights of *Bacillus subtilis* m-type α-amylase. Bacteriology, 156: 327-337.