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"Research Note"

THERMAL DENATURATION OF α-AMYLASE FROM BACILLUS AMYLOLIQUEFACIENS IN THE PRESENCE OF SODIUM DODECYL SULPHATE*

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Abstract – The thermal denaturation of α-Amylase from Bacillus amyloliquefaciens has been investigated in the presence and absence of sodium dodecyl sulphate (SDS) over the temperature range (293-373) K in 20 mM sodium phosphate buffer, pH 6.9, using temperature scanning spectroscopy. The presence of SDS caused the destabilization of α-Amylase resulting in a decrease in the temperature of unfolding with an increase in SDS concentration. The thermodynamic parameters for unfolding of α-Amylase were determined in terms of the two-state model, using the temperature dependence of the spectroscopic behaviour of the enzyme at 280 nm. The result from specific activity measurements in the presence of SDS is in conformity with the results from thermal studies.

Keywords – Stability, denaturation, thermodynamic, SDS, α-Amylase

1. INTRODUCTION

α-Amylases (α-1, 4-glucan–glucanohydrolase) are widely distributed in microorganisms, plants, and animals. These enzymes have been investigated most thoroughly and used most widely in the starch industry [1]. They catalyze the hydrolysis of internal α(1, 4)–glycosidic bonds with net retention of the anomeric configuration in starch, amylose, amylopection, glycogen and other polysaccharides through multiple attacks toward the non reducing end [2]. In general, bacterial α-Amylases have been classified into two types, one is saccharifying and the other liquefying soluble starch. α-Amylases of *B. amyloliquefaciens* belong to the latter type [1, 3]. Sequence comparison of the α-Amylases has shown that their core structure were highly conserved among bacterial strains, indicating that they originated from a common ancestor gene [4]. The conformational stability of most globular proteins is surprisingly low, generally 5-15 kCal mol^{-1} [5]. The conformational stability of a globular protein may be defined as the free energy change for the reaction:

$$\text{folded} \longleftrightarrow \text{unfolded}$$

Estimates from SDS denatured curves are designated $\Delta G_D^\circ(H_2O)$ and estimates that form thermal denaturation curves are designated ΔG_D° [5]. Ionic surfactants like sodium n-dodecyl sulphate disrupt the native structure of most globular proteins at very low concentration [6]. The mechanism of the denaturation of proteins by surfactants involves the binding of surfactant ions to sites on the protein molecules. The initial interaction between surfactants and proteins is predominantly ionic, as the

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surfactant ions bind to groups of opposite charge on the protein [6]. These initial interactions cause the protein to unfold, resulting in the exposure of more binding sites, and as the surfactant concentration is increased, binding becomes co-operative, and ultimately, saturation occurs [7]. A number of studies on the thermodynamics of the interaction between surfactants and globular proteins have been reported during recent years [8-10]. In the present paper, a thermal stability study of *B. amyloliguefaciens* α-Amylase in the presence of sodium dodedcyl sulphate (SDS) as an anionic detergent is reported to obtain some thermodynamic parameters of unfolding as well as the midpoint of the thermal unfolding curves, (T_m). *a*-Amylase is an important industrial enzyme. As well as being used as an additive in detergents, it can be used for such things as the removal of starch sizing from textiles, the liquefaction of starch, and the proper formation of dextrin in baking. The thermostability of the *a*-amylase must be matched to the application. For example, thermostable *a*-amylases are used for the liquefaction of starch at high temperatures and thermolabile *a*-amylases are used for the saccharification of starch in baking [1-3].

2. MATERIALS AND METHODS

α-Amylase from *Bacillus amyloliquefaciens* and Sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Company. Sodium phosphate 0.02 M pH 6.9 was used as the buffer.

a) Determination of α-Amylase stability

Denaturation curves of α-Amylase (0.5 mg) from 293 to 373 K at 280 nm were made using Pharmacia Biotech–4000 UV–Visible spectrophotometer having a cell holder whose temperature was regulated by an external thermostat. The heating rate was 1 K/min. Protein concentration was measured by the Lowry method [11].

b) Effect of SDS on α-Amylase stability

To assess the effect of SDS on the enzyme stability, samples were made in sodium phosphate buffer 20 mM, pH 6.9, and SDS concentration over the range of 1–8 mM below the critical micelle concentration (CMC) [10]. Denaturation curves were made at 280 nm at the desired temperatures.

c) Assay of α-Amylase activity

α-Amylase activity was measured in a 1–ml reaction mixture using soluble potato starch as the substrate in a 20 mM sodium phosphate buffer pH 6.9. The concentration of reducing sugars obtained from the catalyzed reaction was measured by the dinitrosalicylic acid method according to Bernfeld [12]. Specific activity of enzyme (SPA) was calculated using:

$$unit/mg = \frac{\mu\ moles\ of\ maltose\ released}{mg\ enzyme\ in\ reaction\ mixture\ \times\ 3\ min}$$

d) Effect of SDS on α-Amylase activity

The activity of α-Amylase was measured at 335 K (as the optimum temperature) in 20 mM sodium phosphate buffer pH 6.9 and SDS concentration over the range of 1–8 mM.

e) Results and Discussion

Thermal denaturation curves for α-Amylase in the presence and absence of sodium dodecyl sulphte (SDS) are shown in Fig. 1. The fraction of the denatured protein, $F_{d,}$ was calculated using the relation [13]:

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

where Y_N and Y_D are respectively the optical properties (absorbance) of the native and denatured molecules under the conditions in which Y_{obs} has been determined.

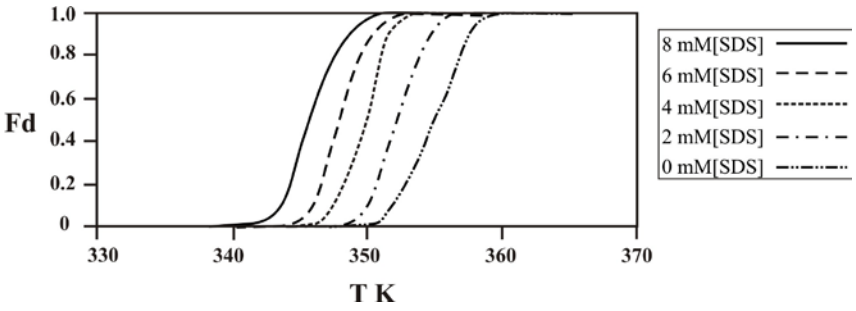

Fig. 1. Denaturation curves of α – Amylase at various SDS concentrations in 20mM sodium phosphate buffer, pH 6.9

It is obvious that increasing the concentration of SDS decreases the stability of α -Amylase to thermal denaturation. The thermal denaturation of small globular proteins closely approach a two-state, mechanism [13]. By assuming a two-state mechanism, the difference in free energy between the folded and unfolded conformation, ΔG°_D, can then be calculated using:

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

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 SDS 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 $T_m (\Delta S^\circ_m)$ and the enthalpy change at $T_m (\Delta H^\circ_m)$. ΔS°_m can be calculated from the slope of denaturation curves at T_m (5). ΔH°_m was calculated using:

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

It is clear that on increasing the concentration of SDS the ΔG°_D curves shift to lower temperatures (Fig. 2). Thermodynamic data of T_m, ΔH°_m and ΔS°_m at various concentrations of SDS are tabulated in Table 1. As indicated in Table 1, increasing the concentration of SDS decreases ΔH°_m, ΔS°_m and T_m. ΔH°_m and ΔS°_m can be shown using:

$$\Delta H^\circ_m = H^\circ_m(D) - H^\circ_m(N) \quad (4)$$

$$\Delta S^\circ_m = S^\circ_m(D) - S^\circ_m(N) \quad (5)$$

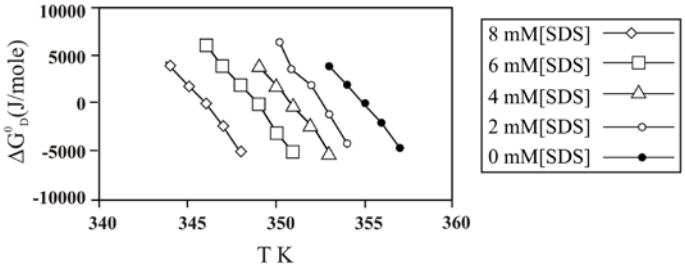

Fig. 2. ΔG°_D versus temperature at various SDS concentration in 20 mM sodium phosphate buffer, pH 6.9

Table 1. Thermodynamic data of Tm, $\Delta S^{\circ}_{m}, \Delta H^{\circ}_{m}$ at various SDS concentrations

[SDS] mM	T_m		ΔS°_{m}	ΔH°_{m}
	K	°C	$Jmol^{-1}K^{-1}$	$kJmol^{-1}$
0	355	82	2000	710
1	352.5	79.5	1800	634.5
3	351	78	1300	456
6	349	76	1250	436.2
8	346	73	1100	380.6

As the denatured state of the protein is random coil, a decrease of ΔS°_{m} and ΔH°_{m} in the presence of SDS implies unfolding of the native conformation of the enzyme. T_m for native α-Amylase is 355 K. This enzyme is more thermostable than other mesophilic enzymes. α-Amylase from *B. amyloliquefaciens* has 10.3 percent positively charged amino acids, 12.3 percent negatively charged amino acids and 33 percent hydrophobic amino acids [14]. All evidence indicate that the enhanced stability of enzymes cannot be attributed to a common determinant, but is the result of a variety of stabilizing effects including hydrophobic interaction, ionic and hydrogen binding [15-17]. It seems that hydrophobicity plays an important role in the α-Amylase thermostability. Data emerged from Fig. 1 can be used to plot the SDS unfolding curves which is shown in Fig. 3, where the free energy of denaturation, ΔG°_{D}, is plotted against SDS concentration. ΔG°_{D} was found to vary linearly with SDS concentration according to the equation:

$$\Delta G^{\circ}_{D} = \Delta G^{\circ}_{D}(H_2O) - m[SDS] \qquad (6)$$

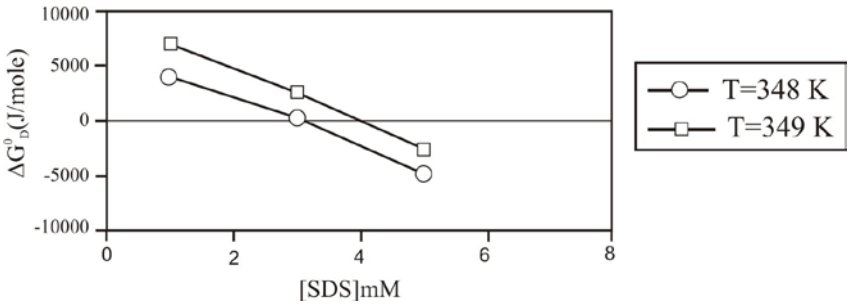

Fig. 3. ΔG°_{D} versus SDS concentration in 20mM sodium phosphate buffer, pH 6.9

where $\Delta G^{\circ}_{D}(H_2O)$ is the value of ΔG°_{D} in the absence of SDS and m is a measure of the dependence of ΔG°_{D} on SDS concentration. The value of $\Delta G^{\circ}_{D}(H_2O)$ at temperatures of 348 K and 349 K are 10 $kJmol^{-1}$ and 6.4 $kJmol^{-1}$ respectively, corresponding to the decreasing stability of α-Amylase. Plotting α-Amylase activity (%SPA) over the 300-370K temperature range at pH 6.9 shows an activity maximum at 335 K (Fig. 4). Under the conditions employed the activity optimum is in a rather broad range of 7-8 (Fig. 5). The activity of α-Amylase decreased dramatically below pH 5. At pH 3, the enzyme lost 100 percent of its initial activity. Formation of positive charges on the surface of α-Amylase and titration of α-Amylase active site (porotonation of Glu and Asp) are two important factors for deactivation of the enzyme [18]. α-Amylase activity also decreased over pH 10. This is due to the deporotonation of basic amino acids such as Lys and Arg in α-Amylase active site [18]. In the presence of SDS the enzyme retained 80 percent of its initial activity (Fig. 6). Thus at the activity optimum temperature (335 K) SDS has no dramatic effect on enzyme stability. This is in conformity with the results of thermal studies.

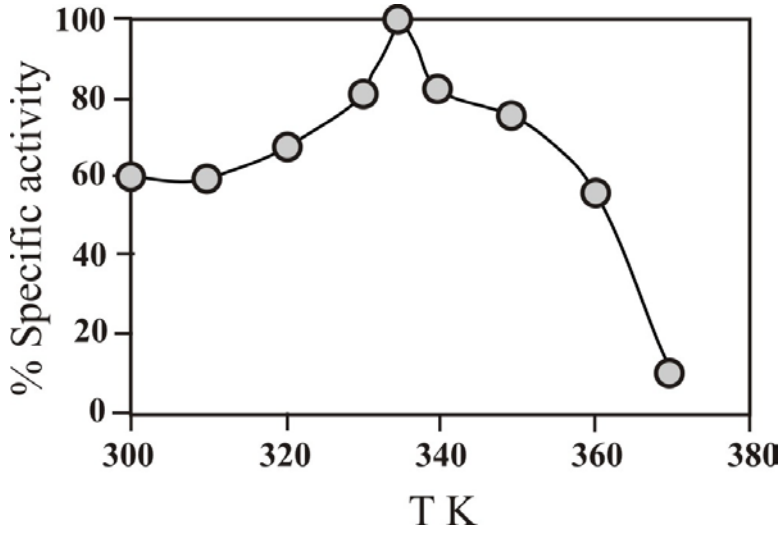

Fig. 4. Specific activity of a-Amylase versus temperature, in 20 mM Sodium phosphate buffer, pH 6.9

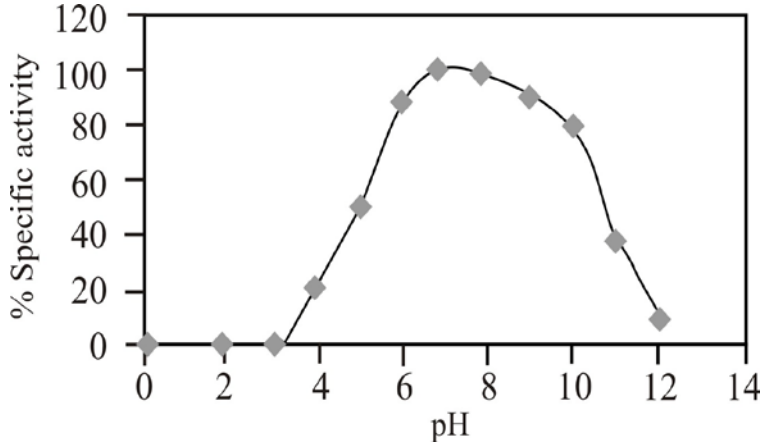

Fig. 5. Specific activity of a-Amylase versus pH in 20mM TMCA buffer

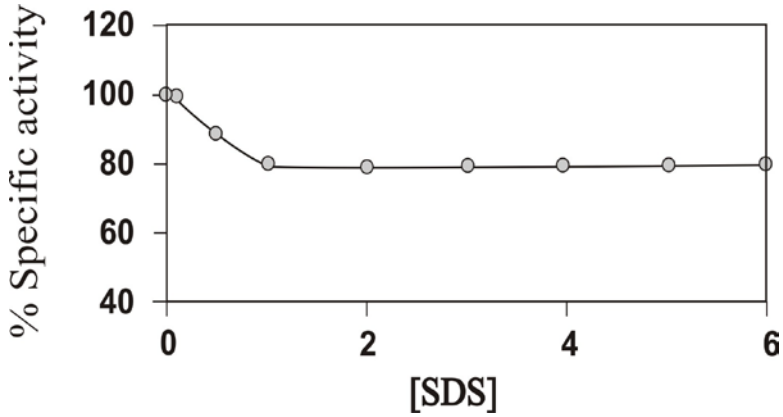

Fig. 6. Specific activity of a-Amylase versus SDS concentrations, in 20 mM sodum phosphate buffer, pH 6.9

3. CONCLUSIONS

T_m for native α-Amylase is 355 K. This enzyme is more stable than other mesophilic types.The interaction of anionic surfactant SDS with α-Amylase at higher temperatures than the optimum temperature of maximum activity was indicated by the unfolding of α-Amylase, which is equivalent to decreasing the stability (diminishing Tm).

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