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Bovine Serum Albumin a Potential Thermostabilizer: a Study on *α*-Amylase

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Abstract Bovine serum albumin (BSA) as a modifier was used with glutaraldehyde as a binder to study the activity and thermal stability of *α*-amylase. The optimum temperature of the enzyme was found to be 50°C ± 2°C. Further increase in temperature resulted in irreversible thermal inactivation of the enzyme. On modification of the enzyme with BSA, the rate of thermal inactivation was found to be significantly reduced. BSA modified *α*-amylase was found to retain its activity at 80°C even after 3 h of incubation. The apparent thermal inactivation energy (E_d) of *α*-amylase was found to be significantly increased on modification with BSA. The half-life of BSA modified *α*-amylase at both 70°C and 80°C was found to be 2.5 times higher than the native *α*-amylase. Thermodynamic parameters, ΔH°, ΔS° and ΔG°, were determined as a function of temperature. The kinetic constants K_m and V_{max}, using starch as substrate, were determined to study the effect of BSA conjugation on *α*-amylase.

Keywords: *bovine serum albumin, α-amylase, half-life, modification, thermal inactivation*

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1. Introduction

Amylase being the first partially isolated enzyme is one of the most significant products obtained for human needs through plants, animals and microbial source. *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 which has wide range of applications in food, beverages, textile, leather and paper industries. Irrespective of the wide range of applications there are some limitations of the amylases, one major being relatively low thermal stability in the operating temperatures of various processes in industries [1,2,3,4]. As a function of stabilizing force, thermostability includes hydrogen bonding, hydrophobic bonding, metal binding, ionic interactions and/or disulfide bridges contributing to the long-term stability of an enzyme [5]. With respect to this most studies are focused on the effect of various chemical modifiers, aqueous co-solvent systems, surfactants and proteins whereas on thermal stability of enzymes only few reports are available for the use of BSA as a thermostabilizer [6-14]. So in this investigation, *α*-amylase, a well-studied molecule, was used as a model enzyme to study the effects of thermal stabilization with BSA as an enzyme modifier.

Bovine serum albumin is a large globular protein made up of single polypeptide chain consisting of about 583 amino acid residues and no carbohydrate residues having molecular weight of 66 kDa. It is reported that BSA can alter the heat denaturation of the protein by partial unfolding between 40°C and 50°C, exposing the non-polar residues on the surface and facilitating reversible protein-protein interactions [15]. Thus the unknown nature of interaction and the extent of effects of BSA are of considerable interest, since it could reveal a specific mechanism by which proteins can stabilize enzymes and would predict whether BSA/protein could be good enzyme modifier. We have therefore examined the effects of BSA on amylase with a view of finding features or properties which would indicate how stabilization has occurred and the extent of stabilization by studying the thermal stability of the native and modified *α*-amylase. The mechanism and the effect of BSA as a modifier on the thermal stability of the enzyme were determined by spectroscopic measurements, Kinetics of thermal inactivation, thermodynamic parameters and kinetic constants.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of analytical grade purchased from standard chemical companies. α-Amylase, starch, maltose, NaCl and Dinitrosalisylic acid were obtained from Hi-Media laboratories, Mumbai. Bovine Serum

Albumin, Glutaraldehyde and Glycine were purchased from Sigma-Aldrich chemical company.

2.2. Estimation of α-Amylase Activity

α-Amylase activity was measured by estimation of reducing sugar [16]. The enzyme solution was prepared in 20 mM Phosphate buffer saline, pH 6.9. The reaction mixture (2 mL), containing 1 mL of 0.5 % starch solution and 1 mL of enzyme solution, were incubated for 10 min at 28°C. The enzymatic reaction was stopped by the addition of 3 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. Then reaction mixture was diluted three times using distilled water, mixed thoroughly, and the absorbance recorded at 540 nm in Elico BL-198 UV-Visible spectrophotometer. 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 µmol, equivalence of maltose at assay conditions.

2.3. Preparation of Bovine Serum Albumin (BSA)-α-Amylase Bio-Conjugates

α-Amylase BSA polymer was produced by incubating 3 mg of α-Amylase and 30 mg of BSA with 3 mg of Starch and volume was made up to 120 mL by Phosphate Buffer Saline (20 mM, pH 6.9). The mixture was mixed properly and chilled to 4°C for 20 min. 50 µL of 25 % glutaraldehyde was added followed by gentle mixing for 3 to 4 h [17]. Cross linking reaction was stopped by the addition of 30 mg glycine followed by a 24 h dialysis against mixture of glycine (1 %) and NaCl (1 %) solution at 4°C. The modified enzyme thus obtained was stored at 4°C.

2.4. Determination of Kinetic Constants

Steady-state kinetic measurements were performed to obtain kinetic constants K_m and V_{max}, of the native and modified Amylase were determined [18] at different concentrations (0.01-1 %) of L-asparagine as a substrate at room temperature using sodium phosphate buffer saline (20 mM, pH 6.9).

2.5. Thermal Stability of α-Amylase

Enzyme solutions (1 mL) were added to 1 mL phosphate buffer saline (20 mM, pH 6.9). The mixtures were incubated at 50°C, 60°C, 70°C and 80°C for 0 to 180 min [19]. After incubation for different time intervals, Starch substrate (1 mL, 0.5 %) was added to each reaction tube. The assay was done as given under "Materials and Methods 2.2." section. The product formed Maltose was estimated at 540 nm. Relative activities were calculated by taking the highest enzyme absorbance value as 100 %.

2.6. Kinetics of Thermal Inactivation and Estimation of Inactivation Energy

In order to study the thermal inactivation kinetics of α-Amylase and BSA-α-Amylase, both were incubated at different temperatures in the absence of the substrate. At periodic intervals, aliquots were withdrawn and assay was performed as described under "Materials and Methods 2.2." section. The residual activity was expressed as percent of the initial activity. The inactivation rate constants (k_d) were calculated from slopes of a semi-logarithmic plot of residual activity versus time and apparent half-lives were estimated using Eq. (1). The time where the residual activity reaches 50 % is known as the half-life.

$$t_{1/2} = \ln(2)/k_d \quad (1)$$

The temperature dependence of k_d was analyzed using the Arrhenius plot [20]. The inactivation energy was calculated from the Arrhenius equation as

$$\ln(k_d) = \ln(k_0) - (E_d/R)1/T \quad (2)$$

The values of E_d and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus 1/T, respectively.

2.7. Estimation of Thermodynamic Parameters

The thermal stability of α-Amylase in the presence and absence of selected additives was determined by the inactivation rate constant (k_d) as a function of temperature at 50°C, 60°C, 70°C & 80°C [21]. The temperature dependence of k_d was analyzed from Arrhenius plot (natural logarithm of k_d *versus* reciprocal of the absolute temperature); the activation energy (E_d) was obtained from the slope of the plot. Activation enthalpy ($\Delta H°$) was calculated according to the equation

$$\Delta H° = E_a - RT \quad (3)$$

where, R = universal gas constant, and T is the absolute temperature. The values for free energy of inactivation ($\Delta G°$) at different temperatures were obtained from the equation

$$\Delta G° = -RT\ln(k_d h/kT) \quad (4)$$

where, h is the Planck constant and k is the Boltzmann constant. Activation entropy ($\Delta S°$) was calculated from equation

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (5)$$

3. Results and Discussion

3.1. Effect of Temperature on Activity and Stability of BSA Modified α-Amylase

The ability of an enzyme to resist thermal unfolding in the absence of substrates is termed as thermostability [22]. The thermostability of the native *α*-Amylase and BSA modified *α*-Amylase was measured using the residual activity of the enzyme after incubation at various temperatures ranging from 50°C – 80°C for 30 to 180 min (Figure 1). The native and BSA modified enzyme was stable at 50°C ± 2°C but significant changes were observed at higher temperature of incubation when kept for 1 h of incubation. After 2 h of incubation at 60°C, 70°C and 80°C, BSA modified enzyme showed 2.4, 3.4 and 4.8 folds more activity respectively to that of native enzyme. The residual activity of the native enzyme was completely lost at 70°C and 80°C whereas activity was

retained in BSA modified amylase even after 3 h of incubation. After 2 h of incubation, it was observed that residual activity of α-Amylase was decreasing rapidly with respect to increase in temperature whereas in BSA modified α-Amylase there was a gradual decrease in the residual activity and it had retained 1.6 folds more residual activity even at 80°C compared to that of native enzyme (Figure 2). This confirms that BSA modified enzyme favors higher temperatures as that can be observed from the stability data. The thermostability of α-amylase is an important parameter, especially in starch based industries. It finds wide range of applications for liquidizing in the industry of alcohol, beverages, lactic acid, detergent, etc. and for desizing in textiles industry.

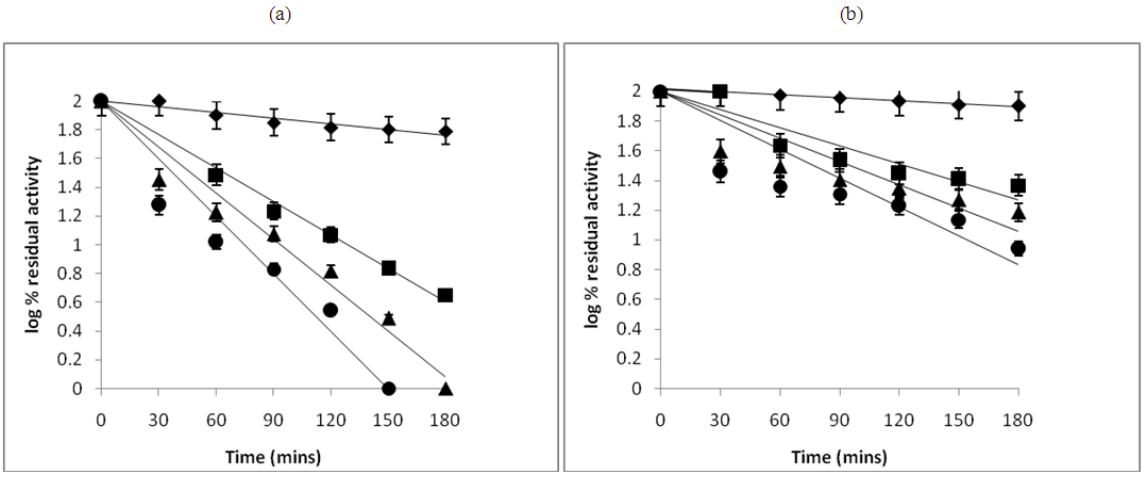

Figure 1. Effect of Thermal Inactivation of (a) α-Amylase and (b) BSA modified α-Amylase assayed at various temperatures (◆) 50°C, (■) 60°C, (▲) 70°C and (●) 80°C with time of incubation range from 30 to 180 min

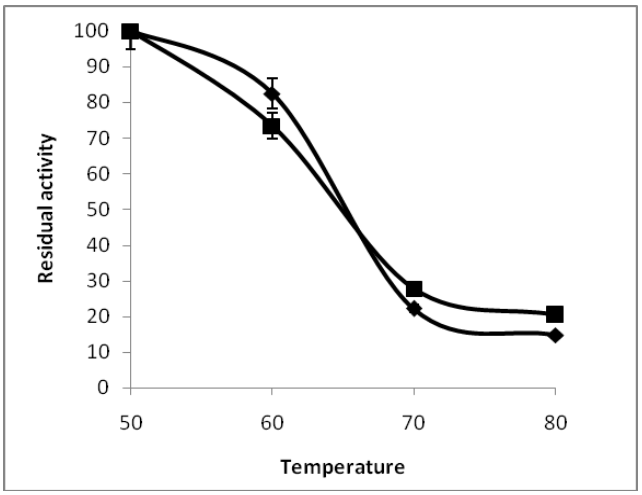

Figure 2. Residual activity vs Temperature for α-Amylase (◆) and BSA modified α-Amylase (■) was studied after 2 h of incubation and plotted for temperature range from 50°C to 80°C

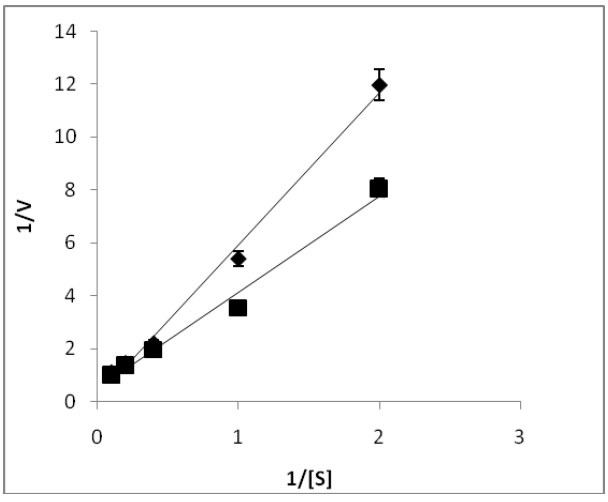

Figure 3. Lineweaver-Burk plot for α-Amylase (◆) &BSA modified α-Amylase (■) where V is Rate of the reaction and [S] is Substrate Concentration

3.2. Kinetic Parameters Estimation of Enzyme

K_m value of the BSA modified amylase was found to be 7.62 mg/mL with a V_{max} value of 22.088 U/mL. Likewise for the native amylase, the K_m and the V_{max} values were 35.43 mg/mL and 6.15 U/mL respectively (Figure 3). On comparing these systems, we observed that there was a 3 fold decrease in V_{max} with 4.65 fold decrease in K_m for the BSA modified amylase than the native amylase. The decrease in the K_m value of the modified enzyme with respect to the native enzyme suggests that BSA could activate the enzymes at the higher temperatures by increasing the affinity towards the substrate while simultaneously increasing its conversion rate whereas decrease in V_{max} is dependent on the concentration of the substrate not on the affinity. Thus at temperatures which normally favours denaturation of the enzyme system with loss of activity, whereas modification with BSA stabilizes the enzyme. The functional impairment of the native enzyme was thus an outcome of a combination of diminished substrate affinity and conversion kinetics.

3.3. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

Inactivation is defined as process in which secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds [21]. Inactivation rate constants (k_d) of native and BSA modified α-amylase at 50°C, 60°C, 70°C and 80°C (Table 1), were calculated from as per "Materials and Methods 2.6." section. Likewise, the half-life values estimated using these constants and Eq. (1), are presented in the same (Table 1).

Table 1. Half - life and Inactivation rate constant of α-Amylase and BSA modified α-Amylase

Type of Enzyme	Temp. (°C)	Inactivation rate const. kd	Half life
Native Amylase	50	0.0013	533.1
	60	0.0084	82.5
	70	0.0099	70
	80	0.0118	58.7
BSA modified Amylase	50	0.0007	990
	60	0.0037	187.3
	70	0.0039	177.7
	80	0.0047	147.4

*Units of half-life were expressed in min and Inactivation rate constant k_d were expressed in min^{-1}.

The half-life of BSA modified α-amylase at 50°C, 60°C, 70°C and 80°C was 1.8, 2.3, 2.5 and 2.5 times higher than the half-life values of native amylase. Inactivation energy (E_d) of the BSA modified α-amylase and native amylase was determined from the slopes of the linear curve plotted by 1/T versus ln(k_d) using Eq. (2) and was found to be as 284.81 kJ/mol and 235.82 kJ/mol. The higher E_d value of thermal denaturation of BSA modified α-amylase show that the enzyme is more stable in the presence of the modifier. The increasing slope of the Arrhenius plot in the BSA modified α-amylase indicates enhancement in the thermal stability of the enzyme. It is clear that the E_d (Table 2) of 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. These results shows that BSA modified α-amylase may be considered as a potential candidate for various industrial applications.

Table 2. Thermal Inactivation kinetics of α-Amylase and BSA modified α-Amylase

Enzyme	Inactivation energy Ed (kJ/mol)	Temp. (°C)	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol)
α-Amylase	235.82	50	235.72	3.99	4634.47
		60	235.70	4.58	3852.04
		70	235.68	5.32	3290.83
		80	235.66	6.06	2870.02
BSA modified α-Amylase	284.81	50	284.71	4.05	5613.04
		60	284.69	4.67	4666.92
		70	284.67	5.45	3988.84
		80	284.65	6.20	3480.57

3.4. Estimation of Thermodynamic Parameters

The changes in enthalpy (ΔH°) and entropy (ΔS°) are calculated using transition state theory [22] according to Eqs. (3) and (5) for the thermal inactivation of BSA modified α-amylase (Table 2). A positive ΔH° and ΔS° were determined in the temperature ranges studied. With increase of temperature, a slight decrease in ΔH° and a marked decrease in ΔS° were observed in native enzyme and as well as BSA modified α-amylase, whereas values of ΔH° and ΔS° of BSA modified α-amylase were higher than that of native enzyme. This suggested decrease in the surface hydrophobicity of BSA modified enzyme stabilizes the reaction and is a major determinant of the extent of stabilization by a protein [23,24]. Therefore this indicates that the hydrophobic interactions of BSA modified enzyme when heated at elevated temperatures does not favor the denatured state and hence stabilizes the enzyme. With decrease in entropy decrease in unfolding of the enzyme structure and disorder of inactivation was confirmed [23]. The decrease in ΔS° also indicated decrease in number of protein molecules in transition activated stage, resulting in higher values of ΔG°. The numerical values of ΔH° and ΔS° are reported to be influenced by Structural changes. Predominantly, ΔS° values are known to provide information regarding the

degree of solvation and the degree of compactness of protein molecule [25]. Also the increase in the ΔH° with respect to temperature reveals that the conformation of the enzyme was altered.

In enzymatic reaction first step would be enzyme substrate binding, it is known to be affected by temperature which has an effect on the higher order of protein structure and their interaction with substrate. The second step of the catalytic process is the conversion of the enzyme-substrate complex into an "activated complex," a high-energy complex that can decay into product and free enzyme. The free energy of activation (ΔG°) must be added to the enzyme-substrate complex to generate the activated complex. The magnitude of ΔG° is, in effect, the "energy barrier" to the reaction, and by significantly reducing the ΔG° values of chemical reactions, enzymes enable metabolic reactions to occur at high rates at biological temperatures. Where as in our study we found that the values of ΔG° of modified enzyme was found to be higher than that of native enzyme. Significant increase in the ΔG° values implies that it requires more free energy to form activated enzyme substrate complex which is gained by increased heat content due to increase in temperature. This indicates that the BSA modified enzyme does not favor the denatured state and hence stabilizes the protein.

4. Conclusion

The activity and thermal stability of BSA modified *α*-amylase was studied. The increase in temperature resulted in irreversible thermal inactivation of the enzyme which was significantly reduced with BSA modification. The apparent thermal inactivation energy (E_d) and half-life of *α*-amylase was found to be significantly increased on modification with BSA. Based on the obtained thermodynamic parameters ΔH°, ΔS° and ΔG°, it is clear that the thermal denaturation of the BSA modified *α*-amylase was reduced and the nature of interactions between BSA and *α*-amylase is such that it favors stability of the protein.

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