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Thermal stability of α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*: A comparative study

Jay Kant Yadav[1,2]

[1]Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore - 570 020, (INDIA)

[2]Max-Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, 06120, Halle (Saale), (GERMANY)

E-mail: jky.bio@gmail.com

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ABSTRACT

Differences in thermal stabilities of proteins are very common and determination of nature of non covalent interactions that contributes such differences is of immense importance in protein science. This study is an attempt to identify the intrinsic physicochemical factors that contributes toward stabilization of protein under various denaturing conditions. α-Amylases from *Bacillus licheniformis* (BLA) and *Bacillus amyloliquefaciens* (BAA), significantly differing in their thermal stability, were use in this investigation. The thermal stabilities of both the enzymes were compared by using activity measurement, thermal unfolding and determination of transition temperature (T_m). Formation of salt bridge was found to be an important factor that confers stability to both the enzymes. The analysis of amino acid sequences of both the enzymes indicated that BLA has more hydrophobic score compared to BAA. Based on this study, it is suggested that the additional hydrophobicity of BLA intensifies hydrophobic interactions in the core region and form rigid and compact molecular structure.

KEYWORDS

Thermal stability;
α-amylase;
Thermal transition temperature;
Salt bridge;
Hydrophobic score.

INTRODUCTION

α-Amylases play an important role in starch based industries. They have been isolated from various sources. Among all the sources, the microbial α-amylases have been utilized maximally due to their suitable characteristics. Among the microbial α-amylases the bacterial α-amylases, especially from the genus *Bacillus*, are widely used in various industries. They have a wide range of temperature, pH and other physicochemical conditions in which they exhibit their optimum activity. Since majority of industrial applications require their use at higher temperatures (up to 110°C), it is imperative to have the enzymes with higher thermal stability[1]. There are a number of enzymes available which show the optimum activity at very high temperature. Such enzymes are generally isolated from thermophilic organisms growing in extreme habitat having temperature in the range of 90 - 120°C. In addition, a number of efforts have been made to modify the mesophilic enzymes to enhance their structural stability and functionality over a wide range of temperature. The rational designing of protein/enzyme with

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improved functionality would require knowledge about the nature of interactions and other factors, which confer structural stability and functionality over a wide range on temperature and pH. The identification of intrinsic and extrinsic factors that contribute to the stabilization of thermophilic proteins has provided valuable information for stabilizing proteins under different conditions, and for designing more stable mutant proteins. In order to obtain the information about structural stability, many efforts have been made to elucidate the nature of interactions that confer addition stability to a particular protein. The differences in structural stability of several proteins are generally assed by several methods including thermal inactivation, thermal or chemical denaturation and deduction of T_m to define their relative stabilities. The determination of three-dimensional structure and characterization of structure stabilizing intramolecular interactions are of great interest in enzymology. The present study is an attempt to discover the structural attributes, which are responsible the differences in the structural stability of α-amylases from *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA). Structure wise, both the enzymes are highly homologous, but they vary in their thermal stability and the catalytic efficiencies[2-8]. The amino acid sequence alignments of BLA and BAA revealed at least 80% homology and the three dimensional structure of both the enzymes are expected to be similar[9].

Several explanations are suggested for difference in the thermal stability of above mentioned α-amylases. Although, both the enzymes reveal significant level of structural homologies, it is anticipated that the numbers of intramolecular ionic interactions differ between BLA and BAA[10]. In the present study it was revealed that BLA and BAA both have structure stabilizing salt bridges that can be dissociated in the presence of salt, however, the propensity to form non covalent hydrophobic interactions were found to be more prominent in the primary structure of BLA compare to BAA.

MATERIALS AND METHODS

Chemicals and reagents

α-Amylases (BLA and BAA), starch, dinitrosalisylic acid, bovine serum albumin (BSA), $CaCl_2$, acrylamide, citric acid and disodium citrate were procured from Sigma chemicals company, St. Louis, MO, USA. All chemicals were of analytical grade. Quartz triple distilled water was used for all the experiments.

Protein estimation

Protein concentration was determined by recording absorbance of the enzyme sample at 280nm on shimadzu UV- spectrophotometer model UV-1601 and using extinction coefficient of α-amylase as 14.46[11]. Alternatively the protein concentration was determined by Lowry method using standard of BSA[12].

α-Amylase assay

α-Amylase activity was measured by using Bernfeld method[13] for estimation of reducing sugar. The enzyme solution (1 μg/ml) containing 1 mM of CaCl2 was prepared in 0.02M buffer, having different pH ranging from 1.5-10.0. Reaction mixture, containing 1 ml of 1%(w/v) starch solution and 1 ml of enzyme solution in buffer, incubated for 5 min at 37°C. The reaction was terminated by addition of 2 ml of 1% (w/v) alkaline dinitrosalicylic acid solution. The whole solution was then subjected to heat in boiling water bath for 10 min. After cooling the solution was diluted five times using triple distilled water, mixed properly and absorbance was recorded at 540 nm. The activity was calculated by using maltose standard. One unit of α-amylase was defined as amount of enzyme required to hydrolyze starch to produce 1μmol of maltose under given condition.

Thermal denaturation of α-amylases

Thermal denaturation of the enzymes was performed by recording the absorbance spectra in a Cary Varian 100-bio UV-Vis spectrophotometer (Mulgrave Victoria, Australia) at 287 nm in quartz cuvets, path length of 1 cm using scan rate of 1ºC per min in the temperature range of 40 - 90ºC. The concentration of protein solution was taken as 8.5μM, throughout the experiment. The fractions unfolded (F_u) of the enzymes at each temperature were calculated using equation[14];

$$\mathbf{Fu} = \frac{\mathbf{A_T - A_N}}{\mathbf{A_D - A_N}} \quad (1)$$

where A_N and A_D are the absorbance of native and denatured state of protein and A_T is the absorbance of

protein at a given temperature T (ºC). The T_m of the enzymes was obtained after normalizing the absorbance of native and denatured state (between 0 and 1.0) and the fraction unfolded was plotted against temperature.

Intrinsic fluorescence and acrylamide quenching

Fluorescence measurement was performed on a Shimadzu spectroflurophotometer model RF- 5000 equipped with temperature-control device. Intrinsic fluorescence spectra of the enzyme were recorded at 25°C. For intrinsic fluorescence excitation was set at 280 nm and emission was recorded in the range of 300 – 400nm using slit width of 10 and 5nm for excitation and emission respectively. Protein concentration for all fluorescence experiment was 50µg/ml. Fluorescence quenching was performed by sequential addition of 10 µl acryamide stock solution (2M) to 2 ml of α-amylase solutions and recording of emission spectra. Excitation was set at 280 nm and emission spectra were recorded in the range of 300-500 nm after each addition of acrylamide stock. The acrylamide fluorescence quenching of protein samples were analyzed by Stern-Volmer equation[15].

$$F_0/F = 1 + Ksv[Q] \quad (2)$$

where the F_0 and F are the fluorescence intensity at 340nm (for BAA) and 337nm (for BLA), in the absence and presence of quencher (acrylamide) respectively. Ksv is Stern-Volmer constant, which can be obtained from the slope of the curve of F_0/ F versus quencher concentrations.

RESULTS

In spite of enormous stability difference between BAA and BLA, they share a number of commonalities with respect to activities pH and mechanism of catalysis. The activities of both the enzymes were measured at different pH in the acidic and alkaline range. The optimum pH range of both the enzyme was found to be in the range of pH 5.0 - 8.0. As shown in Figure 1, with increasing or decreasing the pH and beyond the optimum range, results in inhibition of enzyme. In the acidic region the enzyme activity sharply drops below pH 5, where as in alkaline range the marginal loss in the activity was observed above pH 9.

Every enzyme has an optimum temperature at which

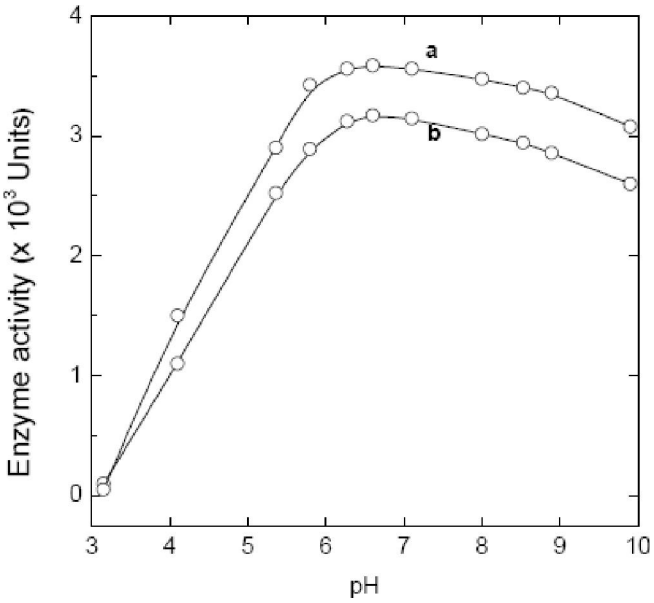

Figure 1 : Activity profile of α-amylases as a function of pH. The curves (a) and (b) represent the activity profile of BLA and BAA, respectively.

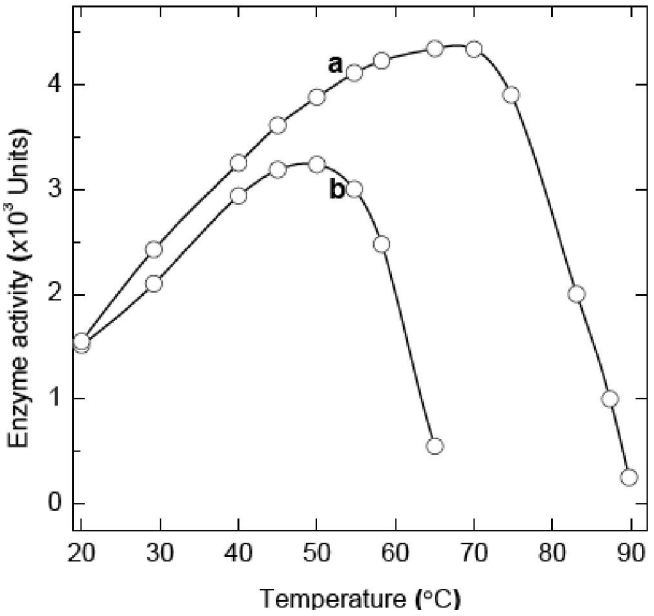

Figure 2 : The activity profile of α-amylases activity as a function of temperature at pH 5.9, in 0.02M citrate buffer. The curves (a) and (b) represent the activity of BLA and BAA, respectively.

the activity is found to be maximum. Deviation from the optimum temperature leads to loss of enzyme activity and subsequently denaturation. The activities of both the enzymes were determined at different temperature and they were found to differ significantly at each temperature. As shown in Figure 4, BLA was shown to have the optimum temperature approximately at 75±

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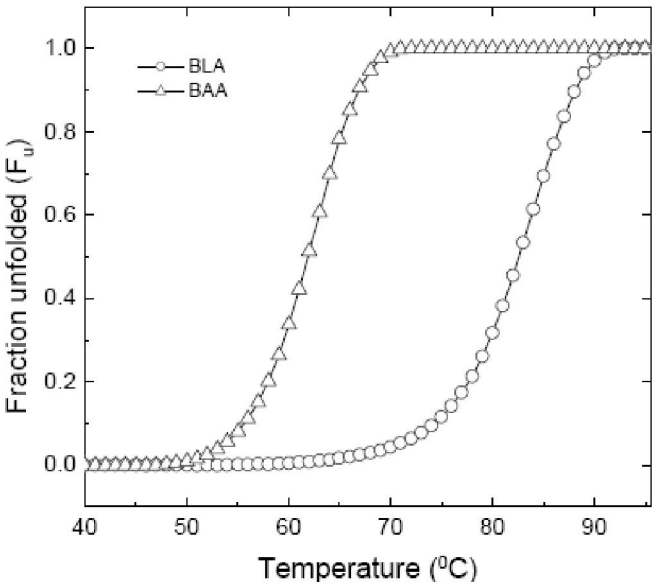

Figure 3 : Thermal denaturation profile of BLA and BAA.

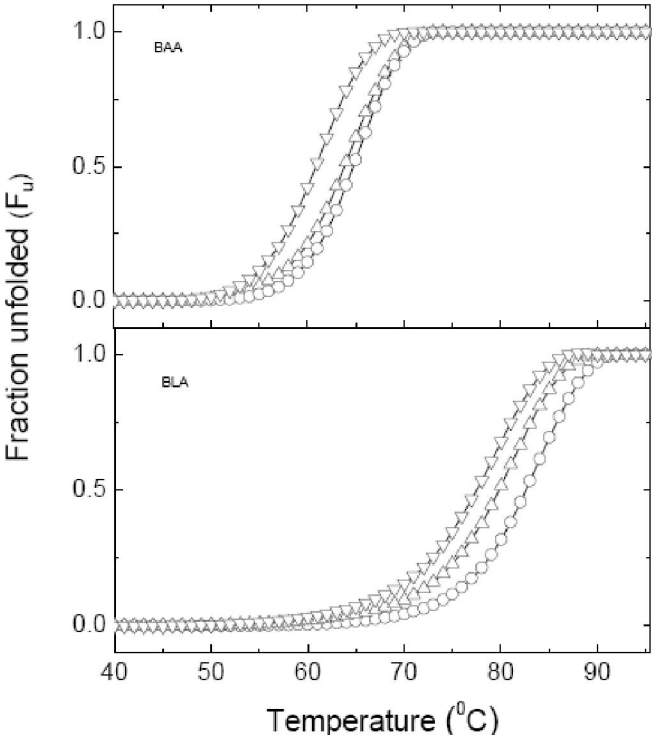

Figure 4 : Effect of KCl on the thermal denaturation profile of BLA and BAA. The protein solutions were incubated in the presence of different concentration of KCl for 4 hrs before measurements. (○) Control (the enzyme in buffer only), (Δ) in 200 mM and (▽) in 400 mM KCl.

5°C, where as BAA displayed the optimum activity in the temperature range of 45 ± 5°C. At lower temperatures, both the enzymes display similar behaviour, as their catalytic activity continued to increase with increase in temperature, at least up to 50°C. Upon further increasing the temperature, a significant difference was observed in the catalytic efficiency between two the enzymes. Quantitatively, BLA was shown to have optimum activity approximately 1.5 fold higher than the optimum activity of BAA.

The difference in the optimum temperature for maximum activity was found to be significant for both the enzymes. This indicates that these two enzymes have different structural and thermodynamic characteristics, which enable BLA to be more active at higher temperatures. The rate of thermal inactivation was found to be significantly lower for BLA compared to BAA. The study was further expanded to evaluate the differences in the thermal stability by carrying out thermal unfolding assay monitored by UV-visible spectrophotometry. This experiment facilitated the determination of T_m of both the enzymes. T_m is an important parameter which is usually defined as the temperature at which native and unfolded populations of a given protein remain in equilibrium. As depicted in Figure 3, both the enzymes have significant difference in their ability to resist heat treatment. For BLA and BAA the T_m values were determined to be 61.5 and 84ºC.

After having established the differences in temperature optima and thermal stability, investigation was further extended to elucidate the nature of intramolecular interactions that confer structural stability. It is reported that the higher thermal stability of BLA is essentially due to the presence of additional salt bridges[2, 3]. The additional salt bridges rigidify the enzyme structure and thereby shifting the thermal unfolding curve towards higher temperatures[15]. It is understandable that the presence of appropriate salts, such as NaCl or KCl may interfere with salt bridges on the protein surface[16]. In the light of this fact thermal unfolding assay was carried out in presence of different concentrations of KCl. It is expected that if the presence of additional salt bridges is the major responsible factor for higher stability of BLA, the disruption of salt bridges may reduce the stability. As evident from Figure 4, addition of KCl resulted in decrease in T_m of both the enzymes in a concentration dependent manner. In presence of 400 mM KCl the T_m of BLA and BAA was found to be reduced by 5ºC and 4ºC, respectively. However, the decrease

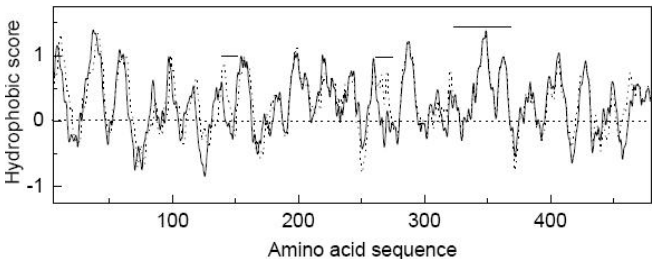

Figure 5 : Hydrophobicity distribution of BLA (—) and BAA (–) in their primary structure.

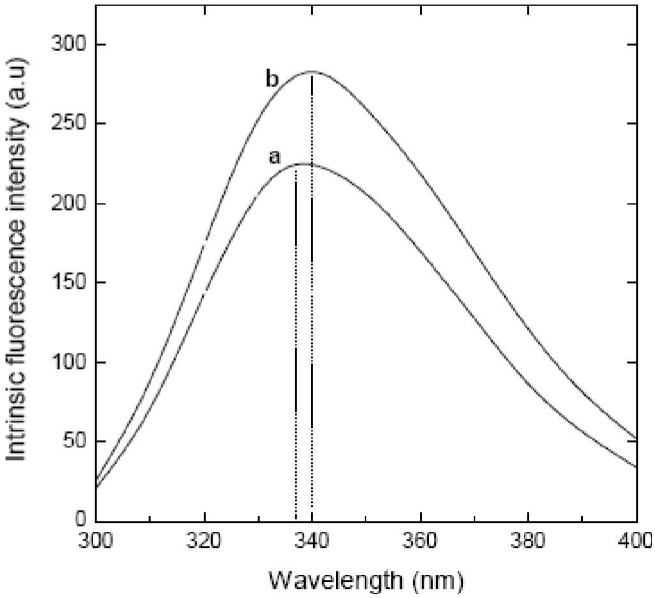

Figure 6 : Intrinsic fluorescence emission spectra of α-amylase from (a) BLA and (b) BAA in 0.02M citrate buffer at pH 5.9. The fluorescence emission maximum is found to be 337 nm for BLA and 340 nm for BAA.

in T_m values in the presence of KCl was evident for both the enzymes and not selective for BLA. This finding establishes that both the enzymes have equivalent amount of salt bridges and their disruption results reduction in stability.

Since, bacterial α-amylases are devoid of disulphide bridges, the next possible contributing factor for enhanced stability of BLA might be the additional hydrophobic interactions. It is well evident that the thermal stability for a number of proteins is contributed by hydrophobic interactions[17, 18]. Therefore, it was imperative to have a comparative look at the primary structures and average hydrophobicities of BLA and BAA. The amino acid sequences of both the enzymes were withdrawn from protein data bank and hydrophobicity score was estimated using a computational algorithm "ProtScale". This algorithm computes hydrophobic profile of an amino acid sequence of a selected protein. As shown in the Figure 5, the hydrophobic score and number of residue in the given amino acid sequences were plotted on the y and x axes, respectively. From Figure 5 it is evident that the average hydrophobicity along the amino acid sequence is variable between two enzymes, however BLA has more hydrophobic region compared to BAA. The additional hydrophobic residues might enhance the non covalent hydrophobic interactions in the core region and also the packing density of BLA. The intrinsic fluorescence and quenching studies suggested that the difference in the thermodynamic stability of both the enzymes are also reflected in their degree of molecular compactness. As shown in the Figure 6 the wavelength of maximum intrinsic fluorescence emission (λ_{max}) was found to be 337 nm and 340 nm for BLA and BAA, respectively. This indicates that the aromatic amino acid residues in BLA are located in relatively more hydrophobic environment compared to BAA. The acrylamide quenching study reproduce that BLA is more rigid and compact compared to BAA. As shown in Figure 7, the slope of Stern - Volmer plot is higher for BAA compared to BLA, probably due to the high penetration of acrylamide and quenching of intrinsic fluorescence. The findings from intrinsic fluorescence and

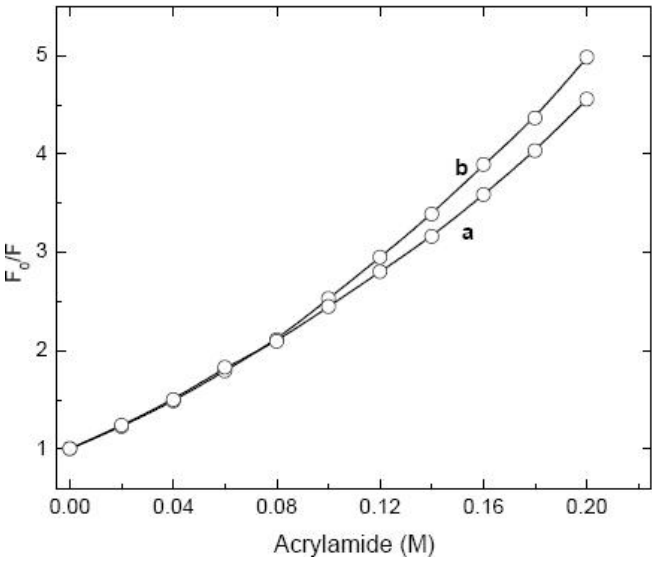

Figure 7 : Stern-Volmer plot for acrylamide quenching of α-amylases from (a) BLA and (b) BAA in 0.02M citrate buffer at pH 5.9. F_0 and F are the fluorescence intensities in the absence and presence of acrylamide, respectively.

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acrylamide quenching studies suggest that BLA has more hydrophobic and compact core structure compared to BAA.

DISCUSSION

From the above experimental data it is evident that BLA is more stable and resistant against the thermal denaturation and inactivation compared to BAA. TABLE 1 summarizes the comparative differences in the biophysical parameters of both the enzymes. The reduction in activity as a function of pH depends on the various factors such as acid induced unfolding due to alteration in the ionization of active site residues, altered stability, changes in the molecular compactness and exposure of hydrophobic clusters.

Although both the enzymes catalyze the same reaction, they differ in the rate of reaction and thermal stability. The thermal denaturation profile indicated that BLA structure is more stable. The decrease in the T_m of both the α-amylases in presence of KCl might be due to disruption of salt bridges under high ionic concentration. Although the reduction in T_m of both the enzymes in the presence of salt, it is not very significant. Therefore presence of salt bridges alone cannot explain the differences in thermal stabilities of both the enzymes. These findings suggest a possibility of involvement of additional non covalent interactions, which might be operating synergistically, with the formation of salt bridges, to enhance stability of BLA.

The hydrophobic score obtained from the amino acid sequence of both the enzymes showed that BLA has more hydrophobic localizing domain compared to BAA. Intrinsic fluorescence and acrylamide quenching studies indicated that the BLA has more nonpolar environment compared to BAA. However, a previous study has shown that the thermal stability determinants in BLA are concentrated in a particular region that depends on the pattern of protein folding and triadic metal binding site[6]. Based on the above results and previously published data, it can be concluded that additional salt bridges and hydrophobic interactions simultaneously confer higher thermal stability to BLA.

TABLE 1 : Comparison of biophysical parameters of α-amylases from BLA and BAA

Parameters	α-Amylases	
	BLA	BAA
Optimum temperature (ºC)	60 - 70ºC	45 - 55ºC
T_m	61- 62ºC	84ºC
Optimum pH	5.5 – 9.0	5.5 – 9.0
Emission maxima (λ_{max})	337 nm	340 nm
Absorption maxima	280 nm	280 nm
Optimum concentration of $CaCl_2$	1 – 2 mM	1 – 2 mM

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