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Assessment of protein stability by measuring the thermal denaturation temperature (T_m): Key considerations

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ABSTRACT

Assessment of thermodynamic stability of a protein is an essential step toward its characterization. The present study elucidates the significance of incubation conditions, such as retention time, on the accurate determination of thermal denaturation temperature (T_m) of protein. A reciprocal relationship was evident between scan rate and value of calculated T_m of two α-amylases differing in their thermal stability. Based on this observation it is suggested that the scan rate should be considered as a vital parameter while performing thermal denaturation and calculating the T_m and further analysis of data.

KEYWORDS

Thermal transition temperature;
Scan rate;
Protein unfolding;
Protein stability.

INTRODUCTION

The measurement of thermodynamic stability of proteins is an essential practice very often used to examine the affect of various mutations and a variety of extrinsic factors[1]. The simplest way to detect the stability of a protein is to detect its biological activity as a function of denaturing conditions. However, such applications are limited only with biologically active proteins and they do not provide enough information about the structural alterations in response to altered stability. Therefore, a parallel analytical approach is always necessary to assess the thermodynamic stability of proteins. To date there are several methods available. Many physical techniques can be used to monitor an unfolding event, including circular dichroism (CD), fluorescence, optical rotation, or UV-visible spectrophotometry[2-4]. The signal of hydrogen exchange (HX) in proteins, as monitored by nuclear magnetic resonance spectroscopy (NMR), can also be used in these studies[5, 6]. These techniques are based on signal differences between native and unfolded states. Traditionally protein denaturation is monitored either by involving chemical denaturants or temperature to obtain two useful parameters, such as, C_{half}, (or T_m), the concentration of a denaturant (temperature) causing unfolding of 50% protein molecules, and $\delta G_{f \rightarrow u}$, the free energy change of unfolding. These two parameters are of vital importance in defining the thermodynamic stability of protein under given conditions. Particularly, with temperature induced unfolding of an enzyme/protein it is important to consider the significance of retention time of at each temperature, and its effect on accurate estimation of T_m. Considering this point of view an investigation was conducted to evaluate the effect of scan rate on temperature induced unfolding of two microbial α-amylases and

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on accurate calculation and extraction of T_m.

MATERIALS AND METHOD

Materials

α-Amylases (BLA and BAA), starch, dinitrosalisylic acid, bovine serum albumin (BSA), CaCl2, 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[7]. Alternatively the protein concentration was determined by Lowery method using standard of BSA[8].

Thermal denaturation

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[9];

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

Where A_N and A_D are the absorbances of native and denatured states of protein and A_T is the absorbance of protein at temperature T (ºC). The Tm of the enzymes was obtained after normalizing the absorbance of native and denatured state (between 0 and 1.0) and the plotting the fraction unfolded against temperature.

RESULTS

Thermal denaturation temperature is usually defined as the temperature at which the native folded and unfolded population of a protein remain in equilibrium. When a protein subjected to higher temperature, it begins to lose its native structure due to disruption of intramolecular noncovalent interactions[8]. In the present investigation two α-amylases (from *Bacillus amylolequifaciens* (BAA) and *Bacillus licheniformis* (BLA)), differing in their thermal stability, were used to assess the effect of scan rate on the calculated value of T_m. The thermal denaturation profiles of both the enzymes were obtained in the temperature range of 40 – 90ºC. As shown in Figure 1, the calculated value of T_m of BAA and BLA were found to be 65.5 and 82.6ºC, respectively, using scan rate of 1ºC/min. Increasing or decreasing the scan rate was shown to have significant effect on the observed value of T_m. For example at scan

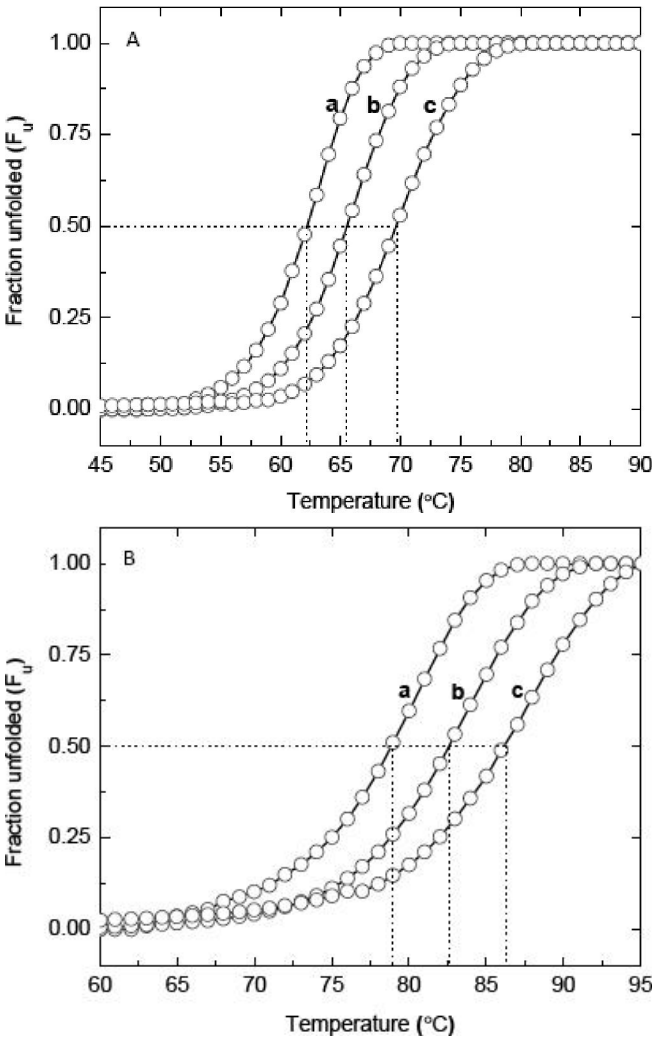

Figure 1 : Effect of scan rate on thermal denaturation profile of (A) BLA and (B) BAA. The enzyme solutions were dialyzed against 0.02M citrate buffer pH 5.9 containing 4.0 mM $CaCl_2$. The Thermal unfolding profiles both the enzymes were obtained by using three different scan rates; (a) 0.5ºC/min (b) 1.0ºC/min and (c) 2.0 ºC/min.

rate 0.5ºC /min, the T_m was found to be 62.4ºC for BAA and 79ºC for BLA. Furthermore the observed T_m at 2ºC /min scan rate was found to be 69.8ºC for BAA and 86.4ºC for BLA. From these data it is obvious that the alteration of scan rate has significant effect on estimation of T_m. In solution, the folded state of any protein is not infinitely stable[10]. It may unfold/ denature into an inactive form through a various types of conformational changes. Although, at higher temperature the rate of unfolding or inactivation of protein is use to be rapid, it also depends on the retention time of the given protein at a particular temperature. At partially denaturing temperature a protein or enzyme may undergo unfolding albeit slowly, however, quantitavely it can be enhanced by increasing the retention time (or decreasing the scan rate) at that temperature. It is worth discussing the importance of temperature range preceding the transition zone. Basically the major conformational preparedness of a protein molecule to undergo denaturation occurs in this range. Therefore the retention time at the temperature prior to the transition range truly influences the transition zone and therefore the evaluation of T_m. The analysis can be complex in case of irreversible unfolding and aggregation. Based on the above observation it would be worth saying that thermodynamic stability can be monitored by using thermal denaturation curve, however, the calculated T_m is not a concrete value and subject to change depending upon the variation in the retention time at temperature particularly in the pre-sigmoidal regions. The data would be comparable only when the retention time is identical in all thermal transitions.

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