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Biophysical insight into structure-function relation of *Allium sativum* Protease Inhibitor by thermal, chemical and pH-induced modulation using comprehensive spectroscopic analysis

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ABSTRACT

In this study, we have analyzed the structural and functional changes in the nature of *Allium sativum* Protease Inhibitor (ASPI) on undergoing various denaturation with variable range of pH, temperature and urea (at pH 8.2). ASPI being anti-tryptic in nature has native molecular mass of ~15 kDa. The conformational stability, functional parameters and their correlation were estimated under different conditions using circular dichroism, fluorescence and activity measurements. ASPI was found to fall in belongs to α+β protein. It demonstrated structural and functional stability in the pH range 5.0-12.0 and up to70 °C temperature. Further decrease in pH and increase in temperature induces unfolding followed by aggregation. Chemical induced denaturation was found to be cooperative and transitions were reversible and sigmoid. T_m (midpoint of denaturation), ΔC_p (constant pressure heat capacity change) and ΔH_m (van't Hoff enthalpy change at T_m were calculated to be 41.25 ± 0.2 °C, 1.3 ± 0.07 kcal mol^{-1}K^{-1} and 61 ± 2 kcal mol^{-1} respectively for thermally denatured ASPI earlier. The reversibility of the protein was confirmed for both thermally and chemically denatured ASPI. The results obtained from trypsin inhibitory activity assay and structural studies are found to be in a significant correlation and hence established structure-function relationship of ASPI.

1. Introduction

Protease Inhibitors (PIs) are one of the major proteins being worked upon recently as they are of great biological importance. PIs arrest, balance and counteract the protease enzymes to keep off their excess proteolytic activity so that they may not possibly harm the host organism [1]. PIs suitably control functioning of these natural proteases just by letting the enzyme idle by locking it up. PIs diminish the function of proteases exclusively while the other proteins remain unaffected, for this reason they are verified to be exceptionally explicit in their character [2]. A well certified literature on physiological significance being present on modulation of synthesis, enzymatic characteristics, functional and structural specifications of PIs [3,4],but only a slight information is present on the stability and folding system of PIs.

To fill in the gap between gene sequence and functional 3D structure of protein, a combined study of protein folding and stability is of an immense value and capacity. As a result, analyzing the protein folding mechanism is usually said to be the extension of genetics where amino acid sequences create platform for proper folding of protein and assists its natural correspondence which is key prototype for self assembly of biological macromolecules [5]. Hence, the information and understanding about this complex process offers a clear vision to the molecular systems where advancement selection has modified its properties for practical benefits [6]. The stability of any protein depends on its intramolecular bonds, charge strength and integration which rely on the adjacent environmental states such as temperature, pH and ionic strength of solvent composition [7]. The proper folding of proteins is the secret for their long survival in biologically packed environment and their interaction with specific natural ligands. Failure to desired conformational fold leads to inactivation and hence destruction of these biological macromolecules causing various neurodegenerative diseases such as Huntington Disease, Alzheimer's Disease etc [8]. Hence, an understanding of relationship between environmental factors and protein stability is crucial for production and biotechnological applications of properly folded proteins at industrial level.

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Folding is a natural phenomenon where protein releases its free energy and attains a natural stage of least free energy level. The process involves metastable folding intermediate hosting, local minima while undergoing equilibrium unfolding induced by extreme pH, temperature and chemical denaturants [7,9–11]. This vision can only be cleared by intensive studies of numerous proteins present globally.

As, it is clear till now that an understanding of structure-function relationship of proteins under various environmental factors is necessary for theoretical as well as application perspectives. Therefore, an approach towards the finding of a novel protein with crucial biotechnological applications has compelled us to study the molecular basis of protein stability and its structure-function relationship. ASPI was purified from garlic bulbs. It is a low-molecular-weight (~15 kDa) protein with high medicinal values. In this observation we have done a thorough enquiry of conformational changes in the ASPI at different pH, temperature and [urea]. Our investigation of ASPI is an initiative for studying the folding-unfolding and establishing structure-function relationship.

2. Materials and methods

2.1. Reagents

ANS (8-anilino-1-naphthalene sulfonate), Urea, SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), BAPNA (N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride) and Lowry reagents were purchased from Sigma Aldrich (St. Louis, Missouri, United States). All the other chemicals for buffer preparation were of analytical grade procured from Merck (Kenilworth, New Jersey, United States) and Thermo Fischer Scientific (Waltham, Massachusetts, United States).

2.2. ASPI purification

Allium sativum Protease Inhibitor (ASPI) was purified as per our previously published protocol [12]. Homogeneity of purified ASPI was assessed by SDS-PAGE as per the protocol of Laemmli [14] and concentration of ASPI was determined by the Lowry method [15].

2.3. Assessment of residual trypsin inhibitory activity

The residual inhibitory activity against trypsin was estimated by incubating ASPI in high temperature, extreme pH and high molar concentrations of chemical denaturants for ~12 h. BAPNA was used as substrate using the protocol of Erlanger et al. [13].

2.4. Circular dichroism measurements

Secondary structure of ASPI (in Tris buffer, pH 8.2) was determined by Heat-induced denaturation carried out in Jasco spectropolarimeter (J-1500) at a heating rate of 1 °C min^{-1}. The ASPI concentration (0.2 mg/ml) and cell path length (0.1 cm) were optimized for the measuring conditions. Each spectrum was the average of three scans. A change in CD signal at 222 nm was recorded with respect to change in temperature ranging 20–80 °C. At the end of each experiment, reversibility was confirmed by cooling the denatured protein and then matching the CD spectrum of the protein with the native spectrum taken before heating the ASPI. The raw CD data were converted into $[\theta]_{222}$ using equation [1]. In order to obtain values of *Tm* (midpoint of denaturation), ΔC_p (constant pressure heat capacity change) and ΔH_m (van't Hoff enthalpy change at *Tm*) entire transition curve of each protein was fitted according to the relation [16,19].

$$y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T)\cdot \exp\left\{\left(\left[\Delta C_p \cdot \left(\left(T_m/T - 1\right) + \ln\left(T/T_m\right)\right)\right] - \left[\Delta H_m \cdot \left(T_m/T - 1\right)\right]\right)/R\right\}}{1 + \exp\left\{\left(\left[\Delta C_p \cdot \left(\left(T_m/T - 1\right) + \ln\left(T/T_m\right)\right)\right] - \left[\Delta H_m \cdot \left(T_m/T - 1\right)\right]\right)/R\right\}} \quad (1)$$

where y_{obs} is the experimentally observed optical property of the protein at temperature T (K), y_n and y_d are the optical properties of the native (n) and denatured (d) molecules at same temperature, and *R* is universal gas constant.

2.5. Measurement of fluorescence spectra

Fluorescence spectra of ASPI in pH denaturation conditions were studied on Jasco fluorimeter (FP-6200). The path length of 3 mm,ASPI concentration of 0.2 mg/ml and incubation of 2 h were optimized for best results. Both excitation and emission slits were set at 5 nm. For fluorescence measurements, the excitation wavelength was set at 280 nm and Trp-emission spectra were taken in the range of 300–400 nm.

The fluorescence measurements were also done for urea induced denaturation studies. The stock of 10 M urea was prepared and ASPI was incubated with final concentrations of range of 0.5–8 M urea at native pH condition (8.2). To obtain protein stability parameters, the entire (y(u), [u]) data of each urea-induced transition curve, shown in Fig. 8 were analyzed using a non-linear least-squares method [33].

$$y(u) = yN(u) + yD(u) * Exp\left[-\left(\Delta GD^0 + m[u]\right)/RT\right] / \left(1 + Exp\left[-\left(\Delta GD^0 + m[u]\right)/RT\right]\right) \quad (2)$$

where y(u) is the observed optical property at [u]; y_N(u) and y_D(u) are respectively optical properties of the native and denatured protein molecules under the same experimental conditions in which y(u) was measured; $\Delta G_D{}^0$ is the value of Gibbs free energy change (ΔG_D) in the absence of the denaturant; m is the slope ($\partial \Delta G_D/\partial$[u]); *R* is the universal gas constant; and *T* temperature in Kelvin. It should be noted that Eq. (2) assumes that a plot of ΔG_D versus [urea] is linear, and the dependencies of y_N and y_D on [urea] are also linear (i.e., $y_N(u) = a_N + a_N[u]$, and $y_D(u) = a_D + b_D[u]$, where a and b are [u]-independent parameters, and subscripts N and D represent these parameters for the native and denatured protein molecules, respectively).

2.6. Determination of extrinsic fluorescence using ANS probe

A fresh stock solution of ANS was prepared in double distilled water and filtered with a 0.2 μm Millipore filter. The concentration of ANS was measured using a molar extinction coefficient εM = 5000 M^{-1} cm^{-1} at 350 nm [52]. ANS binding studies with ASPI were done by incubating 0.2 mg/ml ASPI in pH range of 0.5-12.0 for 2 h at room temperature. Post incubation, the samples were supplemented with ANS solution in a ratio of 1:20 and were incubated for a further 30 min in the dark. The ANS emission spectra were detected using an excitation wavelength of 380 nm, and the spectra were recorded using a Shimadzu (RF-5301PC) fluorescence spectrophotometer from 390 to 600 nm. The excitation and emission slit widths were both set at 5 nm.