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A differential scanning calorimetric study on the irreversible thermal unfolding of concanavalin A

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Abstract

A differential scanning calorimetry study on the thermal denaturation of concanavalin A at pH 5.2 where it exists in the dimeric form was carried out. The calorimetric transitions were observed to be irreversible and the transition temperature of the protein increased with increasing scan rate, indicating that the thermal denaturation process is under kinetic control. The thermal unfolding, and its scan rate dependence could be explained according to the kinetic scheme $N_2 \xrightarrow{k} 2U$ with k as first-order kinetic constant whose change with temperature is given by the Arrhenius equation. Using this model, rate constant as a function of temperature and activation energy of the process have been calculated. The average activation energy of the kinetic process using different approaches is $129 \pm 10\,\mathrm{kJ\,mol^{-1}}$. The differential scanning calorimetric results on transition temperatures and calorimetric enthalpies supported by intrinsic fluorescence indicate that the irreversibility in the calorimetric transitions of concanavalin A includes a combination of post-transition aggregation, chain separation and loss of cofactor.

Keywords: Differential scanning calorimetry; Concanavalin A; Irreversible thermal denaturation; Enthalpy; Activation energy

1. Introduction

Concanavalin A is a lectin isolated from jack beans [1] that exists as a tetramer at physiological pH and as a dimer at pH values less than 6 [2,3]. Each monomer ($M_r = 26500$) possesses one saccharide binding site as well as a transition metal ion site S1 that typically binds Mn^{2+} and a site S2 that binds Ca^{2+} [4]. The three dimensional structure of the lectin at 1.75 Å resolution has been determined by X-ray diffraction analysis [5] that was further refined at 1.2 Å [6]. The lectin dimer, termed 'canonical dimer', is characterized by a large 12-stranded β-sheet resulting from the anti-parallel side-by-side alignment of the two six-stranded back sheets.

Differential scanning calorimetry (DSC) is a powerful technique that allows investigators to obtain valuable information on thermodynamics and kinetic features of the thermal unfolding of proteins under investigation [7–10]. Under certain conditions, the thermal denaturation profiles of many small globular proteins [7] and some complex proteins [11] have been observed to be irreversible. This is reflected by the absence of thermal transition in the DSC thermogram corresponding to the second heating of the protein sample. Methods have been described in literature [12–14] to extract the kinetic parameters from the irreversible calorimetric transitions. Although extensive studies have been performed on lectin–carbohydrate interactions, information on the thermodynamics and kinetics of the thermal unfolding of concanavalin A using non-calorimetric methods is rarely available in literature [15,16]. The present work describes thermal unfolding of concanavalin A using micro-differential scanning calorimetry: the process is irreversible and provides fundamental information about denaturation.

2. Materials and methods

Concanavalin A was obtained from Sigma Chemical Company, USA. Sodium acetate, sodium chloride, calcium chloride and manganese chloride were extrapure analytical reagent grade, obtained from Merck Limited. The water used for preparing the solutions was double-distilled and then deionized using a Cole-Parmer research mixed-bed ion exchange column. The protein was dialyzed extensively against $20 \times 10^{-3}\,\mathrm{mol\,dm^{-3}}$ sodium acetate buffer containing

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1×10^{-3} mol dm^{-3} calcium chloride, 0.1 mol dm^{-3} sodium chloride and 0.1 mol dm^{-3} manganese chloride at pH 5.2 with at least four changes of the buffer. The reported pH is that of the dialysate measured on a Standard Control Dynamics pH Meter at room temperature. The concentration of concanavalin A was determined spectrophotometrically on a Shimadzu double beam spectrometer UV 265 at 280 nm using $A^{1\%,1cm} = 12.4$ [17–19] at pH 5.2 and expressed in terms of monomer.

2.1. Differential scanning calorimetry

The thermal denaturation experiments were performed on a SETARAM micro-differential scanning calorimeter equipped with removable Hastelloy C-276 fluid tight batch cells of 1 cm^3 capacity. Before loading into the calorimetric cells, all the solutions were degassed. Any loss in water thus evaporated, determined from the mass of the sample before and after degassing, was compensated by addition of appropriate amounts of degassed deionized water. In order to bring the transition temperature of the protein within the detection range of the instrument with appreciable post-transition baseline, all the experiments on concanavalin A were performed in the presence of 2 mol dm^{-3} urea. The volume of the sample solution in the cell was fixed at 0.85 cm^3 and the weights of the sample and reference cells containing respective solutions were always matched to within 0.1 mg. The reference solution in all the calorimetric experiments was 2 mol dm^{-3} urea. An excess power versus temperature scan for the protein transitions was obtained by subtracting the power input of thermal scan of solvent in both the cells from the power input scan of the protein solution in the sample cell and solvent in the reference cell. The excess power thermal scans were also corrected for the thermal lag of the calorimeter and then converted to excess heat capacity versus temperature scan by dividing by the scan rate. The corrected DSC data were analyzed by the EXAM program of Kirchoff [20]. The calorimetric reversibility of the thermal transitions was determined by heating the sample to a temperature that is little over the transition maximum, cooling immediately, and then reheating. All the thermal denaturation transitions were found calorimetrically irreversible as reflected by the lack of transition in the second run of all the samples. The thermal denaturation of concanavalin A was performed at scan rates of 0.1, 0.2, 0.3, 0.5, 0.6 and 0.8 K min^{-1}.

2.2. Fluorescence measurements

The fluorescence measurements were performed on an LS-55 Perkin-Elmer spectrofluorimeter at ambient temperature. For intrinsic fluorescence, the sample was excited at 295 nm, where tryptophan is selectively excited and the emission spectra recorded. Both the excitation and emission slits were set at 5 nm. The emission spectra of the samples containing the protein were always analyzed after subtracting the emission spectra of the reference buffer or buffer containing 2 mol dm^{-3} urea. The emission spectra was studied at five different concentrations of protein, namely 0.5, 1.0, 1.5, 2.0 and 2.5 $\times$ 10^{-6} mol dm^{-3}, containing 2 mol dm^{-3} urea, where specified.

3. Results and discussion

Fig. 1 shows the representative differential scanning calorimetry traces for the thermal denaturation of concanavalin A at different scan rates. The excess heat capacity versus temperature profiles are asymmetric transitions even

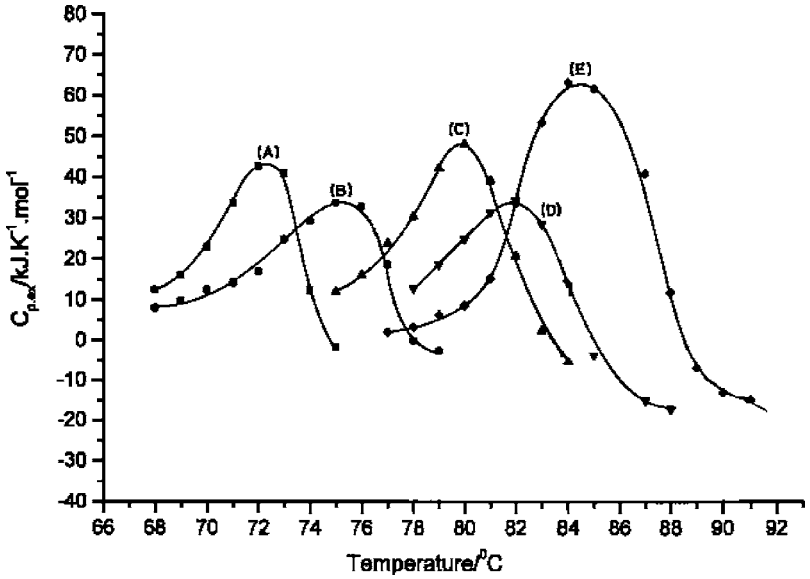

Fig. 1. DSC scans of thermal denaturation of 0.19 $\times$ 10^{-3} mol dm^{-3} concanavalin A in 20 $\times$ 10^{-3} dm^{-3} acetate buffer at pH 5.2 in the presence of 2 mol dm^{-3} urea at different scan rates: 0.1 (A), 0.2 (B), 0.3 (C), 0.5 (D) and 0.6 K min^{-1} (E).

Table 1
Thermodynamic parameters of thermal unfolding of 0.19×10^{-3} mol dm^{-3} concanavalin A at pH 5.2 (scan rate dependence)

Scan rate (K min^{-1})	Urea (mol dm^{-3})	T_m (K)	ΔH (kJ mol^{-1})
0.1	2	344.7 ± 0.2	200 ± 6
0.2	2	348.3 ± 0.1	210 ± 3
0.3	2	353.3 ± 0.2	241 ± 4
0.5	2	355.5 ± 0.1	252 ± 6
0.6	2	357.4 ± 0.2	321 ± 3
0.8	2	359.2 ± 0.3	355 ± 3

Each value in this table is an average of three to four measurements.

after correcting for the time constant of the calorimeter. Concanavalin A at pH 5.2 in the presence of 2 mol dm^{-3} urea unfolds at 82.3 °C with a calorimetric enthalpy (ΔH) of 252 kJ mol^{-1}at a scan rate of 0.5 K min^{-1}. Since the thermal transitions are observed to be irreversible, the scan rate dependence was studied. The time constant of the differential scanning calorimeter may also produce a scan rate dependence of the heat capacity versus temperature traces. It is seen in Fig. 1 that the instrumental time constant corrected thermal transitions of concanavalin A are highly scan rate dependent. It is therefore established that the observed scan rate dependence is due to chemical kinetic factors. All the thermal denaturation profiles fitted well to the model $N_2 \xrightarrow{k} 2U$ with rate constant k, where N_2 represents dimeric concanavalin A in the native form and U represents the thermally denatured form. Irreversibility in the calorimetric transitions restricts the application of equilibrium thermodynamics; therefore we are reporting only transition temperature T_m (temperature corresponding to maximum excess heat capacity) and calorimetric enthalpy (ΔH) summarized in Table 1. The T_m values have an experimental error of ±0.1 °C and ΔH values have a maximum error of 2% including error in sample preparation, calibration constant and reproducibility.

Lumry and Eyring [21] have proposed that in general the irreversible thermal denaturation of a protein may involve two steps: (i) reversible unfolding of the native protein to the unfolded state D, and (ii) irreversible alteration of the unfolded protein D to yield final step U that is unable to fold back to the native structure: $N \rightleftharpoons D \rightarrow U$. Concanavalin A is a dimeric protein at pH 5.2; therefore we can expect that its denaturation may follow the dissociative mechanism of denaturation as $N_2 \rightleftharpoons 2D \rightarrow 2U$ with k_1, k_2 as rate constants of the forward and reverse $N_2 \rightleftharpoons 2D$ reaction and k_3 as the rate constant of $2D \rightarrow 2U$ reaction. Here N_2 is the native dimer, D and U have the same meaning as above. Since the calorimetric transitions were irreversible even up to and after heating the sample to T_m, the data does not support the pre-equilibrium step ($k_3 \gg k_2$) thus justifying the adopted $N_2 \rightarrow 2U$ model, with k as the effective rate constant of the denaturation process in which the thermally induced disruption of the quaternary structure of the protein kinetically follows all or none of the process. Such irreversible scan rate dependent calorimetric transitions have been in many cases successfully analyzed in terms of a two-state kinetic model originally developed by Sanchez Ruiz et al. [14]. The mathematical elaboration of this model leads to several methods of calculating the activation energy of this kinetic process [14]. To apply the laws of equilibrium thermodynamics, it is necessary that the equilibrium exists throughout the temperature range of the calorimetric transitions. On the other hand, it has been reported that some irreversible calorimetric transitions of thermal denaturation can be analyzed in terms of equilibrium thermodynamics [22–24]. This conclusion was based upon agreement between van 't Hoff enthalpy for the shape of the calorimetric traces and those calculated from the effect of the ligands (nucleosides, nucleotides or glycosides) and protein concentration on the transition temperature. Thus in cases of reversible denaturation, the equilibrium thermodynamics analysis of the DSC thermograms allows us to check the two-state character of the process, and in case of non-two-state denaturation, the number and characterization of the significantly populated intermediate states can be determined. However, our data fits only to a two-state irreversible model.

The rate constant of the denaturation reaction at a given temperature T can be obtained [14] by using

$$k = \frac{vC_{p,ex}}{(Q_t - Q)} \tag{1}$$

where v (K min^{-1}), $C_{p,ex}$ (kJ K^{-1} mol^{-1}), Q_t (kJ), and Q (kJ) represent the scan rate, excess heat capacity, total heat of the process and heat evolved at a given temperature T, respectively. From the values of the rate constant k at several temperatures, the activation energy can be obtained by using the Arrhenius plot, of $\ln k$ versus $1/T$. This plot at all the scan rates employed is given in Fig. 2 and the calculated average activation energy is 126 ± 9 kJ mol^{-1}. It is observed that the values obtained for the activation energy at different scan rates are in excellent agreement.

The two-state kinetic model predicts [14] that the temperature corresponding to the maximum of heat capacity, T_m, changes with the scan rate according to

$$\ln\left(\frac{v}{T_m^2}\right) = \text{constant} - \frac{E}{RT_m} \tag{2}$$

Using the data at different scan rates, the plot of $\ln(v/T_m^2)$ versus $1/T_m$ for the DSC data is linear as shown in Fig. 3 in accordance with Eq. (2). The activation energy calculated from the T_m values obtained at six scan rates is 138 ± 0.2 kJ mol^{-1}.

According to the two-state kinetic model [14], the dependence of the heat evolved on temperature is given by

$$\ln\left[\ln\left\{\frac{Q_t}{Q_t - Q}\right\}\right] = \frac{E}{R}\left[\frac{1}{T_m} - \frac{1}{T}\right] \tag{3}$$

The plot of $\ln[\ln\{Q_t/(Q_t - Q)\}]$ against $1/T$ gives rise to straight lines (Fig. 4) with slope as $-E/R$. The data fit

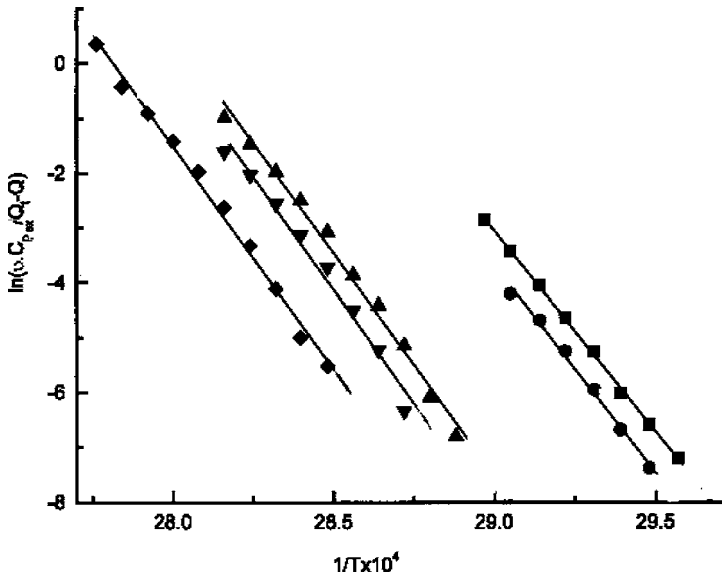

Fig. 2. Plot of $\ln\{vC_{p,ex}/(Q_t - Q)\}$ versus $1/T$ for thermal denaturation of 0.19×10^{-3} mol dm^{-3} concanavalin A in the presence of 2 mol dm^{-3} urea at scan rates 0.1 (■), 0.2 (●), 0.3 (▲), 0.5 (▼) and 0.6 K min^{-1}(◆).

to a straight line and the calculated average activation energy is 124±7 kJ mol^{-1}. It is seen from Eq. (3) that x-axis intercepts in these plots give the values of T_m. The T_m values obtained at the scan rates 0.1, 0.2, 0.3, 0.5, and 0.6 are 344.3, 347.6, 353.4, 354.9, and 358.2 K, respectively. The corresponding T_m values obtained directly from the calorimetric traces are, respectively, 344.7, 348.3, 353.3, 355.5, and 357.5 K. It is seen that both the calculated and experimental values compare well. The validity of the model is proved beyond doubt as all the three plots, corresponding to the governing equations of the model, are linear. It is also seen that different methods involving different approximations and experimental information provide good agreement between the results obtained for the energy of activation, which on average is 129 ± 10 kJ mol^{-1}, thus justifying the validity of the assumed two-state kinetic model $N_2 \rightarrow 2U$.

The unfolding of oligomeric proteins requires disruption of additional molecular interactions over those of monomeric proteins since both inter- as well as intrasubunit interactions make distinct and differential contributions to

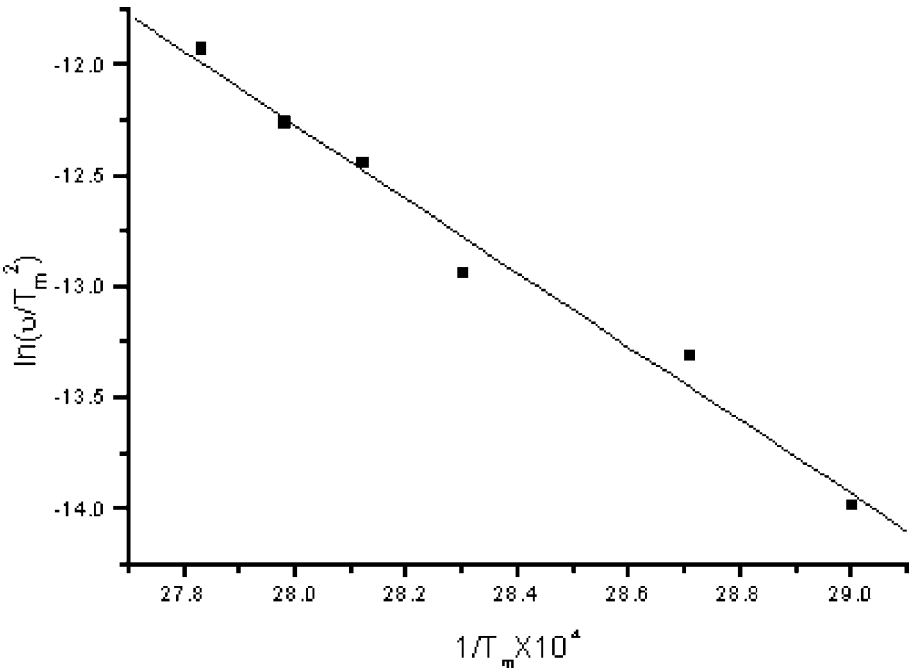

Fig. 3. Plot of $\ln(v/T_m^2)$ versus $1/T_m$ for thermal denaturation of 0.19×10^{-3} mol dm^{-3} concanavalin A in the presence of 2 mol dm^{-3} urea at pH 5.2.

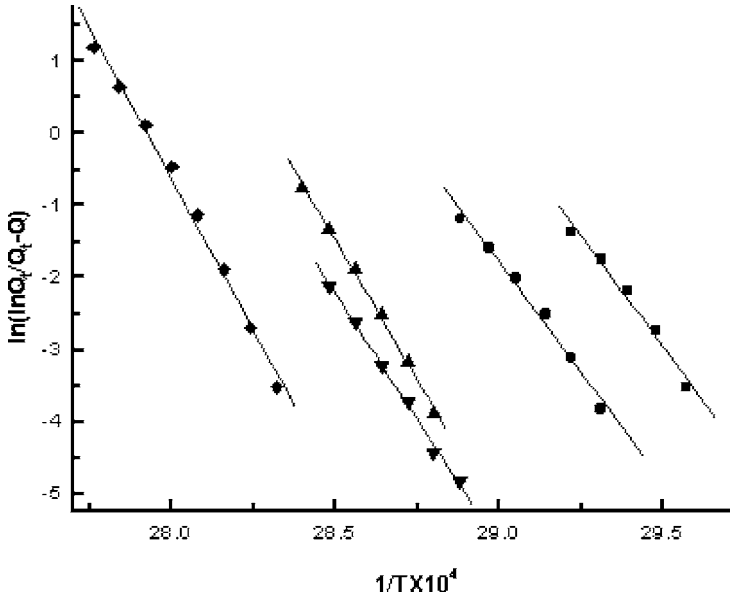

Fig. 4. Plot of $\ln[\ln\{Q_t/(Q_t - Q)\}]$ versus $1/T$ for the thermal denaturation of 0.19×10^{-3} mol dm^{-3} concanavalin A at pH 5.2 in the presence of 2 mol dm^{-3} urea at scan rates: 0.1 (■), 0.2 (●), 0.3 (▲), 0.5 (▼) and 0.6 K min^{-1}(◆).

their overall structure and stability. Whereas cooperative unfolding may be expected of a single-domain monomeric protein, the relative contributions of the inter- and intramolecular forces would govern the degree of cooperativity, the mechanism of unfolding and the overall stability of their oligomeric counterparts [25,26]. Thus the denaturation of oligomeric proteins can include a step of reversible dissociation to monomer [27]. In such cases, the excess heat capacity versus temperature profile can become dependent on protein concentration due to the bimolecular character of the association [27]. It is also possible that aggregation status of a protein may change above the denaturation temperature. The role of dissociation and association reactions in the mechanism of thermal denaturation of dimeric concanavalin A were analyzed by carrying out the DSC experiments at varying protein concentrations.

As seen from Table 2, upon varying the protein concentration from 0.075×10^{-3} to 0.23×10^{-3} mol dm^{-3}, the transition temperature changes only from 356.5 to 357.3 K, a very small range. However, the calorimetric enthalpy increases progressively from 106 to 305 kJ mol^{-1} with increase in protein concentration. Since the data fit to $N_2 \xrightarrow{k} 2U$ model, the increase in the ΔH may indicate a contribution from the onset of the post-transitional aggregation that does not affect the kinetic parameter extracted from the calorimetric data reported here. However, it is possible that the aggregation may start at a temperature which is near the end of the transition denaturation profile. Denaturation of oligomeric proteins can include a step of reversible dissociation to monomers. In such a case, the $C_{p,ex}$ versus T profile will show a protein concentration dependence [27]. Since T_m values do not change with protein concentration in our studies, it indicates that association of monomers does not contribute significantly to the kinetics of the thermal denaturation of dimeric concanavalin A, thus supporting $N_2 \rightarrow 2D$ scheme.

Table 2
Thermodynamic parameters accompanying the thermal unfolding of concanavalin A at pH 5.2 at varying concentrations of protein at a scan rate of 0.5 K min^{-1} in the presence of 2 M urea

Protein (10^{-3} mol dm^{-3})	Urea (mol dm^{-3})	$T_{1/2}$ (K)	ΔH (kJ mol^{-1})
0.075	2	356.5 ± 0.1	106 ± 5
0.113	2	356.8 ± 0.2	219 ± 4
0.150	2	356.6 ± 0.1	245 ± 3
0.188	2	355.8 ± 0.1	252 ± 4
0.226	2	357.3 ± 0.2	305 ± 6

Each value in this table is an average of three to four measurements.

Fig. 5 gives the fluorescence emission spectrum of concanavalin A at varying protein concentrations, and the inset gives the emission spectrum of concanavalin A at varying protein concentrations in the presence of 2 mol dm^{-3} urea. No shift in λ_{max} or deviation of fluorescence intensity at λ_{max} from linearity (Fig. 6) is observed in the intrinsic fluorescence of concanavalin A (both in the absence and presence of 2 mol dm^{-3} urea) with increase in the concentration of the protein. It supports that there is no change in the extent of aggregation with increase in its concentration at ambient temperature. This further confirms that the increase in the calorimetric enthalpy with increase in the concentration of the protein is caused by a post-transitional effect due to the change in the aggregation status of the protein after the denaturation. This effect may cause overlap with the main calorimetric profile towards the end. Protein aggregation, deamination of Asn/Gln residues, isomerization of proline residues, chain separation in oligomeric

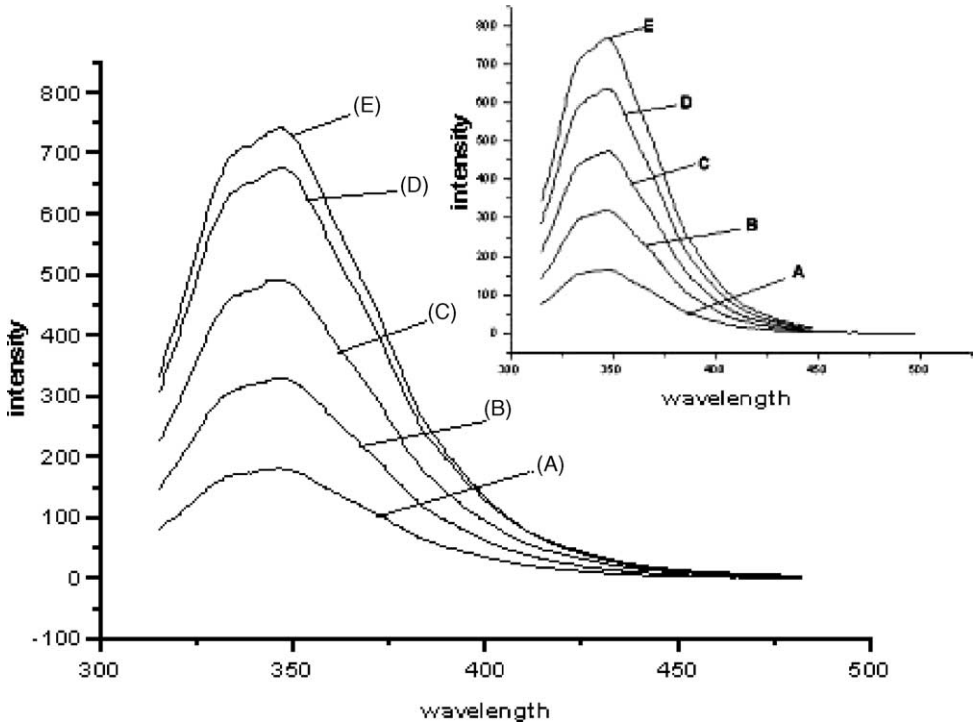

Fig. 5. Emission spectrum of concanavalin A at different protein concentrations at pH 5.2: 0.5 (A), 1.0 (B), 1.5 (C), 2.0 (D) and 2.5×10^{-3} mol dm^{-3} (E). Inset: Emission spectrum of concanavalin A at the same concentrations as above in the presence of 2 mol dm^{-3} urea.

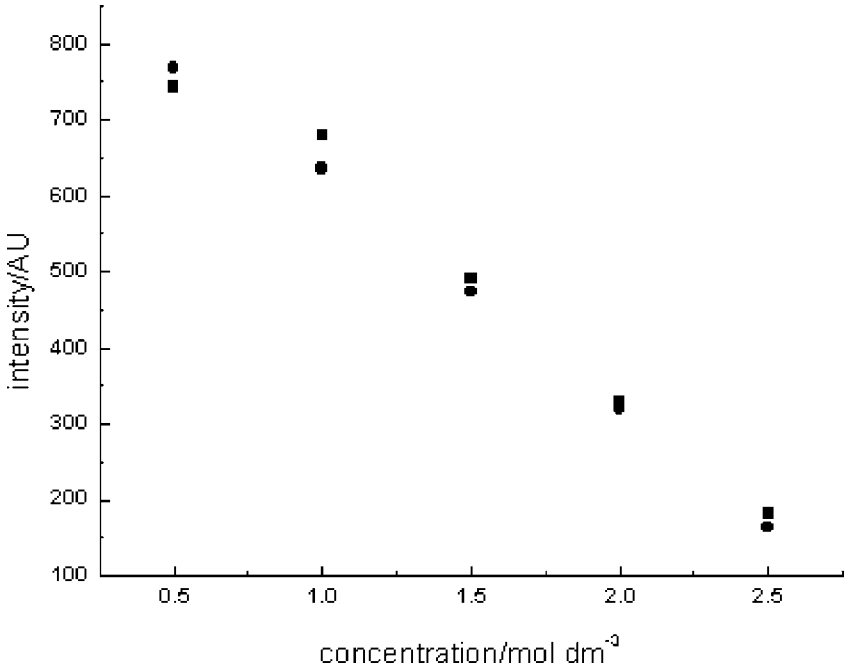

Fig. 6. Fluorescence intensity at λ_{max} plotted against concentration of concanavalin A at pH 5.2.

proteins, loss of cofactor etc. have been reported to be responsible for the irreversibility in the denaturation [28–30]. The well-established reversible thermal denaturation of some proteins such as lysozyme and ribonuclease A lose reversibility after being maintained for a certain length of time at high temperatures [29,31]. This could also be due to the hydrolysis of peptide bonds, deamination of certain residues, β-elimination of cystine residues and/or disulfide interchange. In the case of concanavalin A, the irreversibility in the calorimetric transitions appears to be due to combination of post-transition aggregation, chain separation and loss of cofactor.

Acknowledgements

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