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pH-dependent thermal transitions of lentil lectin

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Abstract The thermal stability of lentil lectin in the 5.0–10.0 pH range was studied by high-sensitivity differential scanning calorimetry and infrared spectroscopy. The thermally induced transitions for protein were irreversible and strongly dependent upon the scan rate at all pH values, suggesting that the denaturation is under kinetic control. It is shown that process of lentil lectin denaturation can be interpreted with sufficient accuracy in terms of the simple kinetic scheme, $N \xrightarrow{k} D$, where k is a first-order kinetic constant that changes with temperature, as given by the Arrhenius equation, N is the native state, and D is the denatured state. On the basis of this model, the parameters of the Arrhenius equation were calculated.

Key words: Lentil lectin; Irreversible thermal denaturation; Differential scanning calorimetry; Infrared spectroscopy; Arrhenius equation

1. Introduction

The mannose/glucose binding lectins comprise a group of agglutinins found in the family Leguminosae. Included in this group are the lectins from the pea (*Pisum sativum*), the lentil (*Lens culinaris*), the fava bean (*Vicia faba*), common vetch (*Vicia cracca*), and the forage legume sainfoin (*Onobrychis viciifolia*) [1]. On the basis of their molecular structure, mannose/glucose-specific lectins may be classified into two groups: those that consist of four identical subunits (concanavalin A) and those composed of two light (α) and two heavy (β) chains having the general composition $\alpha_2\beta_2$ (the pea, lentil, fava bean, etc.) [1]. These lectins require metal ions – e.g. Mn^{2+} and/or Ca^{2+} – for activity and are rich in acidic and hydroxylic amino acids, but devoid of or low in sulfur-containing amino acids. All members of this group are mitogenic for lymphocytes.

The nearly 40 sequences of legume lectins that have been determined to date exhibit identities or similarities close to 40% of the amino acid positions [2]. In addition, the three-dimensional structures of 10 legume lectins have been elucidated by high-resolution X-ray crystallography [3–6]. Although their quaternary structures are sometimes different, the tertiary structures of these proteins are practically identical. They are in the form of elaborated jelly rolls derived from antiparallel β-strands arranged as two β-sheets ('lectin fold' structure). The presence of Mn^{2+} and Ca^{2+} in the vicinity of the putative carbohydrate recognition site is essential for lectin binding activity and the geometry of the binding sites is a highly conserved feature in the different legume lectins.

In spite of the enormous interest in legume lectins owing to their broad practical applications in biotechnology, little is known about their physico-chemical properties. Although there are several publications addressing the thermal denaturation of legume lectins [7–10], in particular using differential scanning calorimetry (DSC) (which is the most useful technique for these purposes), the mechanism of this process remains unclear because of incorrect analysis of the experimental data on the basis of equilibrium thermodynamics. Thermodynamic analysis requires that denaturation should be a reversible equilibrium process. However, it is well known that the thermal denaturation of many proteins (including legume lectins) is irreversible and also strongly dependent on the heating rate. And if the DSC transitions corresponding to irreversible protein denaturation are highly scan rate-dependent, it is clear that the overall denaturation process is controlled kinetically and, therefore, the analysis of thermograms in equilibrium thermodynamics should be ruled out [11]. Accordingly, a more realistic model should include two steps: a reversible unfolding step followed by an irreversible denaturation step:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} U \xrightarrow{k_2} D \quad (1)$$

where N, U and D are the native, unfolded or partially unfolded and denatured states of the protein [12].

The aim of the present study was to investigate the thermal denaturation of lentil lectin within a wide pH region by DSC and infrared spectroscopy using model that is approximation to Eq. 1.

2. Materials and methods

2.1. Materials

DEAE-cellulose, glycine and glycylglycine buffers were purchased from Sigma (St. Louis, MO, USA). Reagents for electrophoresis were from Bio-Rad laboratories (Richmond, CA, USA), Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Silver nitrate, potassium and sodium phosphate were from PROBUS (Barcelona, Spain). All reagents were of the highest purity available. Double distilled water was used throughout.

2.2. Protein purification and determination

Lentil seeds, *Lens culinaris* from the La Armuña area, Salamanca, Spain, were used. Lentil lectin purification was accomplished by DEAE-cellulose chromatography [13] and specific absorption on Sephadex G-100 [14] as it was previously described [15].

Sample purity was checked by gel filtration with fast protein liquid chromatography (FPLC). The chromatographic apparatus was a Pharmacia LKB FPLC system consisting of a 2150 FPLC pump, a 2154 loop injection valve, a 2158 Uvicord SD monitor (set at 280 nm),

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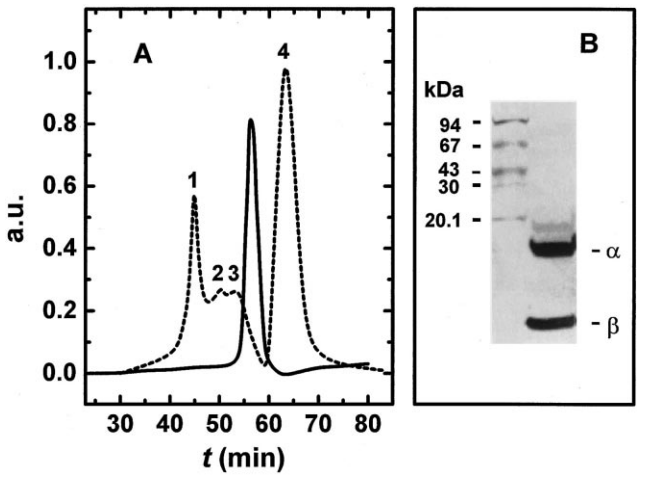

Fig. 1. Chromatographic and SDS-polyacrylamide gel electrophoretic analysis of lentil lectin from lentil seeds, *Lens culinaris* (area La Armuña, Salamanca, Spain). A: FPLC of lentil lectin (solid line). Protein in 10 mM phosphate buffer, pH 7.4, was loaded onto a Superosa 12 column (1 cm×30 cm) and then eluted at a flow rate of 0.25 ml/min. The dashed line is the chromatographic profile of Pierce molecular weight markers obtained under the same conditions as for protein: 1, horse spleen ferritin (450 kDa); 2, hen egg albumin (45 kDa); 3, bovine pancreas chymotrypsinogen A (25 kDa); 4, horse heart cytochrome *c* (12.5 kDa). B: SDS-PAGE of lentil lectin. The left lane is the protein molecular weight markers and the right lane shows lentil lectin bands α and β.

and a 3390A integrator (Hewlett-Packard). One peak of the sample studied was observed (Fig. 1A). This peak was identified to be the lentil lectin with a molecular weight of 24 kDa. SDS-PAGE of this sample revealed two bands with molecular weights of 17 kDa and 7 kDa, designated as the α- and β-subunits (Fig. 1B). Electrophoresis was performed in a flat block with a polyacrylamide gradient of 5–25% by the method of Fairbanks et al. [16]. Protein concentration determinations were performed by the Lowry procedure [17] and by ultraviolet absorption at 280 nm [13].

2.3. Scanning calorimetry

DSC experiments were performed on a MicroCal MC-2D differential scanning microcalorimeter (MicroCal Inc., Northampton, MA, USA) with cell volumes of 1.22 ml, interfaced with a personal computer (IBM-compatible) as described previously [15]. Different scan rates within the 0.2–1.0 K/min range were employed. The reversibility of the thermal transitions was checked by examining the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling from the first scan. The experimental calorimetric traces were corrected for the effect of instrument response time using the procedure described previously [18].

The excess molar heat capacity was calculated taking into account that the molecular mass of lentil lectin is 24 kDa.

The temperature dependence of the molar heat capacity of lentil lectin was further analyzed and plotted using the Windows-based software package (Origin) supplied by MicroCal and original programs developed for IBM-compatible computers by Dr. A.E. Lyubarev and A.V. Malyshev.

2.4. Infrared spectroscopy

Spectra were recorded on a Nicolet Magna II 550 spectrometer equipped with a MCT detector using a demountable liquid cell (Harrick Scientific, Ossining, NY, USA) equipped with calcium fluoride windows and 50 μm teflon spacers. The thermally induced changes in the infrared spectrum were investigated by increasing the temperature at ca. 0.5 K/min in a cell holder controlled thermostatically by a water bath while the spectra were acquired using a rapid scan feature included in the OMNIC software from Nicolet. The actual temperature in the cell was controlled by a tungsten-copper thermocouple placed directly onto the window. Typically, 608 interferograms were averaged each minute and after finishing the measurements these were reprocessed against a specific background obtained previously obtaining the absorbance spectra with a nominal resolution of 2 cm^{-1}. The buffer contribution was subtracted for each spectrum and the absorbances at 1622 and 1634 cm^{-1} were measured after baseline correction.

3. Results and discussion

3.1. DSC studies

The thermal denaturation of lentil lectin gives rise to a well defined DSC transition, whose apparent T_m (temperature at the maximum of the heat capacity profile) depends on pH and scan rate. This effect can be seen in Fig. 2, where the thermal denaturation of lentil lectin (C_p^{ex} versus T) at pH 5.0, 7.4, 8.5 and 10.0 and at three scan rates is shown. This thermal denaturation was always calorimetrically irreversible since in a second heating of the lentil lectin solution no thermal effect was observed. The protein has different stability at different pH; however, regardless of this, the effect of scan rate on the calorimetric profiles clearly indicates that they correspond to irreversible, kinetically controlled transitions. It is thus evident that equilibrium thermodynamics cannot be applied in their analysis. Analysis of the DSC transitions was therefore done taking into account the kinetics of formation of the irreversibly formed protein state. Of course, the most attractive mod-

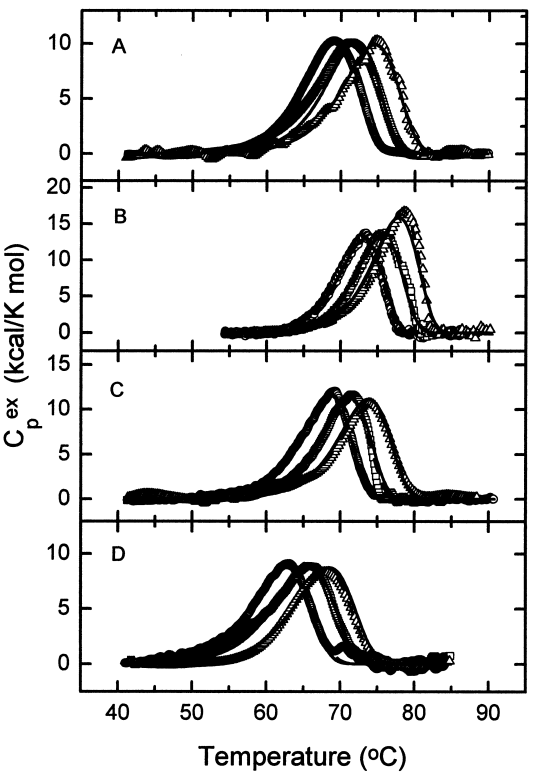

Fig. 2. Temperature dependence of the excess molar heat capacity of lentil lectin at scan rates of 0.21 (circles), 0.5 (squares) and 1.0 (triangles) K/min, and at pH 5.0 (A), 7.4 (B), 8.5 (C) and 10.0 (D). Dashed lines are the best fit using Eq. 5 to each experimental curve and solid lines are the best fit using the same equation simultaneously on all curves for each pH value. The buffers used were succinate for pH 5.0, phosphate for pH 7.4, glycylglycine for pH 8.5 and glycine for pH 10.0 at a concentration of 10 mM. Protein concentrations were ca. 0.04 mM at a scan rate of 1 K/min, ca. 0.06 mM at a scan rate of 0.5 K/min, and ca. 0.08 mM at a scan rate of 0.21 K/min.

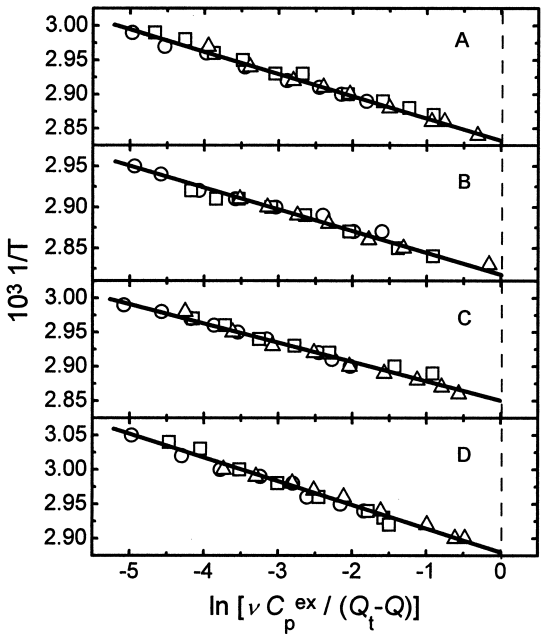

Fig. 3. Dependences of $1/T$ on $\ln[\nu C_p^{ex}/Q_t-Q)]$ of lentil lectin at pH 5.0 (A), 7.4 (B), 8.5 (C) and 10.0 (D) calculated from DSC experimental data shown in Fig. 2. To obtain these dependences the experimental points in range of Q/Q_t values from 5 to 95% were used (for details see [20]).

el for this analysis is Eq. 1, which involves a reversible unfolding step followed by an irreversible denaturation step. However, using the whole Lumry-Eyring model has difficulties, because the system of differential equations describing this model cannot be solved at varying temperature. Therefore, we have used the model, which is a simplest approximation to Eq. 1, the so-called two-state irreversible model of protein denaturation [19,20]:

$$N \xrightarrow{k} D \tag{2}$$

This model considers only two significantly populated macroscopic states, the initial or native state (N) and the final or denatured (D) state. In this case, the process is characterized by a first-order rate constant k which follows the Arrhenius equation:

$$k = \exp\left[\frac{E_A}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right] \tag{3}$$

where E_A is the activation energy of the denaturation process and T^* is the temperature at which k is equal to 1 min^{-1}.

Previously, we have proposed some criteria for the validity of Eq. 2 [21]. One of them plot the values of $\ln[\nu C_p^{ex}/(Q_t-Q)]$ versus $1/T$ according to

$$\ln[\nu C_p^{ex}/(Q_t-Q)] = \frac{E_A}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right) \tag{4}$$

where C_p^{ex} is excess heat capacity, and Q_t and Q are the total and excess heats at a given temperature of the denaturation process. If the data satisfy the model, the experimental points corresponding to all the scan rates should lie on a common straight line. Fig. 3 shows the DSC data for lentil lectin at different pH values in the above coordinates. As can be seen from the plot, the experimental data follow this criterion fairly well in all cases. The parameters of the Arrhenius equation obtained from these dependences are given in Table 1.

A more reliable way of checking Eq. 2 is to estimate the Arrhenius equation parameters for different scan rates using the equation that quantitatively explains the thermal capacity curves as a function of absolute temperature [20]:

$$C_p^{ex} = \frac{1}{\nu} Q_t \exp\left\{\frac{E_A}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right\} \exp\left\{-\frac{1}{\nu}\int_{T_0}^{T}\exp\left[\frac{E_A}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right]dT\right\} \tag{5}$$

The results of fitting Eq. 5 to the experimental data are shown in Fig. 2 (solid and dashed lines) and in Table 2. As can be seen, when fitting was carried out either separately on the individual experimental curves or simultaneously on all the curves, a good approximation was achieved. It is evident that the approximation is not absolutely precise, probably due to differences in the values of the total heat of the denaturation process obtained at different scanning rates. At present the reason for this difference remains unclear, although it could be related to a process of aggregate formation which accompanies protein denaturation [21], and whose form depends on the rate of denaturation [22].

3.2. Infrared spectroscopy

To obtain more information about the mechanism of thermal denaturation of lentil lectin, we carried out an investigation of this process by infrared spectroscopy, studying conformational changes by looking at variations in the amide I band arising mainly from C=O stretching vibrations from the peptide bond [23,24]. In a previous work [25] we have made a detailed analysis of the pH-dependent structural changes of lentil lectin using infrared spectroscopy. It was shown in this work that lentil lectin is composed mainly of β-sheet with the strands connected by turns and long loops and that the protein is particularly compact at a neutral pH. An increase or decrease in pH induced variations in the topology of loops and turns changing the forces stabilizing the strands and the tertiary structure of the protein. These changes did not affect the secondary structure, but the interactions between strands were weakened, thus decreasing the stability. In the present

Table 1
Arrhenius equation parameter estimates for the two-state irreversible Eq. 2 of thermal denaturation of lentil lectin using Eq. 4

Parameter	pH			
	5.0	7.4	8.5	10.0
T^* (K)	352.9 ± 0.4	354.0 ± 0.2	351.3 ± 0.2	347.2 ± 0.5
E_A (kcal/mol)	62.4 ± 1.7	81.8 ± 0.8	68.5 ± 1.4	58.4 ± 1.7

Table 2
Arrhenius equation parameter estimates for the two-state irreversible model (Eq. 2) of thermal denaturation of lentil lectin using Eq. 5

Parameter	pH	Temperature scan rate (K/min)			Fitting to all curves simultaneously
		0.21	0.5	1.0	
Q_t (kcal/mol)	5.0	97 ± 1	108 ± 3	99 ± 2	
	7.4	97 ± 2	106 ± 6	126 ± 8	
	8.5	104 ± 2	94 ± 4	104 ± 2	
	10.0	98 ± 5	100 ± 3	84 ± 1	
T^* (K)	5.0	352.9 ± 0.14	353.3 ± 0.14	352.7 ± 0.07	352.3 ± 0.07
	7.4	353.5 ± 0.1	354.3 ± 0.16	354.0 ± 0.08	353.6 ± 0.09
	8.5	351.3 ± 0.1	350.4 ± 0.16	351.4 ± 0.07	351.2 ± 0.07
	10.0	347.6 ± 0.15	347.6 ± 0.14	346.5 ± 0.04	347.1 ± 0.08
E_A (kcal/mol)	5.0	65.1 ± 0.3	58.0 ± 1.1	63.9 ± 0.5	66.0 ± 0.4
	7.4	85.9 ± 0.3	82.3 ± 0.4	87.2 ± 0.7	85.5 ± 0.3
	8.5	68.8 ± 0.5	73.2 ± 1.0	69.5 ± 0.3	69.5 ± 0.3
	10.0	57.6 ± 0.6	56.0 ± 0.9	60.0 ± 0.3	59.4 ± 0.4
r	5.0	0.9994	0.9995	0.9950	0.9931
	7.4	0.9989	0.9974	0.9936	0.9934
	8.5	0.9983	0.9910	0.9977	0.9923
	10.0	0.9923	0.9974	0.9977	0.9911

r is the correlation coefficient calculated by equation: $r = \sqrt{1-\sum_{i=1}^{n}(y_i-y_i^{calc})^2/\sum_{i=1}^{n}(y_i-y_i^{m})^2}$, where y_i and y_i^{calc} are respectively the experimental and calculated values of C_p^{ex}; y_i^{m} is the mean of the experimental values of C_p^{ex}, and n is the number of points.

work we have estimated the thermal stability of lentil lectin at different pH values by following an empirical parameter, defined as the intensity ratio of the amide I band between the wave number where the band related to protein aggregation is located (1622 cm^{-1}) and the maximum corresponding to the major structure present, in this case the β-sheet (1634 cm^{-1}) (Fig. 4, symbols). This parameter is equivalent to others used previously but is easier to measure [26]. The plots for lentil lectin at pH 5.0, 7.0, 8.5 and 10.0 show a sharp denaturation transition characteristic of a cooperative process with a decrease of loops and β-sheet and increase in the bands related to aggregation and turns [25]. The solid lines in this figure are the result of a non-linear least-squares fit to the experimental data, assuming a two-state irreversible process between the native and denatured states, to the equation:

$$I_{1622}/I_{1634} = (I_{1622}/I_{1634})_d + [(I_{1622}/I_{1634})_n - (I_{1622}/I_{1634})_d]$$
$$\exp\left\{-\frac{1}{\nu}\int_{T_0}^{T}\exp\left[\frac{E_A}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right]dT\right\} \quad (6)$$

which is valid for Eq. 2 and where $(I_{1622}/I_{1634})_n$ and $(I_{1622}/I_{1634})_d$ refer to the ratios of the amide I band at 1622 cm^{-1} and 1634 cm^{-1} of the native and denatured states of protein, respectively. The parameters of the Arrhenius equation obtained from this fitting are given in Table 3, where it can be seen that the Arrhenius equation parameters estimated from the spectroscopic data for pH values of 5.0, 7.4 and 8.5 diverge in comparison with the parameters estimated for the same pH values from the calorimetric data. In our opinion, this divergence is related to the absence of sufficient points from the high-temperature end of the spectroscopic thermal denaturation profiles due to technical reasons. And, as is known, an error in the estimation of the denatured baseline slope can lead to important errors in the fitting parameters [27]. As a proof of this, the fitting parameters for pH 10, where there are enough pre- and post-transition data points, are in good agreement with the parameters obtained from DSC data analysis. It may be seen that there is reasonable agreement between the Arrhenius equation parameters obtained with DSC and infrared spectroscopy methods. Thus, the denaturation of lentil lectin over a broad pH range can be interpreted in terms of a simple two-state first-order kinetic mechanism.

It is known that the thermal denaturation of many proteins is irreversible. This type of denaturation is generally attributed to alterations (such as aggregation, autolysis, chemical alteration of residues [28]) that lock the protein in a final state that is unable to fold back to the native structure. These irreversible alterations are fundamentally kinetic processes that must be analyzed on the basis of kinetic models and described by rate equations [29]. For an equilibrium process of denaturation the protein stability is often defined as the Gibbs energy difference between the denatured and native states [30], but for some practical purposes, the denaturation temperature (T_m) may be a more useful measure of this stability. For two-state reversible denaturation the T_m is simply determined

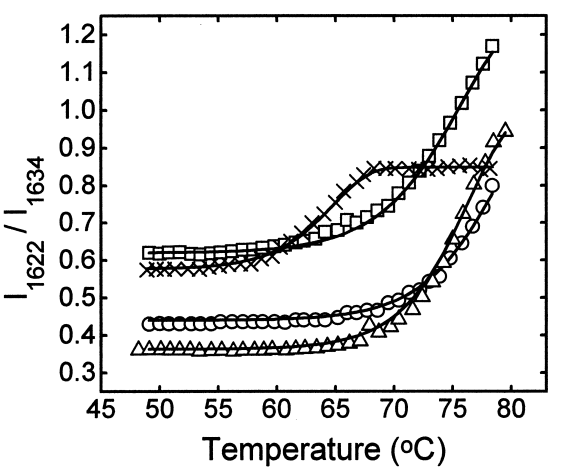

Fig. 4. Ratio of amide I band intensity at 1622 cm^{-1} to that at 1634 cm^{-1} as a function of temperature for lentil lectin at pH 5 (□), 7.4 (○), 8.5 (Δ) and 10.0 (×) obtained at heating with a scan rate of ca. 0.5 K/min. The solid lines are the best fits using Eq. 6.

Table 3
Arrhenius equation parameter estimates for the two-state irreversible model (Eq. 2) of thermal denaturation of lentil lectin using Eq. 6

Parameter	pH			
	5.0	7.4	8.5	10.0
T^* (K)	355.6 ± 1.6	356.0 ± 1.4	354.5 ± 1.4	346.3 ± 0.3
E_A (kcal/mol)	66.2 ± 1.2	78.1 ± 0.9	73.3 ± 1.1	58.4 ± 0.3
r	0.9963	0.9938	0.9989	0.9968

by the stability curve [30], however, the irreversible alterations shift the calorimetric transitions to temperatures significantly lower than that corresponding to the Gibbs energy difference equal zero [29]. Therefore, in many cases of irreversible denaturation, as well as in the case of the data presented in this work, operational thermal stability (as measured by the denaturation temperature under given conditions) may be subject to kinetic constraints.

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