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Spectroscopic and differential scanning calorimetric studies on the unfolding of *Trichosanthes dioica* seed lectin. Similar modes of thermal and chemical denaturation

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Abstract Physico-chemical and unfolding studies have been carried out on *Trichosanthes dioica* seed lectin (TDSL). The lectin exhibited maximum activity between pH 7.0 and 10.0, which decreased steeply at lower pH. The hemagglutination activity of TDSL was unaffected in the temperature range 4–50°C, but decreased rapidly at higher temperatures. Differential scanning calorimetric studies indicate that thermal unfolding of TDSL is an irreversible process, which could be described by a three-state model. The calorimetric scan recorded at pH 7.0 consists of two transitions, occurring at around 338.6 K, and 342.8 K. In the presence of carbohydrate ligands both these transitions shifted to higher temperatures, suggesting that ligand binding stabilizes the native conformation of the protein. The unfolding temperature was highest at pH 5.0 indicating that TDSL is more stable at acidic pH. Gdn.HCl induced unfolding, monitored by following changes in the intrinsic fluorescence properties of the protein, was also observed to be a three-state process involving an intermediate. CD spectroscopy indicates that the secondary and tertiary structures of TDSL are rather similar at different pH values, indicating that the lectin structure remains essentially unchanged over a wide range of pH.

Keywords Agglutinin · Carbohydrate binding protein · Thermal unfolding · Van't Hoff enthalpy · Calorimetric enthalpy · Chemical denaturation

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Abbreviations

TDSL	*Trichosanthes dioica* seed lectin
CD	Circular dichroism
ΔC_p	Change in excess heat capacity
DSC	Differential scanning calorimetry
GalNAc	*N*-acetyl-D-galactosamine
Gdn.HCl	Guanidine hydrochloride
ΔH_c	Change in calorimetric enthalpy
ΔH_v	Change in van't Hoff enthalpy
MeαGal	Methyl-α-D-galactopyranoside
MeβGal	Methyl-β-D-galactopyranoside
T_m	Transition temperature

Introduction

Lectins are proteins of non-immune origin that bind carbohydrates reversibly with a high degree of specificity in a non-catalytic manner. The unusual ability of lectins to specifically recognize carbohydrate structures has made them important tools to study cell surface glycans, glycoconjugates in solution, mitogenic stimulation of lymphocytes as well as in clinical and biomedical applications such as blood typing, enzyme replacement therapy for treatment of Gaucher's disease etc [1, 2]. Among the lectins, legume lectins are well studied and a great deal of information including primary structure and crystal structure of many of them is available [1, 3–7]. Additionally, thermal and chemical unfolding processes of a number of legume lectins have been characterized [8–12]. Studies on lectins from other families are fewer, and there is a need to characterize them in detail to understand their properties in detail. Our laboratory has been working on the purification and characterization of lectins from the Cucurbitaceae with

respect to physicochemical properties, carbohydrate binding and hydrophobic ligand binding [13–18].

One of the Cucurbitaceae lectins that is being investigated in our laboratory is the *Trichosanthes dioica* seed lectin (TDSL), which is a heterodimeric, galactose specific protein with an M_r of 55 kDa [18]. TDSL is a glycoprotein with about 4.9% covalently bound sugar. CD spectroscopic studies have shown that the secondary structure of TDSL contains 13.3% α-helix, 36.7% β-sheet, 19.4% β-turns and 31.6% unordered structure. Chemical modification studies have implicated tyrosine residues in the carbohydrate binding activity of the lectin [18]. Additionally, fluorescence quenching studies employing neutral and charged quenchers suggested that the tryptophan residues are largely buried in the hydrophobic core of the protein and that at least one Trp residue has positively charged residue(s) in close proximity [19].

Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure. The three-dimensional structure of a protein is maintained by covalent and non-covalent interactions. Protein physical and biological properties can be altered by conditions that affect the native conformation of the protein. The conformational stability of proteins is defined as the difference in free energy change between the folded state and unfolded state under physiological conditions. The conformational stability of protein is determined by analysis of denaturant induced or thermal unfolding of proteins determined either by spectroscopy or calorimetry [20–22].

In the present study, with the aim of understanding the stability of TDSL to thermal and chemical denaturation processes, we have carried out differential scanning calorimetric studies at different pH and also investigated the unfolding of the protein by Gdn.HCl. Additionally, the effects of pH and temperature on the hemagglutination activity of TDSL have been investigated. Thermodynamic parameters characterizing the unfolding transition(s) such as transition temperature (T_m), calorimetric enthalpy (ΔH_c), van't Hoff enthalpy (ΔH_v), and the changes in excess heat capacity (ΔC_p) were obtained from the DSC measurements, whereas changes in the secondary and tertiary structures at different temperatures were investigated by CD spectroscopy. Chemical unfolding by Gdn.HCl was studied by fluorescence spectroscopy monitoring changes in the protein intrinsic fluorescence emission characteristics.

Materials and methods

Materials

Trichosanthes dioica seeds were obtained from local vendors. Guar gum, lactose, lactulose, galactose, GalNAc, MeαGal and MeβGal were purchased from Sigma (St. Louis, MO, USA). All the other reagents used were of the highest purity available and obtained from local suppliers.

Purification of *Trichosanthes dioica* seed lectin

TDSL was purified by a combination of ammonium sulfate precipitation and affinity chromatography on cross-linked guar gum as described earlier [18]. Purity of the affinity eluted protein was assessed by polyacrylamide gel electrophoresis, where it moved as a single band [23]. Concentration of TDSL was determined by Lowry assay [24]. For experiments at different pH, the following buffers were used: 20 mM KCl-HCl (pH 1.0–2.0), 20 mM citrate-phosphate (3.0–5.0), 20 mM phosphate (pH 6.0–7.0), 20 mM Tris-HCl (pH 8), and 20 mM glycine/NaOH (pH 9.0–11.0). NaCl (150 mM) was included in all buffers to maintain a constant ionic strength. Protein samples were dialyzed against the buffer of appropriate pH before further experimentation.

Agglutination assay

Agglutination activity of TDSL was assayed by the hemagglutination technique as described previously [18]. A 4% suspension of human O (+) erythrocytes in 20 mM buffer containing 150 mM NaCl was mixed with serially diluted samples of the lectin in a 96-well ELISA plate and incubated at 4°C for 1 h. The agglutination titer was scored visually.

pH dependence and thermal inactivation of TDSL

Agglutination activity of TDSL at different pH was assessed by dialyzing the lectin samples extensively against a buffer of desired pH, followed by the hemagglutination assay at 4°C. The erythrocyte suspension used for the hemagglutination assay was also prepared in the same buffer.

Effect of temperature on the agglutination activity was investigated by incubating lectin samples at the desired temperature for 30 min. The samples were then allowed to cool to room temperature, clarified by centrifugation and the clear supernatants were tested for hemagglutination activity as described above.

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco-J-810 spectropolarimeter equipped with a Peltier thermostat supplied by the manufacturer. Protein samples were taken in a 2 mm path length rectangular quartz cuvette. Spectra were recorded at a scan

rate of 20 nm/min with a response time of 4 sec and a band width of 1.5 nm. Lectin concentrations used were 2.8 and 16 μM, respectively, for measurements in the far UV and near UV regions. Each spectrum was the average of 4 accumulations. CD spectra were recorded at different temperatures in the near UV region and far UV region in order to investigate the effect of temperature on the secondary and tertiary structures of TDSL. Buffer scans recorded under the same conditions were subtracted before further analysis.

Differential scanning calorimetry

DSC measurements were made on a MicroCal VP-DSC differential scanning calorimeter (MicroCal LLC, Northampton, MA, USA) equipped with two fixed cells, a reference cell and a sample cell. DSC experiments were carried out as a function of pH, scan rate, and at different ligand concentrations. Samples were dialyzed extensively against buffers of desired pH before recording the thermograms. Buffer and protein solutions were degassed before loading. All the data were analyzed by using the Origin DSC software provided by the manufacturer.

Fluorescence spectroscopy

Unfolding of TDSL induced by Gdn.HCl was monitored by fluorescence spectroscopy. Fluorescence measurements were performed on a Spex Fluoromax-3 spectrofluorimeter (Jobin Yvon Ltd, Edison, NJ, USA, website: www.jobinyvon.com). Lectin samples were incubated with different concentrations of Gdn.HCl overnight before the measurements were taken. Lectin concentration used for fluorescence measurements was 1.8 μM. Samples were excited at 280 nm, and emission spectra were recorded above 300 nm. The slit widths used were 3 and 5 nm, for the excitation and emission monochromators, respectively.

Results

pH optimum and thermal stability of TDSL

Activity of the TDSL samples dialyzed against appropriate buffers in the pH range 3–10 were tested by hemagglutination assay and the relative activities observed were plotted as a function of the sample pH (Fig. 1a). The results indicate that only 50% relative activity is observed at pH 4, which increases to 75% at pH 6, and to 100% at pH 7. Hemagglutination activity remains unaltered with further increase in pH up to 10 (Fig. 1a).

Thermal inactivation of TDSL was investigated by incubating lectin samples at different temperatures for 30 min followed by the hemagglutination assay to check lectin activity of the heat-treated samples. The results obtained are given in Fig. 1b. It is seen from the figure that lectin activity is unaffected up to 50°C, while at higher temperatures the activity decreases sharply. Incubation at 60°C reduced the activity to 81% as compared to the native protein, whereas incubation at 70°C led to a complete loss in the hemagglutination activity of TDSL.

Far UV and near UV circular dichroism spectra of TDSL recorded at different temperatures are given in Fig. 2a, b, respectively. The far UV CD spectrum of TDSL shows two minima around 208 and 222 nm, which is consistent with earlier reports [18]. It is seen from this figure that the CD spectrum of TDSL incubated at 50°C (spectrum 2) is essentially identical to the spectrum recorded at room temperature (spectrum 1). Significantly larger changes were observed in the CD spectra recorded at 65°C and 70°C, suggesting the occurrence of a thermally-induced unfolding phase transition in this temperature range. This is clearly seen from Fig. 2c, which gives a plot of ellipticity at 206 nm as a function of temperature.

The near UV CD spectrum of TDSL is characterized by a minimum around 283 nm and a shoulder around 293 nm (Fig. 2b), which is again in good agreement with previous results [18]. All the CD spectra of TDSL samples incubated between 30°C and 60°C are very similar, indicating that the tertiary structure of the protein does not change when it is heated up to 60°C, whereas incubation at 70°C led to large changes in the spectral features, indicating a significant loss in the tertiary structure. In order to monitor this more closely, intensity of the CD signal at 283 nm (corresponding to the minimum in the near UV spectrum) was monitored as a function of temperature (Fig. 2d). In this experiment the lectin sample was heated at a scan rate of 1° per min in the temperature range 30°C to 90°C. While only marginal changes are seen in the signal intensity between 30°C and 63°C, a steep increase is observed in the signal intensity between 65°C and 70°C, clearly indicating that a major transition occurs at ca. 67–68°C in the protein tertiary structure. When the heating scan was completed and the sample was cooled to room temperature, a white precipitate was observed in the sample cell, indicating irreversible thermal denaturation of the lectin.

Far UV and near UV CD spectra of TDSL at different pH are shown in Fig. 3a, b, respectively. Only marginal changes are seen in the CD spectra between pH 2 and pH 10, suggesting that the lectin structure is quite stable and is essentially unchanged in this pH range.

Differential scanning calorimetry

A representative DSC thermogram of the *T. dioica* lectin along with the fit of the single transition peak data to a non-

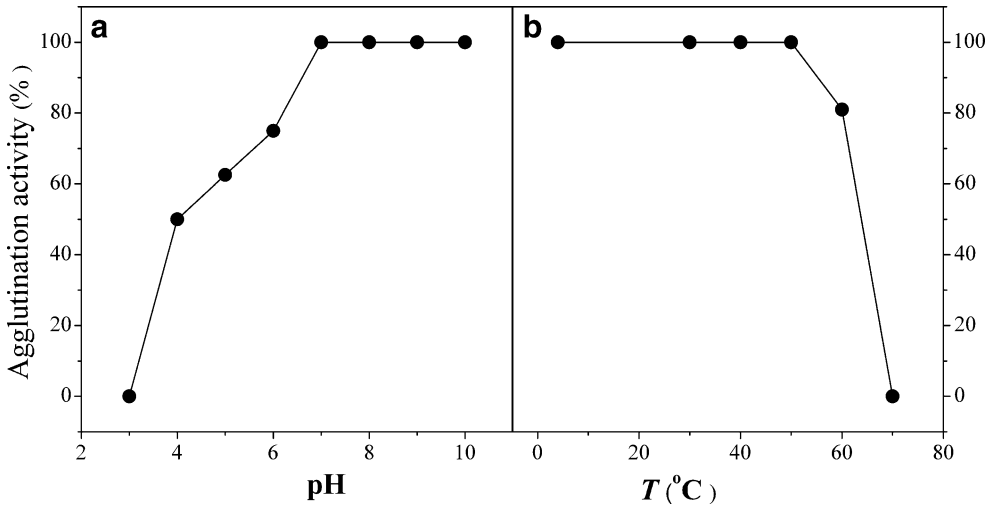

Fig 1 Effect of **a** pH and **b** thermal inactivation on the agglutination activity of *T. dioica lectin*. To study pH dependence TDSL samples were dialyzed against buffers of desired pH. Activity was assessed by the hemagglutination technique at 4°C. The percentage activity was calculated assuming the maximum activity as 100%. To investigate the effect of temperature, lectin samples were first incubated at the desired temperature for 30 min. They were then cooled to room temperature, centrifuged and the clear supernatant was tested for agglutination activity

two-state transition model is shown in Fig. 4. Both two-state method and non-two-state method use the Levenberg–Marquardt non-linear least square method. The two-state model gives calorimetric enthalpy (ΔH_c) change and the thermal mid-point of transition (T_m), whereas the non-two-state model gives van't Hoff enthalpy (ΔH_v) in addition to calorimetric enthalpy and transition mid-temperature. The transition peak consists of two non-two-state transitions melting at different temperatures. The lower-temperature transition has a T_m of 338.42 K (65.27°C) with a ΔH_m value of 474 kJ mol^{-1}, while the higher-temperature transition is characterized by a T_m of 342.6 K (69.45°C) with ΔH_m=385 kJ mol^{-1}. A white precipitate was observed in the cell after the scan, and the peak did not reappear upon a rescan of the sample suggesting that the transition is irreversible. To find out any kinetic effects on the thermal unfolding of TDSL, the data were collected at different scan rates. No change was seen in the T_m values of the lower and higher transition peaks at different scan rates, when the scan rate was increased by a factor of 4. Additionally, calorimetric and van't Hoff enthalpies did not change when scan rate was varied, suggesting that equilibrium thermodynamics can be applied to evaluate the thermodynamic parameters [25, 26]. Ratio of calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) for the first transition peak is considerably greater than 1 for TDSL dimer indicating that the two subunits of the protein unfold as a single entity during denaturation. For the second transition the ratio $\Delta H_c/\Delta H_v$ is about half the value observed for the first transition, which implies that intermediate transitions are observed in the transition profile [26].

Effect of ligand binding

In the presence of saturating amounts of ligands also TDSL exhibits two transition peaks. These peaks are again fitted to a non-two-state transition model. DSC data obtained in the presence of different ligands is shown in Table 1. Both the lower-temperature transition and the higher-temperature transition shift to higher temperatures in the presence of different carbohydrate ligands, suggesting that ligand binding stabilizes the native conformation of TDSL. Among all the carbohydrate ligands used, binding of lactose induced the maximum shift of 5.38 K in the T_m. Shifts induced in the T_m of TDSL by other sugars are: lactulose (4.8 K), MeβGal (4.11 K), MeαGal (3.18 K) and GalNAc (3.53 K). From the ligand-induced shifts in the T_m, the association constants at the denaturation temperature [$K_b(T_c)$] were calculated according to equation (1) [27]:

$$K_b(T_c) = \{\exp[(T_c - T_m)\Delta H_c/nRT_cT_m] - 1\}/[L] \qquad (1)$$

where T_c and T_m are denaturation temperatures in the presence and absence of lactose, respectively, ΔH_c is the calorimetric enthalpy of the carbohydrate ligand–protein complex, [L] is the ligand concentration and n is the number of binding sites on the dimeric protein.

From the above analysis, values of the binding constant for various ligands calculated at denaturation temperature are 188, 133, 87.5, 60 and 48 M^{-1} for lactose, lactulose, MeβGal, GalNAc and MeαGal, respectively. The ratio of $\Delta H_c/\Delta H_v$ in the presence of different ligands is similar to that obtained with the native lectin for both transitions indicating that the unfolding behavior of both the liganded form and unliganded form of TDSL are similar. The

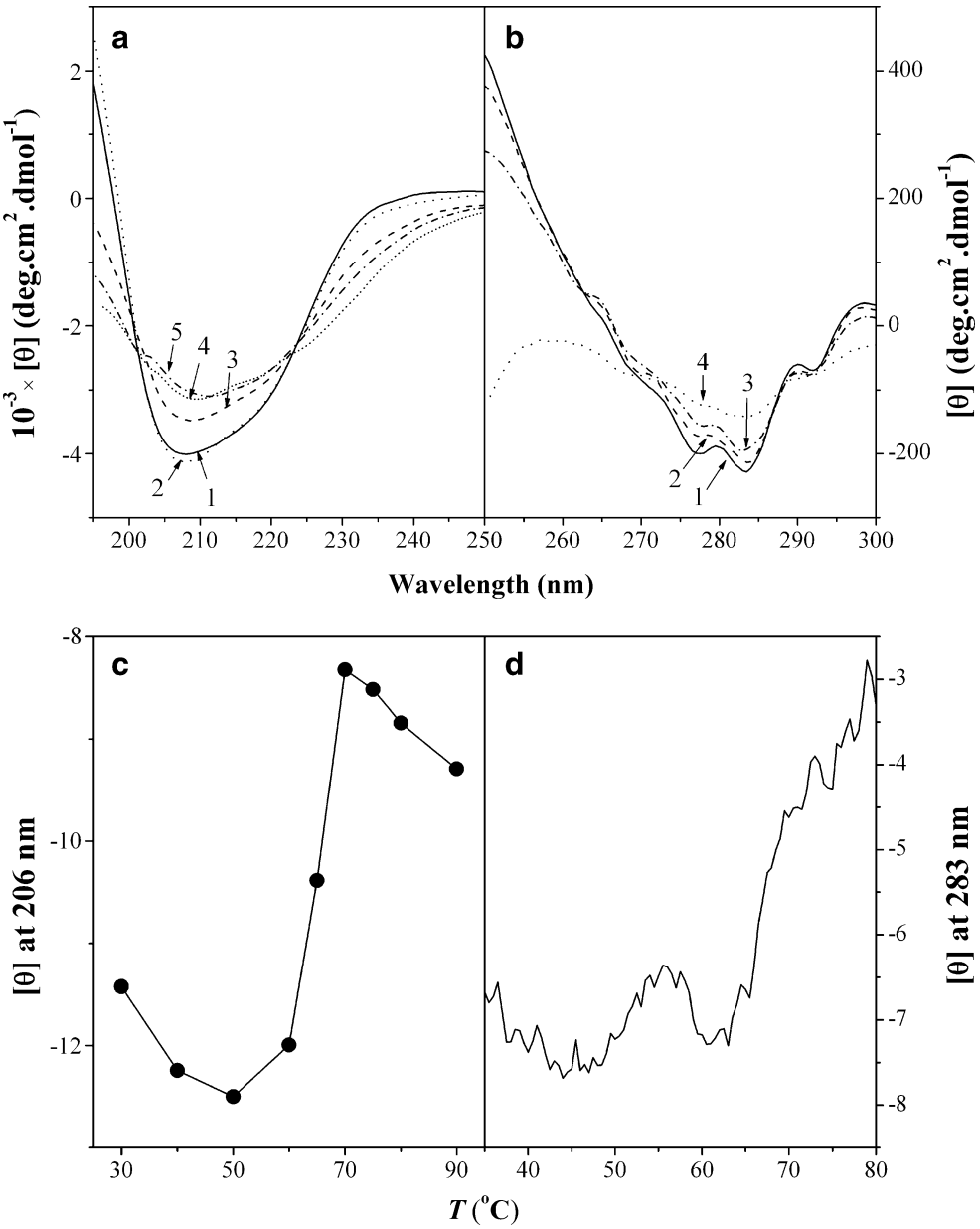

Fig 2 Effect of thermal inactivation on the CD spectrum of TDSL. **a** Far UV CD spectra of TDSL. Samples that were incubated at 30°C, 50°C, 65°C, 70°C, and 90°C were numbered as *1–5* respectively. **b** Near UV CD spectra of TDSL samples incubated at 30°C, 50°C, 60°C and 70°C were numbered as *1–4*, respectively. **c** A plot of ellipticity (Θ) at 206 nm *versus* temperature. **d** The ellipticity (Θ) in the near UV region of the TDSL was monitored at 283 nm as a function of temperature

denaturation transition in presence of bound ligand can be written as follows [28]:

$$\ln [L] = -\Delta H_v [L] / [RT_p m] + \text{constant} \quad (2)$$

where T_p is the maximum point of the transition peak, $[L]$ is the ligand concentration and m is the number of ligand molecules, bound to each molecule of the dimeric protein.

A plot of ln $[L]$ *versus* $1/T_p$ for lactose is shown in Fig. 5. From the slope of this plot using the value of m as 2, van't Hoff enthalpy for the lower-temperature transition was estimated as 768±140 kJ mol^{-1}, whereas the ΔH_v value for the higher-temperature transition was calculated as 954±118 kJ mol^{-1}. These values are close to the values obtained by non-two-state fit of the transition data in the presence of lactose. Since T_p is not determined from the fits of non-two-state method, the agreement between the values ΔH_v and $-\Delta H_v$ $[L]$ is an independent conformation. Therefore, it can be concluded that two lactose molecules bind to each TDSL dimer.

Effect of pH

DSC data obtained for the thermal transition of TDSL at different pH (2–10) is presented in Table 2. CD studies in this pH range (Fig. 3) show that the secondary and tertiary structures of TDSL are very similar, indicating that TDSL is stable over this wide range of pH. At all pH values in this range the calorimetric data could be satisfactorily fit by a

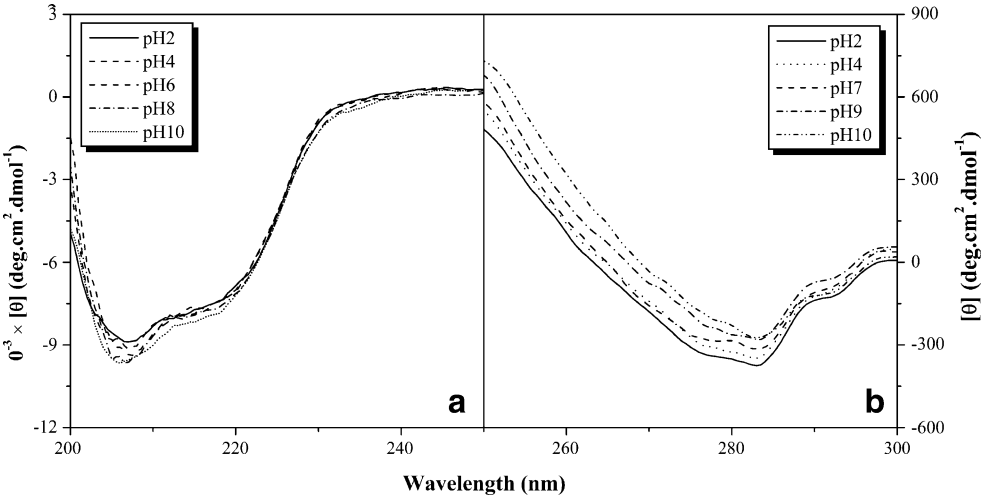

Fig 3 Effect of pH on the CD spectrum of TDSL. **a** Far UV CD spectrum. **b** Near UV CD spectrum. The pH at which each spectrum was recorded is indicated in the figure. See text for details

non-two-state, two-peak model. From the data presented in Table 2 it is seen that at pH 2 the T_m is 326.5 K, which increases with pH up to pH 5. The T_m values indicate that the thermal stability of TDSL is highest around pH 4–5. Ratio of $\Delta H_c/\Delta H_v$ increases along with pH. Between pH 7 and 10 both T_{m1} and T_{m2} remain nearly constant, suggesting that the lectin stability is comparable in this pH range.

Chemical denaturation

Denaturation of TDSL with Gdn.HCl was monitored by following changes in the intrinsic fluorescence characteristics of the protein when the concentration of the denaturant was varied. Tryptophan fluorescence emission λ_{max} of native TDSL is seen at 328 nm (Fig. 6a), which is in agreement with previously published results and suggests that the tryptophan residues are well buried in the interior of the protein [19, 29]. The emission maximum increases with increase in the concentration of the denaturant up to 2.0 M, with a clear plateau being seen between 2.0 and 2.5 M Gdn. HCl, where the λ_{max} remains constant at 340.5 nm (Fig. 6b). As the concentration of the denaturant is increased further the emission λ_{max} again increases gradually and reaches a maximum of 349 nm at 4.5 M Gdn.HCl and remains constant thereafter. The emission λ_{max} of 349 nm indicates complete exposure of the Trp residues to the aqueous medium (solvent), suggesting complete unfolding of the protein. The unfolding of TDSL is also associated with a quenching of the fluorescence intensity (Fig. 6a). A maximum of 48.2% quenching was observed around 5 M Gdn.HCl. The observation of a plateau in the emission λ_{max}, as alluded to above, is consistent with the presence of a stable intermediate structure in the unfolding process at 2.0–2.5 M of the denaturant. At higher concentrations of Gdn.HCl, a shoulder is observed in the fluorescence spectrum near 307 nm, which may be attributed to the tyrosine residues (Fig. 6a). This shoulder is not due to the solvent Raman band as the spectra were corrected by subtracting buffer blanks recorded under identical conditions. Since resonance energy transfer between Tyr and Trp residues is not expected to occur in the denatured protein this observation is consistent with complete unfolding of TDSL [30].

From the fluorescence emission spectra the fraction unfolded (f_U) was calculated according to the expression:

$$f_U = (F_F - F_o)/(F_F - F_U) \quad (3)$$

where F_F is the fluorescence intensity of the fully folded protein (*i.e.*, in the absence of denaturant), F_o is the fluorescence intensity at any given concentration of the denaturant and F_U is the fluorescence intensity of the fully unfolded protein. A plot of f_U as a function of the denaturant concentration describes the denaturation profile

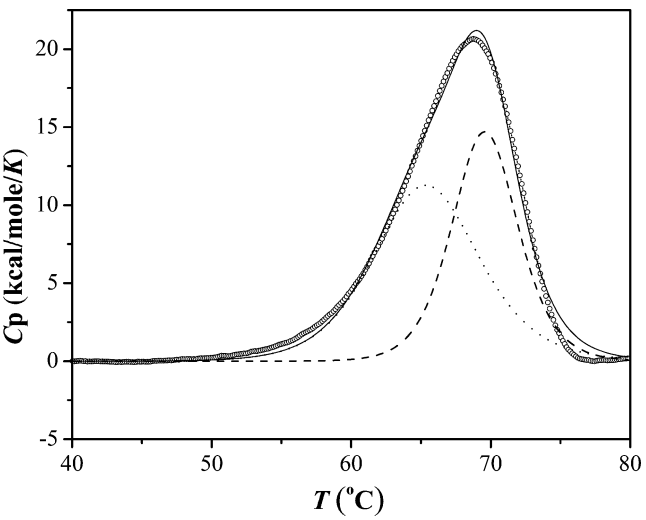

Fig 4 DSC scan of *Trichosanthes dioica* seed lectin in 20 mM phosphate buffer containing 150 mM NaCl at pH 7.4. The scan rate was 30 K h^{-1}. The data points are shown as *open circles*, and the *solid lines* are the best fits of the DSC data to the non two state transition model. Concentration of *T. dioica* lectin used was 0.0163 mM

Table 1 Thermodynamic parameters from DSC measurements on the thermal transition of TDSL in presence of ligands

Ligand	T_{m1} (K)	ΔH_{c1} (kJ mol^{-1})	ΔH_{v1} (kJ mol^{-1})	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} (kJ mol^{-1})	ΔH_{v2} (kJ mol^{-1})	$\Delta H_{c2}/\Delta H_{v2}$
–	338.42	474	378	1.25	342.6	385	618	0.62
MeβGal	343.35	577	456	1.26	347	373.5	727	0.51
MeαGal	341.9	527	418	1.26	345.9	420	664	0.63
GalNAc	342.6	568	426	1.3	346.4	439	651	0.67
Lactulose	344.8	392	576	0.68	347.9	209	900	0.23
Lactose								
10 mM	340.8	564	481	1.17	344.7	457	687	0.66
20 mM	341	683	428	1.59	345.2	574	675	0.84
30 mM	342.5	713	463	1.5	346.2	522	717	0.72
50 mM	344.28	614	560	1.1	347.6	351	836	0.42
100 mM	344.9	518	493	1.05	348.4	296	819	0.36

DSC scan rate was 30 K h^{-1}. Concentration of TDSL used is 0.0163 mM per dimer. Ligand concentration used for ligands except lactose is 0.1 M. The errors were less than 0.02% for T_m, less than 10% for ΔH_c and ΔH_v, respectively

of the protein. A denaturation curve obtained for the unfolding of TDSL is shown in Fig. 6c. From the curve it is seen that unfolding follows a three-state mechanism with an intermediate. Gdn.HCl induced denaturation of TDSL is qualitatively consistent with DSC data, presented above.

Discussion

In previous studies we reported the isolation and characterization of the *T. dioica* seed lectin with respect to macromolecular properties, saccharide specificity, and the amino acid side chains involved in its carbohydrate binding activity [18]. Additionally, we have also investigated the exposure and accessibility of the tryptophan residues of this protein [19]. In the present study this lectin has been further characterized with respect to its activity at different pH as well as incubation at different temperatures. The thermal, chemical, and acid-induced unfolding of TDSL has been investigated by differential scanning calorimetry, fluorescence spectroscopy and CD spectroscopy. The results obtained are discussed here.

Thermal stability and pH-dependence of TDSL

The hemagglutination activity of TDSL is unchanged in the pH range 7.0–11.0. At lower pH the activity decreases steeply. This is somewhat similar to the activity of other Cucurbitaceae seed lectins. MCL and TCSL exhibits maximum activity in the pH range from 8.0–11.0 [14, 15], whereas another cucurbit seed lectin, SGSL exhibits maximum activity in the pH range of 6.0–10.0 [17]. The data presented in Fig. 1b clearly shows that the hemagglutination activity of TDSL was unaffected by increase in the temperature up to 50°C, with close to 20% decrease at 60°C, whereas incubation at 70°C resulted in a total loss of the activity. In comparison, the activity of TCSL was unaffected up to about 60°C, and decreased to 50% at 70°C, indicating that it is thermally more stable than TDSL. However, TDSL appears to be more stable than MCL and SGSL, which were inactivated by incubation above 50°C and 40°C, respectively [14, 15, 17].

It is interesting to note that while the DSC and CD studies indicate that TDSL undergoes thermal unfolding in the temperature range of 65–70°C, the activity studies on samples incubated at different temperatures show that the lectin activity starts to decrease from 60°C onwards. This

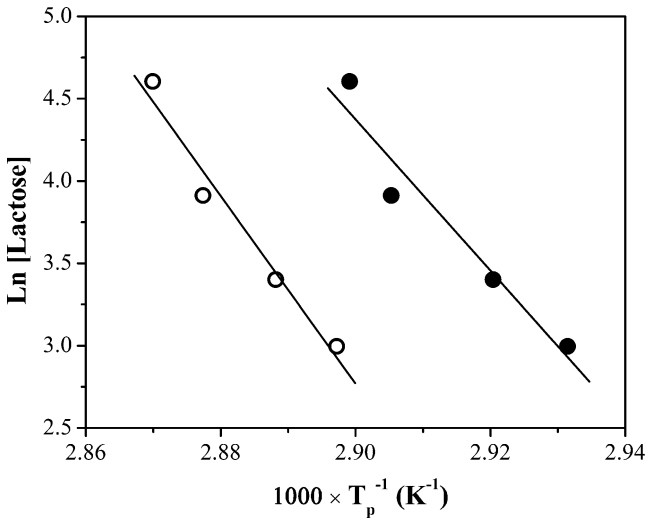

Fig 5 Plots of ln[Lactose] *versus* $1/T_p$ for TDSL. The *lines* are best linear least-square fits of ln [Lactose] against $1/T_p$, for the lower temperature transition peak (*closed circle*) and higher temperature transition peak (*open circle*). Scan rate was 30 K h^{-1}. The correlation coefficients are 0.96 and 0.98 for the lower-temperature and higher-temperature transition peaks, respectively

Table 2 Thermodynamic quantities from DSC measurements on the thermal transition of *Trichosanthes dioica* seed lectin as a function of pH

pH	T_{m1} (K)	ΔH_{c1} (kJ mol^{-1})	ΔH_{v1} (kJ mol^{-1})	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} (kJ mol^{-1})	ΔH_{v2} (kJ mol^{-1})	$\Delta H_{c2}/\Delta H_{v2}$
2	326.5	461	444	1.04	330	348	706	0.49
3	333.66	465	420	1.1	337	402	669	0.6
4	340	647	638	1.01	343	501	973	0.51
5	340.5	526	555	0.947	344	310	1049	0.3
6	339.6	785	547	1.4	343	668	869	0.76
7	338.6	610	379	1.6	342.8	568	601	0.9
8	338.4	756	434	1.74	343.15	501	693	0.72
9	338.8	598	412	1.45	343	447	664	0.67
10	338.6	706	361	1.95	343	606	610	0.99

T_{m1}, ΔH_{c1}, ΔH_{v1} are the thermodynamic parameters corresponding to lower-temperature transition, where as T_{m2}, ΔH_{c2}, ΔH_{v2} corresponds to higher temperature transition. Concentration of TDSL used was 0.0163 mM in dimer. DSC scan rate was 30 K h^{-1}. The errors were less than 0.05% for T_m and less than 10% for ΔH_c and ΔH_v respectively

apparent discrepancy is due to the fact that for carrying out the thermal inactivation studies the lectin samples have been incubated for 30 min at different temperatures before cooling to room temperature followed by the hemagglutination assay. It is well known that rate of thermal inactivation of proteins increases with increase in temperature and incubation for 30 min at 60°C led to a partial inactivation (ca. 19%) of TDSL. Longer incubation periods would be required to decrease the activity at lower temperatures.

DSC studies indicate that the pH at which TDSL is most stable is ~5.0 whereas the highest activity is seen at pH 7.0–11.0. This again may appear contradictory. However, it must be noted that for the lectin to exhibit activity the amino acid residues that are directly involved in its interaction with the ligand should be in the correct charge state. Previous chemical modification studies have implicated the side chains of tyrosine residues in the hemagglutination activity of TDSL while modification of lysine, histidine, cysteine and tryptophan residues did not affect the activity [18]. Since tyrosine side chains do not undergo any protonation/deprotonation processes between pH 5.0 and 7.0, it is unlikely that the decrease in the activity is due to the change of charge on tyrosine residues. On the other hand, it is possible that carboxy side chains, which undergo protonation below pH 7.0 may be important for the activity of TDSL. This appears to be the most likely reason, because the lectin activity of TDSL at pH 4.0 was about 50% of the maximal activity (see Fig. 1a), and the side chain carboxy groups of aspartic and glutamic acids titrate around this pH. This is consistent with the results of detailed three-dimensional structures of a number of plant lectin–monosaccharide complexes and site-directed mutagenesis studies, which have shown that in most cases an aspartic acid, an asparagine and an aromatic amino acid participate

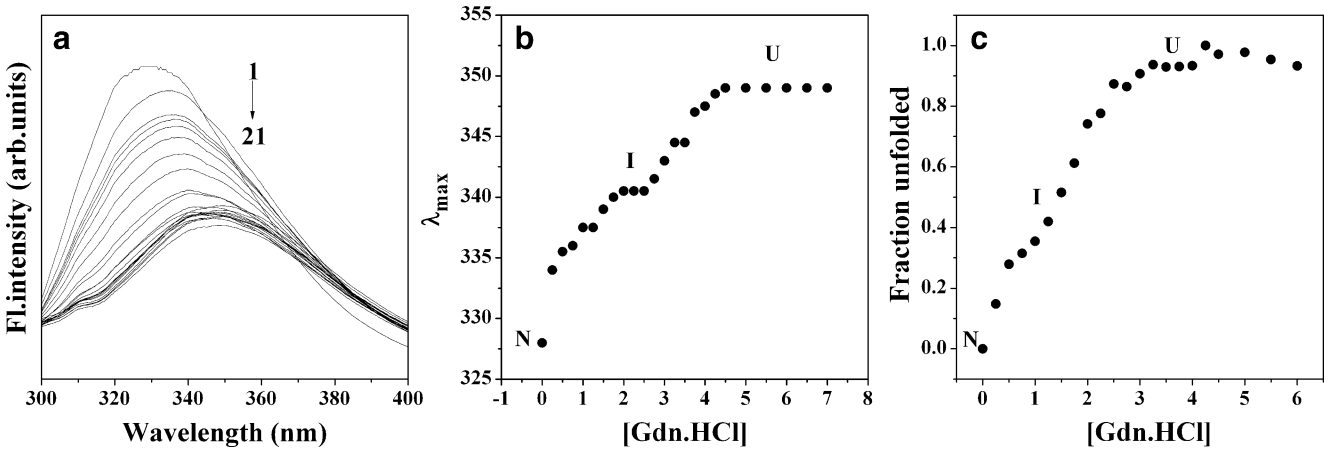

Fig 6 Equilibrium unfolding of TDSL in the presence of Gdn.HCl. **a** Fluorescence spectra of TDSL in presence of increasing concentrations of Gdn.HCl. Spectrum *1* corresponds to native lectin, *2–21* correspond to spectra in the presence of increasing concentrations of Gdn.HCl. **b** Plot of emission λ_{max} as a function of Gdn.HCl concentration. **c** Unfolding curve for Gdn.HCl induced denaturation of TDSL. *N*, *I*, and *U* denote native, intermediate and unfolded states of the protein. The fraction unfolded was calculated from fluorescence spectra recorded at pH 7.2. See text for details

in direct interaction with the ligand [1]. However, further studies are required before this possibility can be confirmed.

Differential scanning calorimetry

The DSC scan of TDSL shows two peaks suggesting that the lectin contains two entities melting at different temperatures. This is similar to the ribosome inactivating protein abrin II and winged bean basic lectin which also unfold as two entities [11, 31]. $\Delta H_c/\Delta H_v$ ratio for the lower-temperature transition is ~1.0 at lower pH (Table 1) and as pH increases the ratio also increases and becomes close to 2.0 at pH 10.0. $\Delta H_c/\Delta H_v$ ratios greater than one may indicate the presence of an intermediate state. Gdn.HCl denaturation studies on TDSL by fluorescence spectroscopy also show that unfolding of TDSL is a biphasic process. Peanut agglutinin also shows biphasic denaturation [9]. The ratio of 1 shows unfolding of lower-temperature transition is more cooperative, indicating that two domains interact more strongly with each other at lower pH. $\Delta H_c/\Delta H_v$ ratio is greater than 1 for unfolding of ConA tetramer, indicating the presence of an intermediate state [12]. Binding of carbohydrate ligands to TDSL increase the thermal stability of the protein as indicated by increased T_m values for both transitions, suggesting that ligand binding occurs preferentially to the folded state of the lectin. Shifts of T_m in presence of lactose and lactulose are higher when compared to MeβGal and GalNAc, suggesting that the second sugar moiety of these β-linked disaccharides may be involved in some additional interaction with the lectin combining site, which is similar to another cucurbit seed lectin, MCL [14]. Shift of T_m is higher for MeβGal when compared to MeαGal, which is in agreement with the preference of the lectin for the β-anomeric derivatives of galactose.

The binding constants determined at denaturation temperature with different carbohydrate ligands show that the disaccharides, lactose and lactulose bind with higher affinity than the monosaccharides. Further, the preference of TDSL for β-linked sugars is reflected in the higher affinity exhibited by the lectin for MeβGal compared to MeαGal. These results are in agreement with the relative inhibitory potencies determined by hemagglutination-inhibition assays [18]. Another cucurbit seed lectin, MCL exhibits a similar trend in its recognition of various mono- and disaccharides [14]. The binding of two lactose molecules to one molecule of TDSL dimer was concluded from the plot of ln [lactose] against $1/T_p$ for both lower and higher transitions. Ratio of $\Delta H_c/\Delta H_v$ remains unaltered in the presence of ligand, suggesting that the mechanism of unfolding is not influenced by ligand binding. DSC studies at different pH suggest that TDSL is more stable at pH 4 and 5, and that the secondary and tertiary structures of the protein do not change when the pH is varied, which shows that TDSL is stable over a wide range of pH. Gdn.HCl induced denaturation of TDSL, monitored by fluorescence spectroscopy shows that unfolding of TDSL is also a three-state process with an intermediate.

In summary, the results of the studies reported here indicate that TDSL is maximally active in the pH range 7.0–10.0 and is stable up to 60°C. Thermal unfolding of TDSL is an irreversible, three-state process. Chemical denaturation also follows a three-state unfolding mechanism. TDSL is more stable at lower pH. Binding of carbohydrate ligands stabilizes the native conformation of the protein and increases the denaturation temperature of TDSL. Binding constants calculated for different carbohydrate ligands at denaturation temperature indicate that TDSL preferentially binds to β-linked galactopyranosides, with the disaccharides possibly exhibiting additional interactions with the lectin.

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