CHAPTER : 5

DENATURATION AND UNFOLDING
OF *FUSARIUM* LECTIN
SUMMARY

The conformational stability of *Fusarium* lectin was determined with chemical, thermal and pH denaturants. Equilibrium unfolding with guanidine thiocyanate showed that the values of $D_{1/2}$ (0.49 M) and $\Delta G^{H_2O}$ (5.24 kJ/mol) were lowest at pH 12. The maximum conformation stability of *Fusarium* lectin was observed at pH 8.0 near its pI. At pH 12, the $\Delta G^{H_2O}$ and $D_{1/2}$ values of urea induced unfolding was higher than that of guanidine thiocyanate. Curves of fraction unfolded ($f_U$) obtained with fluorescence and CD measurements overlapped at all the pH studied. Moreover, the denaturation of the lectin was irreversible at higher denaturant concentration. At acidic pH, *Fusarium* lectin showed a flexible tertiary structure with pronounced secondary structure and retention of its hemagglutinating activity. Thermal denaturation did not result in aggregation or precipitation of the protein even at high temperatures. ANS binding studies revealed no exposure of hydrophobic patches.

INTRODUCTION

Specific biological functions of proteins emerge directly from their unique and highly individualistic three-dimensional structure, attained in a very short time after their synthesis. The three dimensional structure of a protein is held together by non-covalent interactions viz. hydrogen bonds, ionic interactions, hydrophobic interactions, van der Waals forces and covalently by disulfide linkages. Conditions which disturb these stabilizing forces affect the native conformation of the protein by changing a majority of its physical properties apart from its biological activity. The extent and balance between different stabilizing forces, studied under various denaturing conditions, is interpreted in terms of conformational stability. Determination of the conformational stability of a protein is critical for understanding the physical interactions that stabilize the protein. Determination of the stability of a protein is generally based on the analysis of denaturant or thermally induced unfolding transition measured either spectroscopically or calorimetrically (1-5).
The folding pathway of a protein, sometimes, involves intermediate states and hence it is important to study such partially folded conformations, to understand the principles governing protein folding/unfolding \((6,7)\). Such stable intermediates have been identified and characterized for several proteins using modern sensitive techniques \textit{viz}. spectroscopy and NMR \((8)\).

The recognition of carbohydrate moieties by lectins, has important applications in a number of biological processes such as cell-cell interaction, signal transduction, cell growth and differentiation \((9)\). The functionality of lectin molecules depends on the specific carbohydrate recognition domain, a part of the three dimensional structure of the protein. A perturbed lectin structure does not allow it to interact with the carbohydrate ligands. Thermodynamic parameters such as \(\Delta G\), \(\Delta H\) and \(\Delta S\), allow to understand the capability of different forces, holding the three dimensional structure of protein.

This Chapter describes studies on the conformational stability of \textit{Fusarium} lectin through chemical, thermal and pH induced denaturation.

**MATERIALS**

Urea, guanidine thiocyanate and 8-anilino-1-naphthalene sulfonate (Sigma Chemical Co., St. Louis, MO, USA) were used. All other chemicals used were of analytical grade.

**METHODS**

**Protein determination**
Protein concentrations were determined according to Bradford \textit{et al.} \((10)\) using BSA as standard.

**Purification of \textit{Fusarium} lectin**
Cultivation of \textit{Fusarium} sp. (LR11) and purification of lectin was carried out as described in Chapter 2.
Buffers and solutions
The buffers used at different pH: Glycine-HCl (pH 2-3), sodium acetate (4-5), sodium citrate-phosphate (5), sodium phosphate (6-7), Tris-HCl (8-9) and glycine-NaOH (10-12). The urea and Gdn-HSCN stock solutions were prepared fresh on the day of use as described by Pace et al. (11).

Equilibrium unfolding studies
Equilibrium unfolding as a function of urea, Gdn-HSCN (guanidine thiocyanate), pH and temperature was monitored by fluorescence. For chemical denaturation, the protein (25 µg/ml) was incubated in presence of various concentrations of the denaturant in 50 mM buffers of various pH, at 25 °C for 24 h. In the thermal unfolding experiments, the spectra were recorded 15 min after the desired temperature was attained. The intrinsic tryptophan fluorescence spectra of the protein were recorded on a Perkin Elmer LS-50B spectrofluorimeter, equipped with Julabo F25 water bath. The protein was excited at 280 nm using a cell of 1.0 cm pathlength and both excitation and emission slit widths were set at 7 nm. CD spectra were recorded on a computer interfaced JASCO J-715 spectropolarimeter using a cylindrical quartz cell of 1 mm.

Refolding of Fusarium lectin
Two hundred microliters aliquot was taken out from the samples treated with different concentrations of Gdn-HSCN, diluted 10 times with 50 mM buffer of the same pH at which the unfolding was done. After 4 h, the fluorescence spectra and hemagglutinating activity of the original (treated with Gdn-HSCN) as well as diluted samples were recorded. Protein samples without Gdn-HSCN treatment under identical conditions were taken as control.

The renaturation of thermally denatured protein was followed by cooling the heated samples to 20 °C, removing any particulate matter by centrifugation, and then monitoring the fluorescence spectra and the activity.
Hydrophobic dye binding studies
A stock solution of ANS was prepared in methanol and the concentration was measured using an extinction coefficient of 5000 M$^{-1}$·cm$^{-1}$ at 350 nm (12). ANS emission spectra were recorded in the range of 400-550 nm with excitation at 375 nm using slit widths of 5 nm for emission and excitation monochromators. Changes in the ANS fluorescence, induced by the binding to the lectin, were recorded at constant concentration of protein (2 µM) and ANS (50 µM). The spectrum of ANS alone in buffer was subtracted from the protein-ANS spectrum to yield the final spectrum.

Light scattering studies
Rayleigh light scattering experiments were carried out with the spectrofluorimeter to follow protein aggregation at different pH with increasing temperature. Both excitation and emission wavelength were set at 400 nm and the time dependent changes in scattering intensity were followed.

Gel permeation-HPLC
Lectin samples (100µg in 100µl), pre-incubated for 24h at different pH (at 25ºC) were injected onto a Protein-PAK SW300 HPLC gel permeation column (300 x 7.8 mm) connected to a Waters HPLC system, at a flow rate of 0.5 ml/ min. The standard molecular mass markers run in the buffer were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

Data analysis
The denaturation curves were plotted, with the fluorescence intensities at the emission $\lambda_{\text{max}}$ of native protein and denatured protein, against denaturant concentration and temperature, and further analysis of the data was performed as described by Pace et al. (11). From the denaturation curves, a two state $F \rightleftharpoons U$ unfolding mechanism was assumed, and consequently, for any of the points, only the folded and unfolded conformations were present at significant
concentrations. Thus, if \( f_F \) and \( f_U \) represent the fraction of protein present in the folded and unfolded conformations, respectively then \( f_F + f_U = 1 \).

\( f_U \) was calculated using the following equation

\[
f_U = \frac{(F_F - F_0)}{(F_F - F_U)}
\]

where, \( F_F \) is the fluorescence intensity of completely folded or native protein, \( F_0 \) is the observed fluorescence intensity at point of denaturant concentration or temperature, \( F_U \) is fluorescence intensity of the completely denatured or unfolded protein.

For a two state F\( \rightleftharpoons \)U unfolding mechanism, the equilibrium constant \( K \) and \( \Delta G_U \), the free energy of unfolding was calculated using Equation 2 and 3 respectively.

\[
K = \frac{f_U}{(1-f_U)} \quad (2)
\]

\[
\Delta G_U = -RT \ln K \quad (3)
\]

where, \( R \) is the gas constant, and \( T \) is the absolute temperature. It is assumed that the free energy of unfolding, \( \Delta G_U \), has a linear dependence on the concentration of the denaturant \([D]\).

\[
\Delta G_U = \Delta G^{H_2O} + m[D] \quad (4)
\]

\( \Delta G^{H_2O} \) and \( m \) are therefore the intercept and the slope respectively, of the plot of \( \Delta G_U \) versus \([D]\). \( \Delta G^{H_2O} \) corresponds to the free energy difference between the folded and unfolded states in the absence of any denaturant and \( m \) is a measure of the cooperativity of the unfolding reaction. The concentration of denaturant at which the protein is half unfolded (when \( \Delta G_U = 0 \)) is given by \( D_{1/2} \) and from Equation 4, \( \Delta G^{H_2O} = -m D_{1/2} \).

The data from the thermal unfolding curves were obtained under the same conditions as those for denaturant unfolding curves. Values of \( f_U \), \( K \) and \( \Delta G_U \) were calculated using Equations 1, 2 and 3. The midpoint of thermal (\( T_m \)) denaturation was obtained as the temperature at which \( \Delta G_U = 0 \) from the plot of \( \Delta G_U \) versus \( T \). The slope of such a plot at \( T_m \) yielded \( \Delta S_m \), the change in entropy. The enthalpy change for unfolding at \( T_m \), \( \Delta H_m \), was calculated using the equation,
\[ \Delta H_m = T_m \Delta S_m \]  

\( \Delta C_p \), the change in heat capacity that accompanies protein unfolding was obtained from the slope of the plot of \( \Delta H_m \) versus \( T_m \), where \( T_m \) was varied as a function of Gdn-HSCN concentration. \( \Delta G_U \) at 25°C was calculated using equation,

\[ \Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln(T/T_m)] \]
RESULTS AND DISCUSSION

A protein molecule has a biological activity conferred by its three dimensional structure. Perturbation of any of the forces holding the protein three dimensional structure results in loss of its structure and biological functionality. Such perturbants provide a way to estimate conformation stability of a protein in comparison with others. Denaturation of *Fusarium* lectin was followed by monitoring the changes in physical properties of the protein *viz.* intrinsic fluorescence and circular dichroism. Tryptophan fluorescence has been used to study protein denaturation for several proteins (13-15), which is greatly influenced by the environmental factors such as polarity, pH, temperature and composition of solvent (16).

**Chemical denaturation**

Denaturation of *Fusarium* lectin with urea and Gdn-HSCN at various pH was monitored by following the changes in intrinsic fluorescence of the protein on excitation at 280 nm. Considerable change in fluorescence spectra, in presence of urea, was observed only at pH 12, whereas it was observed at all the pH studied, with Gdn-HSCN. At higher concentration of Gdn-HSCN, the fluorescence quenching was upto 80 %, whereas it was 32 % in case of urea (Fig. 5.1 and 5.2). In the presence of denaturant, a decrease in fluorescence intensity (Fig. 5.1A) accompanied with red shift in $\lambda_{\text{max}}$ towards 356 nm (Fig. 5.1B) was observed, a characteristic for the fluorescence spectra of Trp residues in an aqueous environment, suggesting unfolded state of the lectin. This conclusion is also supported with the appearance Tyr peak at 307 nm. It has been suggested that under denaturing conditions the energy transfer from Tyr to Trp residues does not occur any more (17). The decrease in fluorescence intensity may be due to the denaturation of protein and can be explained on the basis of increase in the polarity of tryptophan environment, as it becomes fully exposed to the solvent. It has been suggested that the excited singlet states of tryptophan interact with water molecules to form...
excited state complexes and such a process competes with the radiative relaxation and leads to diminution of the fluorescence intensity (18-21).

**Fig. 5.1**: Equilibrium unfolding of *Fusarium* lectin in the presence of Gdn-HSCN at pH 8.0. (A) Fluorescence spectra (B) Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 348 and 356 nm as a function of Gdn-HSCN concentration. The spectra were recorded after 24 h incubation of the lectin in different concentration of Gdn-HSCN at 25 °C.
**Fig. 5.2 A:** Fluorescence spectra of *Fusarium* lectin in the presence of varying concentration of urea at pH 12

**Fig. 5.2 B:** $f_U$ curves for urea induced denaturation. The fraction unfolded ($f_U$) from fluorescence were calculated using Eq. 1 and plotted as a function of urea concentration at pH 12.0
The denaturation of *Fusarium* lectin was also monitored by following the changes in the far-UV CD spectra (Fig. 5.3). The denaturation curves were prepared by plotting $\theta_{220}$ as a function of denaturant concentration and the $f_U$ was calculated using Equation 1. The $f_U$ plots obtained from CD data were in agreement with those obtained by fluorescence spectroscopy (Fig. 5.4). The majority of the proteins exhibit a single step denaturation curve and so are assumed to follow a two-state N$\rightleftharpoons$D mechanism in which only the native state and denatured states are present at significant concentrations in the transition region (11,22,23). Superimposition of $f_U$ curves, obtained by fluorescence and CD measurements for Gdn-HSCN induced denaturation, supports this assumption (Fig. 5.4).

![Fig. 5.3: Far-UV CD spectra of *Fusarium* lectin in the presence of Gdn-HSCN at pH 8.0. Concentration of denaturant is indicated in figure.](image-url)
Chapter 5: Denaturation

Fig. 5.4: $f_U$ curves for Gdn-HSCN induced denaturation. The fraction unfolded ($f_U$) from fluorescence (■) and far-UV CD (○) were calculated using Eq. 1 and plotted as a function of Gdn-HSCN concentration at pH 8.0.

The data obtained from fluorescence spectra was used to prepare the denaturation curves, which were then analyzed to obtain $\Delta G_U$, using Equations 1, 2 and 3. The $\Delta G_U$ was plotted as a function of denaturant concentration to obtain $D_{1/2}$ and $\Delta G_{H_2O}$(Fig. 5.5). The change in free energy ($\Delta G$), associated with *Fusarium* lectin unfolding, varied linearly ($r > 0.99$) with the increase in urea and Gdn-HSCN concentrations. The values of $D_{1/2}$ (3.88 M), $\Delta G_{H_2O}$ (9.38 KJ/mol) and $-m$ (2.4 kJ/mol/deg) were obtained for urea denaturation at pH 12 (Table 5.1). The denaturation was observed at lower concentrations of Gdn-HSCN at pH 12 than at pH 6, 8 and 10 and the value of $\Delta G_{H_2O}$ (5.24 kJ/mol) and $D_{1/2}$ (0.49 M) were lowest at pH 12 (Table 5.2). Moreover, the value obtained with Gdn-HSCN (0.49 M) at pH 12 was approximately eight times lower than that with urea. The maximum value of $\Delta G_{H_2O}$ (8.45 kJ/mol) with Gdn-HSCN was obtained at pH 8.0. In general, proteins are most stable at their isoelectric pH since the electrostatic interactions among the charged groups will tend to favor the maximum conformational stability.
occurring near the pI (24). Similar observations also have been made in some other proteins in presence of chaotropes (25,26).

![Graph](image)

**Fig. 5.5**: $\Delta G_U$ as a function of Gdn-HSCN concentration at pH 8.0. $\Delta G_U$ was calculated using Equation 3, intercept and slope of the graph equals to $\Delta G^{H2O}$ and $m$, respectively.

**Table 5.1**: Parameters for urea denaturation of *Fusarium* lectin$^a$

<table>
<thead>
<tr>
<th>pH*</th>
<th>$\Delta G^{H2O}$ (kJ/mol)</th>
<th>$-m$ (kJ/mol/deg)</th>
<th>$D_{1/2}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>9.38</td>
<td>2.4</td>
<td>3.88</td>
</tr>
</tbody>
</table>

$^a$ The values represent the mean of three independent sets of experiments with SD of less than 10%.

At pH 6, 8 and 10 no change in fluorescence spectrum was observed.

$D_{1/2}$, $m$, and $\Delta G^{H2O}$ values were obtained with linear extrapolation method from plots of $\Delta G_U$ as a function of urea concentration using Eq. (4).
Table 5.2: Parameters for guanidine thiocynate denaturation of *Fusarium* lectin$^a$.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\Delta G_{H_2O}^{U}$ (kJ/mol)</th>
<th>$-m$ (kJ/mol/deg)</th>
<th>$D_{1/2}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>7.46</td>
<td>7.86</td>
<td>0.95</td>
</tr>
<tr>
<td>8</td>
<td>8.45</td>
<td>8.05</td>
<td>1.05</td>
</tr>
<tr>
<td>10</td>
<td>7.55</td>
<td>7.63</td>
<td>0.99</td>
</tr>
<tr>
<td>12</td>
<td>5.24</td>
<td>10.7</td>
<td>0.49</td>
</tr>
</tbody>
</table>

$^a$ The values represent the mean of three independent sets of experiments with SD of less than 10%.

$D_{1/2}$, $m$, and $\Delta G_{H_2O}^{U}$ values were obtained with linear extrapolation method from plots of $\Delta G_{U}$ as a function of urea concentration using Eq. (4).

Influence of pH revealed that the lectin showed minimum stability at pH 12. Higher pH causes ionization of tyrosine and histidine residues, which are generally packed in the hydrophobic interior of the protein. At high pH, therefore, ionization of these groups make the protein more susceptible to urea and Gdn-HSCN induced denaturation. The *Fusarium* lectin showed marginally lower value of $\Delta G_{H_2O}^{U}$ (6-9 kJ/mol) as compared to other proteins. The conformational stability of almost all naturally occurring globular proteins has been observed to be in the range of 20-60 kJ/mol (24). In addition, no ANS binding and protein aggregation was observed in chemical denaturation and the unfolded lectin did not exhibit hemagglutinating activity. Refolding was measured as the extent of reappearance of the original spectra and recovery of the hemagglutinating activity. However, no considerable refolding was observed in denatured *Fusarium* lectin.
**pH dependent denaturation**

Tryptophan fluorescence, which is influenced by environmental factors like polarity, composition, pH and temperature of the solvent, has been used to study protein denaturation of several proteins (13-16). Influence of pH on denaturation of *Fusarium* lectin showed quenching at lower pH without any shift in the $\lambda_{\text{max}}$ (Fig. 5.6). The $\lambda_{\text{max}}$ at 348 nm, indicated relatively exposed tryptophan residues since completely exposed tryptophan has a $\lambda_{\text{max}}$ between 355-360 nm, characteristic of totally unfolded protein (18,27-29). Thus the acid induced unfolding of *Fusarium* lectin does not cause Trp to come in direct contact with water. However, the Trp fluorescence and its dependence on pH agrees well with the results of fluorescence studies performed with some other proteins and model substances (27,30). Emission intensity observed at 348 nm remained constant over pH 6-10 while it gradually decreased below pH 5. The maximum decrease in fluorescence emission intensity was 95 % at pH 2.

![Fluorescence spectra of *Fusarium* lectin at different pH](image)

**Fig. 5.6 : Fluorescence spectra of *Fusarium* lectin at different pH.** The lectin was incubated at different pH (as denoted by numbers) for 24 h, at 25 °C.
In most of the proteins the Trp fluorescence dominates the Tyr fluorescence even in the proteins containing more Tyr than Trp. This is caused by relatively high absorbance of Trp residues and relatively low fluorescence efficiency of most Tyr residues, and often by an efficient transfer of excitation energy from Tyr to Trp (27,30). Furthermore, it has also been shown that the observed reduction of fluorescence at low and high pH values cannot be explained simply in terms of partial denaturation of the protein in acid or alkaline environment. The reduction of fluorescence intensity at low pH values can be caused by quenching of fluorescence by hydrogen ion (acid quenching) and neutralization of COO\(^-\) groups on acidic amino acid in vicinity of fluorophores (30).

The far-UV CD spectra of the native and acid perturbed lectin showed that both the forms possess considerable secondary structure (Fig. 5.7). The molar ellipticities at 220 nm were 7.46, 7.47 and 8.13 deg·cm\(^2\)·dmol\(^{-1}\) at pH 2, 4 and 6 respectively.

**Fig. 5.7 : pH dependent denaturation of *Fusarium* lectin.** (A) far-UV CD spectra. The symbols used are: (—) pH 2, (---) pH 6, (····) pH 8, and (·-·-·) pH 12. (B) near-UV CD spectra (pH denoted by numbers). The spectra were recorded at different pH after 24 h incubation at

The lectin retained approximately 92\% of its secondary structure between pH 2-4. On the other hand, the near-UV CD spectrum showed distinct positive peaks at 266 and 296 nm and a negative peak 283 nm for phenylalanine,
tryptophan and tyrosine, respectively. The molar ellipticity in the near-UV region at pH 2 was lower than that observed at pH 6, suggesting that the low pH provides a more flexible environment for the aromatic residues (31).

8-Anilino-1-naphthalene sulfonate (ANS) does not fluoresce in aqueous solution but does in organic solvents or when exposed to hydrophobic environment. ANS does not bind to native or completely unfolded polypeptide chain but it binds to intermediates formed during the unfolding. ANS bound to *Fusarium* lectin between pH 2-4 with an increase in the fluorescence intensity accompanied by blue shift in the emission maximum (Fig. 5.8). The fluorescence intensity of ANS was 6.8 and 3.8 times higher at pH 2 and 4, respectively from those between pH 6-10. *Fusarium* lectin showed considerable secondary structure between pH 2-4, with an exposure of large hydrophobic patches to the solvent.

![Fig. 5.8 : ANS binding to *Fusarium* lectin.](image)

Change in ANS fluorescence (50 µM) was recorded in the presence of *Fusarium* lectin (2 µM) at different pH (as denoted by numbers) on excitation at 375 nm.
Gel permeation of the *Fusarium* lectin showed single peak corresponding to molecular mass of 26 kDa at pH 6. However, at pH 3 and 4, no change in elution profile was observed, which excluded the possibility of monomerization or aggregation of the lectin (Fig. 5.9). In the hemagglutination assays, the lectin was found to have hemagglutinating activity in a broad range of pH varying from 2-12.

![Gel Permeation-HPLC of Fusarium lectin at different pH](image)

**Fig. 5.9 : Gel Permeation-HPLC of *Fusarium* lectin at different pH.** The lectin (100 µg) was incubated for 24 h at respective pH and 25 °C, injected on HPLC (Waters Corporation) gel permeation column (Protein-PAK SW300, 300×7.8 mm).

The molecular mass of acid denatured lectin suggested its presence in the dimeric form. At lower pH, it retained approximately 92 % and 70 % of its secondary and tertiary structure respectively. The ANS binding data suggested that it was highly solvent exposed. This type of structure of *Fusarium* lectin does not fit into the classical concept of molten globule as the
latter represents a compact native-like structure of the protein with pronounced secondary structure devoid of any rigid tertiary and quaternary structure. The acid perturbed state of *Fusarium* lectin is represented by its dimeric form with pronounced secondary structure and its hemagglutinating activity. Whereas, the molten globule state, reported for dimeric, peanut lectin (32) and galectin-1 (14), are represented by monomeric carbohydrate binding entities.

The pH induced unfolding of the proteins is achieved by protonation or deprotonation of relatively few discrete sites (-OH, -COOH and -NH$_2$) (33). Thus, unlike temperature and chaotropes, pH induced unfolding of the proteins is achieved by perturbation of few residues and a complete unfolding of the protein cannot be expected. In the present studies, the acid induced loss of tertiary structure of *Fusarium* lectin can be due to perturbation of the ionic and/or hydrogen bond interactions. However, this perturbation does not appear to affect the interactions involved in hemagglutination activity.

**Thermal denaturation in presence of low concentration of Gdn-HSCN**

Incubation of the *Fusarium* lectin at various temperatures up to 80 °C resulted in fluorescence quenching without any shift in $\lambda_{\text{max}}$ indicating that the lectin retains its conformational integrity even at high temperature. Moreover, the far-UV CD spectra at 80 °C also did not show any loss of ellipticity. Hence, thermal denaturation was carried out in the presence of low concentrations of Gdn-HSCN (0-0.4 M), which did not result in unfolding of the lectin at low temperatures. Thermal denaturation in the presence of Gdn-HSCN resulted in fluorescence quenching with a shift in emission $\lambda_{\text{max}}$ towards 356 nm (Fig 5.10) as well as loss of ellipticity in far-UV CD spectrum (Fig. 5.11). The fluorescence data was used to prepare the denaturation curves at varying concentrations of Gdn-HSCN as a function of temperature; and the data was analyzed using Equations 1-3 (Fig. 5.12). $\Delta G_U$ was plotted against T to obtain $T_m$ and $\Delta S_m$ (Fig. 5.13). The change in enthalpies ($\Delta H_m$), determined at $T_m$ for different Gdn-HSCN concentrations were plotted as a function of $T_m$ (Fig. 5.14). The slope of this plot yielded $\Delta C_p$ (3.95 kJ/mol/deg), which was then
used to calculate $\Delta G$ (at 25 °C) from the thermal denaturation curves at different concentrations of Gdn-HSCN (Table 5.3). The value of free energy, $\Delta G$, was approximately half of that required for chemical induced denaturation. The $T_m$, $\Delta H_m$ and $\Delta G$ of thermal unfolding showed a decrease with the increase in the Gdn-HSCN concentration.

Fig. 5.10: Thermal unfolding of *Fusarium* lectin in the presence of 0.1 M Gdn-HSCN at pH 8.0. (A) Fluorescence spectra (B) Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 348 and 356 nm as a function temperature. The spectra were recorded after 15 min incubation of the lectin at different temperature.
Fig. 5.11: Far-UV CD spectra of *Fusarium* lectin in the presence of 0.4 Gdn-HSCN at various temperature (as denoted in the figure).

Fig. 5.12: $f_U$ curves for thermal denaturation in the presence of 0.1 M Gdn-HSCN at pH 8.0. The fraction unfolded were calculated using Eq. 1 and plotted as a function of temperature.
**Fig. 5.13 :** $\Delta G_U$ as a function of temperature. $\Delta G_U$ was calculated using Equations (1)-(3) from the thermal denaturation curves obtained at pH 8 in the presence of Gdn-HSCN (■) 0.05 M, (●) 0.1 M, (▲) 0.2 M, (▼) 0.3 M and (○) 0.4 M.

**Fig. 5.14 :** Determination of $\Delta C_p$. Thermal denaturation experiments were performed in the presence of different concentrations of Gdn-HSCN at pH 8.0 to vary $T_m$. $T_m$, as the transition midpoint and $\Delta S_m$, as the slope were obtained from the plots of $\Delta G_U$ versus $T$. $\Delta H_m$ was determined at each value of $T_m$ using Equation (5).
Table 5.3 : Parameters for thermal denaturation of \textit{Fusarium} lectin in presence Gdn-HSCN at pH 8.0\textsuperscript{a}

<table>
<thead>
<tr>
<th>Gdn-HSCN [M]</th>
<th>$-\Delta S_m$ (J·mol\textsuperscript{-1}·K\textsuperscript{-1})</th>
<th>$T_m$ (°K)</th>
<th>$-\Delta H_m$ (kJ·mol\textsuperscript{-1})</th>
<th>$\Delta G$ (kJ·mol\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>335.2</td>
<td>330.2</td>
<td>108.6</td>
<td>4.17</td>
</tr>
<tr>
<td>0.1</td>
<td>297.3</td>
<td>327.3</td>
<td>97.3</td>
<td>3.35</td>
</tr>
<tr>
<td>0.2</td>
<td>285.7</td>
<td>326.4</td>
<td>93.2</td>
<td>3.07</td>
</tr>
<tr>
<td>0.3</td>
<td>252.1</td>
<td>323.7</td>
<td>81.6</td>
<td>2.33</td>
</tr>
<tr>
<td>0.4</td>
<td>240.1</td>
<td>321.5</td>
<td>77.4</td>
<td>2.10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The value represent the mean of three independent sets of experiments with SD of less than 10%.

$T_m$ was calculated from the plots of $\Delta G_U$ against $T$. $\Delta G$ (25 °C) was calculated using Equation no. (6).

$$
\Delta G (T) = \Delta H_m (1 - T/T_m) - \Delta C_p [(T_m - T) + T \ln (T/T_m)]
$$

$\Delta C_p$ value used for calculation of $\Delta G$ (25 °C) was obtained from the slope of the plot of $\Delta H_m$ versus $T_m$ varied as a function of guanidine thiocynate.

During thermal denaturation, many proteins do not refold, due to aggregation of the unfolded state (34,35). The aggregation usually involves unfolding of the whole protein or of a specific domain and it is often attributed to the association of partially unfolded molecules (36,37). Hence, aggregation stabilizes the unfolded oligomers by reducing the energetically unfavorable interactions between water molecules and exposed hydrophobic patches in the protein. However, in the present studies, static light scattering showed the absence of insoluble protein aggregates at higher temperatures and no ANS binding was observed.

The most important thermodynamic parameter is a large positive change in heat capacity of the two state $F\rightleftharpoons U$ transition in protein denaturation, and within the range of experimental error, it may be assumed to
be a constant for a given protein ($\approx 5 \text{ kJ}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$) (38). This has been suggested to result from the exposure of hydrophobic patches in the process of unfolding due to contact with water (24). Changes in enthalpy of melting ($\Delta H_m$) associated with thermal unfolding of the *Fusarium* lectin, varied linearly ($r= 0.990$) with melting temperature ($T_m$), resulting in constant value of heat capacity, $\Delta C_p$, of the unfolding at different concentrations of Gdn-HSCN. This suggests that the heat capacity of the products (unfolded protein) was same as the heat capacity of the reactants (native protein) (39) and the unfolding did not result in change in the heat capacity of the lectin.

In conclusion, the above studies show that *Fusarium* lectin follows a two-state $F \rightleftharpoons U$ unfolding mechanism during denaturation. The pH dependence of $\Delta G_{H_2O}^{\text{HSS}}$ suggested that the electrostatic interactions among the charged groups make a significant contribution to the conformational stability of the protein. At high pH, the lectin is more susceptible to chemical denaturants, while at low pH it shows flexibility in the tertiary structure with the retention of secondary structure and hemagglutinating activity.
References


