CHAPTER: 4

CONFORMATIONAL TRANSITIONS OF THE RECOMBINANT LECTIN
SUMMARY

Conformational transitions of the recombinant *Cicer arietinum* lectin were studied using fluorescence and circular dichroism spectroscopy. Thermal denaturation of rCAL caused rapid secondary structural rearrangements above 50 °C and transient exposure of hydrophobic residues at 55 °C, leading to aggregation. The denaturation was irreversible. GdnHCl-mediated unfolding of rCAL indicated a progressive unfolding of the protein chain with increasing concentration of the reagent. The unfolding involved several intermediates, also it was irreversible. Treatment of rCAL with GdnHCl resulted in unfolding followed by dissociation of the dimer. The protein structure was drastically affected within one hour in acidic as well as alkaline buffers.

4.1. INTRODUCTION

Specific biological functions of proteins emerge directly from their unique and highly individualistic three-dimensional structure, which is attained in a very short time after their synthesis. The three dimensional structure assumed by a protein can, in general, be considered to be thermodynamically the most stable conformation adopted by the polypeptide chain. This stable 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 its physical properties and biological activity. A polypeptide can also adopt a less rigid or more flexible conformation, different from its functional native form, in response to changes in its environment. Thus, proteins can be considered only marginally stable because of the functional requirement for their inherent dynamic state and due to delicate balancing of interactions involved in stabilizing or destabilizing particular structure [1-4].

Determination of the conformational stability of a protein is critical for understanding the physical interactions that stabilize the protein [5]. The folding pathway of proteins, 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 *viz.* spectroscopy and NMR (8). By recording changes in intrinsic tryptophan fluorescence and the secondary and tertiary structural features of protein in response to tailored changes in
surroundings, one can establish the presence of interesting structural intermediates relevant to structure-function relationship of the protein.

It has been known for many years that proteins can be unfolded in aqueous solution by high concentrations of certain reagents such as guanidine hydrochloride or urea or at higher temperatures. Denaturation with these chemicals is one of the primary ways of measuring the conformational stability of the proteins [9]. The roles and magnitudes of specific electrostatic interactions in determining the stability of a protein can be studied by measuring the dependence of the stability on pH. pH is known to influence the stability of a protein by altering the net charge of the protein. The aim of this study is to understand the conformational stability of rCAL as a function of temperature, pH and chemical denaturants using intrinsic fluorescence and circular dichroism.

The unfolding of oligomeric proteins requires the disruption of additional molecular interactions over those of monomeric proteins, since both inter- as well as intra-subunit interactions would make distinct and differential contributions to their overall structure and stability. In this regard, legume lectins serve as attractive models for studying the folding process of oligomeric proteins [10]. Several plant lectins have been characterized for their unfolding behaviour in presence of denaturing agents like temperature, urea or guanidine hydrochloride [see Table 4.1 for details].

**Table 4.1 Representative examples of plant lectins studied for unfolding**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name and source of lectin and its refs</th>
<th>Main observations</th>
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</thead>
</table>
          |                                       | • Protein undergoes cold denaturation that becomes distinct above 3.8 M urea.  
          |                                       | • Refolding upon thermal- and urea-induced unfolding is reversible. |
| 2.      | Pea lectin[12]                        | • Secondary structure resistant to conformational changes up to pH 2.5.  
          |                                       | • Addition of 80 % TFE retained the residual β-sheet structure, but with a loss in tertiary structure. |
• Transition from β-sheet structure to α-helical structure began in presence of 12 % HFIP, completed in 30 %.
• Occurrence of a common intermediate in the unfolding induced by these two fluoroalcohols (differing in their mode of stabilization of structure).
• TFE was found not to induce α-helical structure, but HFIP induced a structure rich in α-helical contacts

3. Pea lectin (PSL) [13]
- Dimeric PSL formed of two chains – a long β-chain and a short α-chain.
- GdnHCl-induced unfolding opens up the fragments to reveal a β-fragment as intermediate, with molten globule like characteristics.
- Larger fragment of PSL may behave as monomeric or single domain protein undergoing unfolding through intermediate(s).

4. Winged bean acidic lectin from *Psophocarpus tetrogonolobus* [14]
- Thermal denaturation leads to dissociation of dimer into its monomers at denaturation temperature.
- Glycosylation leads to less stable (lower denaturation temperature) structure compared to other legume lectins.
- Inter sub unit interface is less extensive compared to glycosylated lectins like Con A, pea and lentil lectins, hence less thermally stable.

5. Peanut agglutinin (tetrameric) [15]
- Thermal unfolding is reversible, with three states.
- Tetramers unfold to folded monomers, which then unfold into monomers (folded monomers
6. Peanut agglutinin [16]  
- GdnHCl-induced denaturation is completely reversible.  
- Biphasic profile, with a non-two-state unfolding process.  
- Intermediate formed during unfolding has reduced tertiary structure, hence is a molten globule and is more compact.  
- Intermediate retains its carbohydrate activity considerably.

7. Peanut agglutinin [17]  
- At pH 2.5, 15 % TFE induces a molten globule like structure.  
- Increasing TFE concentration leads to increase in α-helical content and compactness of protein.  
- Compact PNA at higher TFE concentration is structurally different than the native protein.  
- TFE at neutral pH does not induce molten globule like state.

8. Peanut agglutinin (PNA) [18]  
- Urea-induced denaturation of tetrameric PNA is three-state, involving molten globule as the intermediate.  
- Refolding involves rapid appearance of intermediate.  
- Tetramerization contributes significantly to stabilization of oligomers.

9. Leucoagglutinin from *Phaseolus vulgaris* [19]  
- Protein is dimeric at pH 2.5 and tetrameric at pH 7.2.  
- Thermal denaturation at neutral pH is irreversible.  
- Extremely thermostable protein, with transition temperature of around 82 °C and above 100 °C.
for pH 2.5 and pH 7.2 respectively.
- Protein remains in compact-folded state, even at pH 2.3, denaturation begins at 60 °C.

10. **Leucoagglutinin (PHA-L) from *Phaseolus vulgaris* [20]**
- Homotetrameric protein refolds at pH 2.5 with the formation of a dimeric intermediate.
- Denaturation kinetics at pH 2.5 followed a single exponential decay pattern; rate of denaturation independent of protein concentration.
- Renaturation kinetics was dependent on protein concentration.

11. **Artocarpus hirsuta lectin [21]**
- Unfolding partially reversible in presence of GdnHCl
- Protein dissociates reversibly into partially unfolded dimer and then irreversibly into unfolded inactive monomer in presence of GdnHCl.
- Thermal denaturation irreversible, lectin loses hemagglutinating activity rapidly above 45 °C.
- Insoluble aggregates during thermal denaturation leads to irreversible denaturation.

12. **Concanavalin A [22]**
- Urea or GdnHCl-induced denaturation exhibits three-state mechanism, involving a structured monomer between native tetrameric and unfolded monomeric states.
- Structural stability maintained by sub unit association.

13. **Concanavalin A [23]**
- Addition of 30 % PEG retains the secondary structure compared to that in 70 % PEG; tertiary structure also retained.
- Tryptophan environment changed in presence of PEG.
- Compact ‘molten globule’ formed in presence
<table>
<thead>
<tr>
<th>No.</th>
<th>Lectin Source</th>
<th>Description</th>
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<tbody>
<tr>
<td>14.</td>
<td>Lentil lectin [24]</td>
<td>- Structure lost at pH 0.8 and more so in presence of GdnHCl compared to native protein at pH 7.0.</td>
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<td></td>
<td></td>
<td>- Presence of intermediate at low pH.</td>
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<td></td>
<td></td>
<td>- Acid-unfolded structure stabilized by addition of fluoroalcohols TFE and HFIP by inducing $\alpha$-helical contacts.</td>
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<td>- This non-native structure (generated by TFE and HFIP) regained more activity than the native protein when treated with bromelain.</td>
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<tr>
<td>15.</td>
<td>Dolichos lablab seed lectin</td>
<td>- Thermally stable protein; unfolds at 75 °C.</td>
</tr>
<tr>
<td></td>
<td>[25]</td>
<td>- Ligand (methyl-$\beta$-D-galactose) binding leads to stabilization of secondary structure.</td>
</tr>
<tr>
<td>16.</td>
<td>Soybean agglutinin (SBA) [26]</td>
<td>- Refolded SBA after urea denaturation exhibits similar quaternary structure as that of native lectin.</td>
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<tr>
<td></td>
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<td>- Dimerization of SBA dimers occurs faster than the dimerization of SBA monomers.</td>
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<td>17.</td>
<td>Erythrina indica lectin [27]</td>
<td>- Monophasic urea-induced unfolding transition from native dimer to unfolded monomers.</td>
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<td></td>
<td></td>
<td>- Rate of unfolding increases several fold with increase in temperature.</td>
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<td>- Hemagglutinating activity and secondary structure not affected at extreme temperature and pH.</td>
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<td>19.</td>
<td><strong>Trichosanthes dioica</strong> seed lectin [29]</td>
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<tr>
<td></td>
<td>• Secondary structure drastically affected in presence of dithiothreitol at and above pH 7.0, indicating role of disulphide linkages in maintaining active conformation of lectin.</td>
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<tr>
<td></td>
<td>• Thermal unfolding is three-state.</td>
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<td></td>
<td>• Ligand binding stabilizes native conformation of protein.</td>
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<td></td>
<td>• Protein more stable at acidic pH.</td>
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<td></td>
<td>• Lectin structure stable over wide range of pH.</td>
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<td></td>
<td>• GdnHCl-mediated unfolding is three-state, involving an intermediate.</td>
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<td>20.</td>
<td><strong>Clitoria ternatea</strong> seed agglutinin [30]</td>
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<td></td>
<td>• Compact molten-globule (MG) conformation at pH 1.2.</td>
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<td>• Two-step non cooperative thermal denaturation of MG compared to cooperative single-step transition of native protein.</td>
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<td>• 72% carbohydrate binding activity retained by MG.</td>
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<td>21.</td>
<td><strong>Sauromatum guttatum and Arisaema tortuosum</strong> lectins [31]</td>
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<td></td>
<td>• Drastic loss in secondary structure of both the lectins at pH 2 and below.</td>
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<td></td>
<td>• Detection of a compact structure between pH 10 – 12.</td>
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<tr>
<td>22.</td>
<td><strong>Ariesaema curvatum</strong> lectin [32]</td>
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<td></td>
<td>• In 0.25 M GdnHCl protein exhibits pronounced secondary structure, hemagglutinating activity and altered tryptophan environment.</td>
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<td></td>
<td>• Detection of acid-induced molten globule with higher thermostability at pH 3.</td>
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<td></td>
<td>• Hemagglutinating activity retained even at 95 °C.</td>
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4.2. MATERIALS

Cultivar BDN 9-3 of chick pea seeds (*Cicer arietinum* L.) was obtained from Badnapur Agricultural University, Jalna, India. Acrylamide, cesium chloride, potassium iodide and guanidine hydrochloride and all other chemicals and reagents as well as buffers used for unfolding studies were from Sigma-Aldrich, USA. The buffers used at different pH were Glycine-HCl (pH 2 – 3), sodium acetate (pH 4 – 5), sodium phosphate (pH 6 – 7), Tris-HCl (pH 8 – 9) and glycine-NaOH (pH 10 – 12). Guanidine hydrochloride was freshly prepared at pH 7.2 and filtered through a 0.22 micron syringe filter before use.

4.3. METHODS

4.3.1. Protein Preparation

The recombinant lectin from *Cicer arietinum* seeds purified as mentioned in Chapter 2. This purified lectin, rCAL, was the subject of the present study. Protein concentrations were determined according to the method of Lowry et al [33] using bovine serum albumin (BSA) as the standard.

4.3.2. Spectroscopic measurements

a) Fluorescence studies: Steady-state intrinsic fluorescence measurements were performed on Perkin Elmer LS 50B luminescence spectrometer at 25 °C. Protein solution of 150 µg/ml was excited at 295 nm (1.0 cm cell path length) and emission was recorded from 310 to 400 nm. Slit widths of 7 nm each were set for excitation and emission monochromators and the spectra were recorded at 100 nm/min. To eliminate the background emission, the signal produced by either buffer solution or buffer containing the desired quantity of GdnHCl or different buffers was subtracted. Rayleigh (light) scattering was measured at 400 nm with the excitation and emission slit widths set at 2.5 and 10 nm, respectively, to follow the aggregation of the protein at different temperatures.

b) Hydrophobic dye binding: The intermediate states during thermal unfolding and GdnHCl-mediated unfolding and refolding of rCAL were analyzed by binding with the hydrophobic dye (8-anilino-1-napthalenesulphonate, ANS). The final ANS concentration used was 50 µM, excitation wavelength was 375 nm and total fluorescence emission was monitored between 400 and 550 nm. Reference spectrum of ANS in either buffer or buffer containing desired concentration of GdnHCl was subtracted from the spectrum of the sample.
c) **CD measurements:** The CD spectra of rCAL were recorded on a J-715 Spectropolarimeter (Jasco, Tokyo, Japan) at 25 °C in a quartz cuvette. Each CD spectrum was accumulated from five scans at 100 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Far UV CD spectra of rCAL (200 µg/ml) were collected in the wavelength range of 190 to 250 nm (for thermal unfolding) and 210 to 250 nm (for GdnHCl-mediated unfolding) using a cell of path length 0.1 cm for monitoring the secondary structure. All spectra were corrected for buffer contributions and observed values were converted to mean residue ellipticity (MRE) in deg cm² dmol⁻¹ defined as

\[
\text{MRE} = \frac{\theta_\lambda}{10 d c r}
\]

where M is the molecular weight of the protein, \( \theta_\lambda \) is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ml and r is the average number of amino acid residues in the protein. Secondary structure elements were calculated using the CDPro software [34]. Near UV CD spectra of rCAL (600 µg/ml) were collected in the wavelength range of 250 to 300 nm using a cell of path length 1.0 cm.

4.3.3. **Thermal denaturation**

Unfolding as a function of temperature was monitored by fluorescence and circular dichroism. Protein samples were incubated at temperatures ranging from 25 to 80 °C for ten minutes each. Protein concentration used was 150 µg/ml for fluorescence studies and 200 µg/ml for CD studies.

4.3.3.1. **Reversibility of thermal unfolding**

To check reversibility of thermal unfolding of rCAL, first the protein was gradually heated from 25 °C up to 50 °C and then allowed to cool to 25 °C. The fluorescence scans were recorded at each temperature.

4.3.4. **GdnHCl induced Unfolding and Refolding.**

Unfolding as a function of GdnHCl was monitored by fluorescence and circular dichroism. Protein samples (150 µg/ml) were incubated in 0–6 M GdnHCl solution at pH 7.2 for 6 h to attain equilibrium. For renaturation, a concentrated sample (600 µg/ml) was denatured in 6 M GdnHCl and then diluted ten times with refolding buffers of 0 to 6 M GdnHCl concentrations. The reaction was maintained for 3 hours before recording the spectra. Respective blanks of GdnHCl were subtracted before analysing the data. Protein concentration of 200 µg/ml was used to study unfolding by CD.
4.3.4.1. Reversibility of GdnHCl-induced unfolding:

To check the reversibility of GdnHCl-induced unfolding, in a second set of experiments, separate aliquots of rCAL were denatured in varying concentrations of GdnHCl, from 0 to 6 M (for 2 hours) and the samples were then diluted ten times. The samples were allowed to renature for 1 hour before recording their fluorescence intensity.

4.3.5. Decomposition of fluorescence spectra

The decomposition of trp fluorescence spectra was carried out using PFAST program (http://pfast.phys.uri.edu/pfast/) based on the SIMS and PHREQ algorithm as described in [35].

4.3.6. Parameter A analysis

Parameter A, the ratio of the intrinsic fluorescence intensity at 320 nm to that at 365 nm (I_{320}/I_{365}), is an attribute of fluorescence spectral shape and position [36]. This analysis was carried out to detect conformational changes of rCAL during GdnHCl-induced unfolding and refolding.

4.3.7. Size exclusion chromatography

Dissociation of oligomeric rCAL in presence of GdnHCl was monitored using WATERS HPLC unit and WATERS gel filtration Protein Pak™ 300SW (7.5 x 300 mm column). 25 mM Phosphate buffer (with 0.15M NaCl), pH 7.2 containing respective concentrations of GdnHCl served as the mobile phase. 100 µl protein sample (600 µg/ml) incubated in 0 to 4M GdnHCl for four hours was injected into the column. Flow rate was maintained at 0.5 ml/min and the elution profile was monitored at 280 nm.

4.4. RESULTS AND DISCUSSION

4.4.1. Secondary and tertiary structure of rCAL

The far UV CD spectrum showed a minimum around 218-220nm, which is characteristic of β-sheet containing proteins (Figure 4.1 (A)). The spectrum was analyzed using the CONTINLL program of the CDPro software [34] to calculate the secondary structure elements. The lectin showed the presence of 3.7% helix, 41.1% sheets, 21.3% turns and 33.9% unordered structure with an NRMSD value of 0.129. The near UV CD spectrum (Figure 4.1 (B)) showed a negative minimum at 284nm and maxima at 255nm and 295nm, indicating an ordered tertiary structure.
4.4.2. Thermal denaturation of rCAL

The fluorescence intensity decreased upon increasing temperature from 25 to 45 °C; a marginal increase was obtained at 50 °C, and a sharp increment in the intensity could be detected at 55 °C (Figure 4.2 (A)).

A slight red shift in the $\lambda_{\text{max}}$ of intrinsic fluorescence was observed for rCAL upon increasing the temperature from 25 to 55 °C (Fig. 4.2 (B)). The light scattering intensity, measured simultaneously, increased suddenly at 55 °C and reached its maximum at 60 °C. This indicated aggregation of the protein involving major structural change.
The minimum in the far UV CD spectrum of rCAL remained stable at 218-220 nm from 25 to 45 °C (Figure 4.3(A)). A distinct alteration in the secondary structure took place at 50, 52.5 and 55 °C; at 50 and 52.5 °C, the minima shifted to 215 nm (Figure 4.3(B)). Interestingly, at 55 °C, the minimum was again centred at 220-222 nm and a marked increase in positive ellipticity within 190-200 nm was observed as compared to the native protein. At 55 °C, the protein began to aggregate and this may have led to the formation of intermolecular β-sheets as reported by Uversky et al [37]. Thus, the protein shows a flexible thermo-labile nature with subtle changes occurring around 55 °C. The structure showed flexibility even between 60 to 75 °C and was completely lost at 80 °C (Figure 4.3 (C)).

An increase in the fluorescence intensity of the hydrophobic dye, ANS, accompanied by a blue shift to 475 nm, was observed at 55 °C (Figure 4.4). Partial unfolding of the protein at 55 °C might have resulted in exposure of hydrophobic sites and simultaneous formation of new intermolecular interactions. This might contribute to formation of aggregates via non-covalent interactions, as described by Vetri et al [38] for BSA.

![Figure 4.3: Thermal unfolding of rCAL. Far UV Mean Residue Ellipticity (MRE) spectra for temperatures (A) 25 to 45 °C, (B) 50 to 60 °C and (C) 55 to 80 °C. 200 µg/ml protein was used.](image-url)
Figure 4.4: ANS binding of the lectin during thermal unfolding of rCAL. The protein sample was incubated at the indicated temperature separately and ANS was added. All the scans were recorded after incubation for ten minutes at indicated temperature. 150 µg/ml protein was used.

4.4.2.1. Reversibility of thermal unfolding

Since rCAL began to aggregate at 55 °C and above, it was interesting to check if the thermal denaturation was reversible when the protein was heated only up to 50 °C and then cooled back to 25 °C. Here, the fluorescence intensity and λ<sub>max</sub> were almost regained after cooling to 25 °C (λ<sub>max</sub> of renatured sample remained same as that of native protein) (Figure 4.5). The renatured sample showed marginal ANS binding, indicating slight exposure of hydrophobic residues upon cooling from 50 to 25 °C.

Figure 4.5: Reversibility of thermal unfolding of rCAL. (A) Fluorescence intensity vs temperature. (B) ANS binding of renatured rCAL. 150 µg/ml protein was used.
4.4.3. Guanidine hydrochloride-induced unfolding and refolding of rCAL

A gradual unfolding of the lectin was observed upon addition of increasing concentrations of GdnHCl, as indicated by a red shift in the $\lambda_{\text{max}}$ (Figure 4.6(A)) from 341 to 356 nm in the vicinity of 6 M denaturant. Parameter A analysis ($I_{320}/I_{365}$) indicated that unfolding of rCAL was multi step, involving several intermediates (Figure 4.6 (B)). Trp conformers of class S and class I was predominant up to 1 M GdnHCl, indicating a hydrophobic environment for the residue. Treatment with 2 M and higher concentration of GdnHCl caused significant unfolding of the protein, with appearance of 100 % of class III conformer, representing complete exposure of trp to solvent.

Unfolding of rCAL was observed to be irreversible, the $\lambda_{\text{max}}$ showed further red shift after providing renaturing conditions to the samples (Figure 4.6 (A)). Formation of more disordered structure takes place when allowed to renature. Class III trp conformer was detected by decomposition analysis of all the above samples. The sample allowed to renature in low concentration of GdnHCl (0.6 M) appeared to be partially reversible as indicated by the appearance of class II conformer ($\lambda_{\text{max}} = 346$ nm).

A gradual but significant loss in the secondary structure as shown by altered far UV CD spectra (decrease in the positive ellipticity in the range of 190-205 nm) was observed in samples treated with GdnHCl after 6 hours (Figure 4.7).
To investigate the dissociation profile of dimeric rCAL during denaturation, HPLC size exclusion chromatography (HPSEC) was performed in the vicinity of 0 to 5M GdnHCl (Figure 4.8). The native rCAL of molecular mass of 53 kDa eluted as a single peak centred at 20.38 min. The retention time decreased slightly with increasing concentrations from 1 to 3M GdnHCl (19.67 min, 19.38 and 19.28 min respectively). However, in presence of 4 M GdnHCl, the above peak (19.28 min) reduced drastically and a new peak was detected at 16.08 min. Further, a single peak appearing at 15.41 min was detected in the presence of 5M GdnHCl. The minor peak corresponding to the retention time of 8.8 min was that of void volume of the column.

Figure 4.8: GdnHCl-mediated unfolding and refolding of rCAL at 25 °C. HPSEC profile of rCAL (600 µg/ml) incubated in 0 to 5 M GdnHCl for 4 hours.
The native dimeric protein began to unfold in the presence of 1M GdnHCl (Figure 4.7 (A)). If dissociation had been the first step, structured monomer would have eluted with higher retention time than that of the native protein. The unfolded (but not yet dissociated) dimer seemed to populate in the presence of 2 and 3 M GdnHCl. Further treatment with 4 and 5M GdnHCl could lead to dissociation of the unfolded dimer to more unstructured monomer, with concomitant loss in the structure and significant reduction in the elution volume. This could be possible if the unfolded monomer is adopting an extensively random coiled conformation compared to the native protein, as has been observed for the Con A lectin [17] and *Erythrina indica* lectin [26].

Taking into account the data from fluorescence, CD and HPSEC (summarized in Table 4.2), we propose the following scheme to delineate the GdnHCl-mediated unfolding of rCAL

\[ N_2 \rightarrow U_2 \rightarrow 2U \]

where N indicates native rCAL and U denotes unfolded dimer, that dissociates into two unfolded monomers (U).

No ANS binding was observed during the unfolding process of rCAL (data not shown). However, under renaturing conditions, protein in 0.6 M to 1.05 M GdnHCl did bind ANS (Figure 4.9), indicating the exposure of hydrophobic residues in an intermediate species formed during refolding in these low concentrations of dilution buffers.

![Figure 4.9: ANS binding of rCAL (600 µg/ml) after 3 hours of renaturation.](image-url)

The far UV CD spectra of the lectin after renaturing in different concentrations of GdnHCl could...
not be recorded, since the lectin has an inherent tendency to aggregate at higher concentrations (more than 700 μg/ml).

### Table 4.2 Comparison of GdnHCl-mediated unfolding of rCAL with different probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Native</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>Intrinsic Fluorescence λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>342</td>
<td>346</td>
<td>350</td>
<td>353</td>
<td>354</td>
<td>355</td>
</tr>
<tr>
<td>Far UV CD θ (MRE&lt;sub&gt;210&lt;/sub&gt;)</td>
<td>-221</td>
<td>-542</td>
<td>-840</td>
<td>-1115</td>
<td>-993</td>
<td>--</td>
</tr>
<tr>
<td>HPSEC Retention time (min)</td>
<td>20.38</td>
<td>19.67</td>
<td>19.38</td>
<td>19.28</td>
<td>16.08</td>
<td>15.41</td>
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</table>

4.4.3.1. Reversibility of GdnHCl after unfolding up to 2M

Figure 4.10: Reversibility of rCAL upon denaturation with different concentrations of GdnHCl. The first set of experiments is represented by samples unfolded in 6 M GdnHCl for 4 hours (unfolded 4 h, open squares) and then allowed to renature for 4 hours in dilution buffers (refolded 4 hours, open triangles). The second set of experiments involved unfolding of samples in varying concentrations of GdnHCl for 2 hours (unfolded 2h, closed squares) and then allowed to renature for 1 hour (refolded 1h, closed triangles).
To check for reversibility of GdnHCl-induced unfolding, separate aliquots of rCAL were denatured in varying concentrations of GdnHCl, from 0 to 6 M (for 2 hours) and the samples were then diluted ten times to check for renaturation. The denaturation was reversible only for samples treated with 2 M GdnHCl (Figure 4.10). Above 2 M, the denaturation was irreversible as obtained before. As seen in Figure 4.7 earlier, the 2 M-denatured sample had begun to lose its secondary structure and it showed the presence of unfolded monomers as seen from Figure 4.8. Hence, this could be the reason why above 2 M denaturant concentration, the protein shows irreversible denaturation and is unable to achieve the conformation of the native protein.

4.4.4. Effect of pH on rCAL

A decrease in fluorescence intensity was observed after incubation of rCAL in buffers of different pH; maximum decrease was obtained on the acidic side.

![Figure 4.11: Effect of pH on rCAL. (A) Fluorescence intensity at different pH. (B) Secondary structure of rCAL after incubation in buffers of pH 2, 7 and 10 for one hour. 200 µg/ml protein was used for fluorescence and 600 µg/ml protein was used for CD.](image)

No ANS binding was observed for samples treated with different buffers of different pH. Hence, indicating no exposure of hydrophobic residues (data not shown). The secondary structure of rCAL was lost after incubation in acidic (pH 2) and alkaline (pH 10) even after one hour of incubation (Figure 4.11 (B)). This indicates that the protein structure is labile in acidic and alkaline pH.

The process of aggregation results from conformational changes of the secondary structure as well as due to the partial unfolding of the tertiary structure. Recent studies by Militello and group [39] have shown that a partial opening of the
protein chain constitutes the first step of aggregation. This, in turn, leads to exposure of hydrophobic sites or free SH groups, hence resulting in aggregate formation [38]. In the case of rCAL also, partial opening of the chain at 50 °C caused exposure of hydrophobic residues (as seen by ANS binding at 50 and 55 °C), further leading to aggregation.

In favour of higher β-sheet structure content, the conformational changes in the secondary structure of many proteins may promote the formation of intermolecular bonds; this can also be one of the first steps for the formation of ordered aggregates in the form of amyloid fibrils. Among such proteins, albumin is well known for its ability to self assemble in the form of aggregates under particular conditions [38, 40]. rCAL is basically an albumin by nature, hence it shows an inherent tendency to aggregate. Another albumin from lentil (Lens culinaris) seeds, albumin-2 was also shown to unfold at 40 °C [41]. Further heating till 60 °C resulted in aggregation and precipitation of the protein. In contrast to rCAL, the Dolichos lablab lectin was found to be highly thermo-stable and unfolded at temperatures above 75 °C [23]. The Leucoagglutinin from Phaseolus vulgaris is also an extremely thermo-stable lectin, unfolding above 100 °C [15].
REFERENCES


