Chapter III

Molten globule-like state of concanavalin A retains its carbohydrate binding specificity

Spectroscopic studies on the protective effect of specific sugar on concanavalin A at acidic, neutral and alkaline pH.

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3.1 **Aim of present study:** Protein folding is the process by which amino acid sequence of a protein determines the three-dimensional conformation of the functional protein. The elucidation of the molecular mechanism of protein folding from a disordered polypeptide chain to specific native state, that is the deciphering of second half of the genetic code [104], remains one of the major challenges in biochemistry [108]. Study of the folding intermediates and denatured states provides an insight to understand how and when various forces come into play in directing protein folding [109-113]. The development of a broad range of techniques has led to the identification and characterization of stable folding intermediates, termed “molten globules”, that have been shown to be compact structure with pronounced secondary structure but lacking rigid tertiary structure [114-115]. Extensive study of sequence homology and 3-D structure of various lectins suggest that they are conserved throughout evolution and thus may play an important role in the physiology of plants. Though their exact role in plants is still unknown, lectins have been implicated in pathogenesis, cell elongation, defense against fungal attack and *Rhizobium* legume symbiosis. Unlike animals, plants have no immune system but lectins are believed to interact with invading microorganism thus providing a line of defense to plants against various bacteria and fungi. The recognition of carbohydrate determinates by lectins plays an important role in mediation of intercellular binding and elicitation of biosignalizing processes [116-118].

Con A is a lectin from jack bean, and is a well-studied protein because of its molecular structure and many medical applications. The predominant secondary structure in con A is β-pleated sheet structure [16]. More than half of amino acid residues of the protein exist in β-conformation. But still a central question in biological chemistry is the minimal structural requirement of a protein that would determine specificity and activity. The underlying basis of the entire structural element of a protein with regards to its activity vis a vis the overall integrity and stability of the protein is lacking. Here novelty lies in the characterization an intermediate of con A with the molten-globule like properties in the presence of polyethylene glycols that retains its carbohydrate binding specificity.
Materials and Methods

3.2.1 Materials: Con A, ovalbumin, ovomucoid and glycogen were purchased from Sigma, St. Louis, MO, USA. Dextran was purchased from BDH chemicals Ltd. Poole, England. PEG 200 and 400 was purchased from Qualigens fine chemicals. All other chemicals used in this study were of analytical grade.

3.2.2 Purification of concanavalin A: The purity of commercial preparation was checked by SDS PAG electrophoresis [95]. Activity of purified preparation was checked by agglutination against 2% trypsinzed rabbit erythrocytes. Alternatively, con A was also purified from jack bean extract following the methods of Agrawal and Goldstein [16]. One hundred grams of jack bean meal was soaked overnight in 500 ml of 0.5 M sodium chloride at 4°C. The suspension was stirred for four hours and filtered through muslin cloth. The residue was extracted with 300 ml of 0.5 M sodium chloride solution. The suspension was again filtered through muslin cloth. The homogenate thus obtained was centrifuged in a Remi centrifuge model RC5C at 6000 rpm for 30 minutes. Solid ammonium sulfate was added to supernatant to achieve 30% saturation. Precipitation was allowed to proceed overnight. The 30% precipitate was removed by centrifugation and the percent saturation raised to 80% by the addition of ammonium sulfate. The pH of the suspension was adjusted to pH 7.0 by the addition of 0.5 M ammonium hydroxide. After twelve hours the precipitate was collected by centrifugation and dissolved in minimal volume of water. It was first dialyzed against water and then against 10 mM Tris/HCl buffer, pH 7.0, containing 0.5 M sodium chloride, 1 mM calcium chloride, 1 mM manganese chloride and 1 mM magnesium chloride. The protein precipitated during dialysis was removed by centrifugation and the clear supernatant was used for further purification of Con A that was carried out by affinity chromatography using mannose CL agarose as affinity chromatography medium.

3.2.3 Affinity chromatography: The fraction which precipitated between 30-80% ammonium sulfate saturation was applied on mannose CL column equilibrated with 10 mM Tris/HCl buffer, pH 7.0, containing 0.5 M sodium chloride, 1 mM calcium acetate, 1 mM manganese chloride and 1 mM magnesium acetate. The unbound proteins were eluted and monitored spectrophotometrically. The column was washed with volume of eluting buffer, which was ten times the total volume of the column. When the eluate
became free of protein, the bound Con A was eluted with 0.1 M glucose in TM-buffer pH 7.0. Glucose was removed from the protein by extensive dialysis at 4°C.

Effect of pH: Solutions for CD and fluorescence were brought to the desired pH by exhaustive dialysis against the appropriate buffer. Buffers were 0.02 M glycine HCl for pH 2-3, sodium acetate for pH 4-5, sodium phosphate for pH 6-8 and glycine NaOH for pH 9-10.

3.2.4 CD measurements: CD was measured with a JASCO J-720 spectropolarimeter calibrated with ammonium D-10-camphorsulphonate. A cell of path length 0.1 and 1 cm was used for scanning between 250-200 nm and 300-250 nm respectively. Protein concentration of the samples was typically 15 µM and 30 µM in 60 mM phosphate buffer of pH 7.0 for the far-UV and near-UV CD studies respectively. The results were expressed as mean residue ellipticity (MRE) in degree cm²/dmol, which is defined as

\[ \text{MRE} = \frac{\theta_{\text{obs}} \times 10 \times n \times C_p \times l}{\text{mdeg}} \]

where \( \theta_{\text{obs}} \) is the observed ellipticity in degrees, \( C_p \) is the molar fraction and \( l \) is the length of light-path in cm [97].

3.2.5 Fluorescence measurements: Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorometer in a 10 mm path length quartz cell. Samples containing con A with different concentration of PEG 200 and PEG 400 were equilibrated at room temperature for 30 minutes before recording for tryptophan fluorescence measurement. The excitation wavelength was 280 nm and emission wavelength was recorded from 300 to 400 nm respectively [98]. The final protein concentration was 15 µM. For each sample proper blank was taken into consideration.

3.2.6 ANS fluorescence measurements: ANS binding was measured by fluorescence emission with excitation at 380 nm and emission was recorded from 400-600 nm respectively [119]. Typically, ANS concentration was 100 molar excess of protein concentration and protein concentration was in the vicinity of 15 µM in 60 mM phosphate buffer, pH 7.0. It should be noted that PEG itself binds to ANS, therefore to avoid anomaly, proper blank was made for every point.

3.2.7 Size exclusion chromatography (SEC): SEC experiments were performed on a seralose 6B (74 X 1.1) cm column. The column was pre-equilibrated with 60 mM sodium phosphate buffer pH 7.0 and 30% PEG 400 or 200. Two milliliters of 3 mg/ml native and
protein in 30%(v/v) PEG 400 or 200 were applied to the column and eluted at 20ml/hr. The eluted fractions were read at 280nm.

3.2.8 GdnHCl stability studies: To determine the GdnHCl stability of the intermediate state in the presence of PEGs relative to the native, changes in absorbance at 292nm, fluorescence at 337nm and CD at 223nm were measured, as a function of GdnHCl. Protein concentration was 15μM for CD and fluorescence respectively and 30μM for absorbance measurements.

3.2.9 Precipitation reaction: The interaction of con A (0.5mg/ml) with polysaccharide or glycoprotein (1.5mg/ml) was studied in 10mM TM (Tris HCl Metal ion [Ca²⁺, Mn²⁺]) buffer (pH 7) by turbidity method at 350 nm on spectrophotometer model Hitachi U 1500 and at 410 nm Shimadzu RF 540 spectrofluorometer [96,99,100]. For each sample proper blanks of native, polysaccharide or glycoprotein were taken into account.

3.2.10 Inhibition reaction: The inhibition of precipitation was measured by incubating con A (0.5mg/ml) with polysaccharide or glycoprotein (1.5mg/ml) in 10mM TM buffer, pH 7 in presence of increasing concentration of PEG 200 and PEG 400 separately and subsequently determining the percent decrease in absorbance at 350 nm and 410nm [100,120].
3.3 Results

3.3.1 Purification of Con A: The elution profile of Con A is shown in figure 13A. Ten gram of crude protein was loaded on sephadex column. The total protein eluted was 0.1 gram and revealed single peak. The percent yield was 1%. The hemagglutinating activity of eluate was checked against trypsinized rabbit red blood cells and the titer was 5. SDS-PAGE of Con A in 0.1% SDS was carried out in Tris-glycine buffer, pH 8.3. Yield of the electrophoretic pattern is shown in figure 13B. Con A migrates as many as five protein bands; the major band with high intensity was the slowest in mobility. The other four minor bands were fast moving and were of low intensity as compared to major band. The presence of minor protein band may be ascribed to the presence of broken polypeptide in segments of Con A. Only the major band corresponding to protein monomer was considered as that of native Con A.

3.3.2 Interaction of Con A with polysaccharides (Dextran (α1-6), Glycogen (α1-4)) and glycoproteins (Ovalbumin (1-2-linked α-D-mannopyranosyl), Ovomucoid (Glc NAc β(1-4) (1-2)-Man α(1-3))): Figures 14A and B show the precipitin reaction curve of dextran, ovalbumin, ovamucoid and glycogen studied by turbidity method at 350nm and 410nm using spectrophotometer and spectrofluorometer respectively. In order to rule out the possibility of aggregation, proper blanks of con A and glycoproteins or polysaccharide were taken into consideration. The turbidity developed due to interaction of con A with the above-mentioned ligands was maximum in glycogen (curve 1) followed by ovalbumin (curve 2), ovamucoid (curve 3) and dextran (curve 4).

Effect of PEGs on the structural properties of Con A as studied by different optical spectroscopic methods

3.3.3 CD measurements: Figure 15A shows the far-UV CD spectra in the 250-200 nm range of con A in the presence of different concentrations (%v/v) of PEG 200 and 400. The absorption of PEG alone was taken into account and here we report the subtracted spectra. As can be seen from the figure the native con A exhibits a trough (negative peak) at 223 nm (curve 4). The position of the trough is a characteristic feature of the β-conformation in lectins. At 30%(v/v) PEG 200 (curve 5) the position of trough remains at 223nm with slight increase in negative MRE while at 30%(v/v) PEG 400
(curve 6), a blue shift of 4nm was obtained with further increase in negative MRE, indicating the existence of a compact state. At 70% (v/v) PEG 200 (curve 3) a red shift of 2 nm with decrease in negative MRE is obtained. PEGs induce an intermediate compact state appearing during the structural transition from the native to an unfolded state. Such types of transitions have also been reported earlier in a number of proteins [121,122]. The effect of PEGs i.e. 200 and 400 on con A was studied by measuring the MRE at 223 nm (fixed wavelength analysis, figure 15B). Upto 30% (v/v) PEG there was more induction of secondary structure while at 70% (v/v) there was a significant loss of secondary structure at this wavelength. An increase in MRE at 30%(v/v) PEGs can be suggestive of an increase in secondary structural content, as polyols induce a higher secondary structure in native state upto a certain concentration. As the concentration of PEGs increases, the polarity of the solvent decreases, creating the hydrophobic environment, which results in disruption of secondary structure. As PEG is hydrophobic in nature, it may interact favorably with the hydrophobic side chains exposed upon unfolding. So, most probably the PEGs perturb the structure of the protein surface, partially modifying the layer of water and the microenvironment of the aromatic residues, which is in agreement with the observed alterations in CD spectrum (see Table V).

The CD spectrum in the near-UV region was used to probe the asymmetry of the proteins aromatic amino acids environment [123]. Figure 16 shows the near-UV CD spectra in the 300-250nm range of native preparation (curve 1), at 30%(v/v) PEG 400 (curve 2), at 30% (v/v) PEG 200 (curve 3), at 70%(v/v) PEG 200 (curve 4), at 70% (v/v) PEG 400 (curve 5), and in the presence of 6M GdnHCl (curve 6). The two prominent positive peaks at 292 and 283nm characterize the near-UV CD spectrum of native con A. As can be seen from figure, at 30%(v/v) PEG 400 and 200 there is loss in MRE values with broaden peaks at 292 and 283nm. Near-UV CD signal show that the spectra at 30%(v/v) PEGs resemble more that of native protein as compared to that at 70%(v/v) PEGs where significant structure is loss or to the completely unfolded state in 6M GdnHCl. At 70%(v/v) PEGs loss of tertiary structure was more in PEG 400 than 200 (curve 5 and 4). From our CD data we conclude that at 30%(v/v) PEGs there was retention of native secondary as well as partial tertiary structure. These results shows that con A at 30%(v/v)
Fig. 13A. Elution profile of mannose-glucose specific lectin from mannose-CL agarose column. Jack bean seed extract prepared in TMB was allowed to bind the column matrix and the lectin was eluted in 0.5 ml fractions with TMB containing 50 mM mannose.
Fig. 13B. SDS-PAGE (15%) of purified lectin under reducing conditions. Lectin sample (15μg) was loaded.
Fig 14A. Quantitative precipitin reaction curve of con A-glycogen (curve 1), con A-ovalbumin (curve 2), con A-ovomucoid (curve 3), con A-dextran (curve 4) at 350 nm on spectrophotometer in TM buffer, pH 7
Fig. 14B. Quantitative precipitin reaction curve of con A-glycogen (curve 1), con A-ovalbumin (curve 2), con A-ovomucoid (curve 3), con A-dextran (curve 4) at 410 nm on spectrofluorometer in TM buffer, pH 7.
Fig. 15A. Far-UV CD spectra of concanavalin A as a function of PEGs in 60 mM sodium phosphate buffer, pH 7.0. Curve 1 represents 6 M GdnHCl denatured con A, curve 2 represents con A with 70%(v/v) PEG 400, curve 3 represents con A with 70%(v/v) PEG 200, curve 4 represents con A at pH 7 alone, curve 5 represents con A with 30%(v/v) PEG 200, curve 6 represents con A with 30%(v/v) PEG 400. The protein concentration was 15 μm and the pathlength was 0.1 cm.
Fig. 15B. Effect of increasing concentrations of polyols (v/v) i.e. PEG 400 (♦) and PEG 200 (▲) on con A at pH 7 as followed by ellipticity measurements at 223 nm.
Fig. 16. Near-UV CD spectra of con A (curve 1), at 30%(v/v) PEG 400 (curve 2), 30%(v/v) PEG 200 (curve 3), at 70%(v/v) PEG 200 (curve 4), 70%(v/v) PEG 400 (curve 5) and 6 M GdnHCl denatured con A (curve 6) in 60 mM sodium phosphate buffer, pH 7.0. The protein concentration was 30 μm and the pathlength was 1cm.
Table V

Structural propensities of intermediate states of concanavalin A as studied by far-UV CD

<table>
<thead>
<tr>
<th>State</th>
<th>Conditions</th>
<th>MRE$_{223\text{nm}}$ (deg.cm$^2$/dmol$^1$)</th>
<th>Cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>pH 7.0, 25°C</td>
<td>-5,100</td>
<td>nd</td>
</tr>
<tr>
<td>PEG 200</td>
<td>pH 7.0, 25°C, 30% (v/v)</td>
<td>-5,610</td>
<td></td>
</tr>
<tr>
<td>PEG 200</td>
<td>pH 7.0, 25°C, 70% (v/v)</td>
<td>-4,602</td>
<td>42% (v/v)</td>
</tr>
<tr>
<td>PEG 400</td>
<td>pH 7.0, 25°C, 30% (v/v)</td>
<td>-7,200</td>
<td></td>
</tr>
<tr>
<td>PEG 400</td>
<td>PH 7.0, 25°C, 70% (V/V)</td>
<td>-1,101</td>
<td>30% (v/v)</td>
</tr>
</tbody>
</table>
PEGs exists as a compact state and is stabilized by inter-chain interactions.

3.3.4 **Intrinsic tryptophan fluorescence:** The intrinsic fluorescence maximum (λ_{max}) is an excellent parameter to monitor the polarity of tryptophan environment in the protein, and is sensitive to the protein conformation [98]. Contribution of PEG alone to the emission spectra was taken into account and we report here the subtracted fluorescence spectra. Figure 17A depicts the tryptophan fluorescence emission spectra of con A in the absence and presence of various concentrations of PEGs. The emission maximum of native protein was obtained at 337nm (curve 1) and a red shift of 9 nm was observed in the completely unfolded state in 6M Gdn-HCl as all four tryptophan are exposed to solvent (curve 6). At 30%(v/v) PEG 200 (curve 2) and PEG 400 (curve 3) there was 11% and 13% increase in fluorescence intensity with 3 nm red shift relative to native preparation. On increasing the concentration of PEG 200 and 400 up to 70%(v/v) (curve 4, 5), there was a further increase in fluorescence intensity, suggesting exposure of tryptophan residues from non-polar to polar environment. Relative to PEG 200, the increase in fluorescence intensity was more in PEG 400. This is possibly due to the presence of more hydroxyl groups in PEG 400 than PEG 200. Figure 17B depicts the fluorescence intensity at 337nm as a function of %(v/v) PEGs. On increasing the concentration of PEGs, there was enhancement in fluorescence intensity. However, at 30%(v/v) PEG, there is only a slight red shift in the wavelength emission maxima (λ_{max}) as compared to a significantly red-shifted spectrum of the completely unfolded state in 6M GdnHCl, indicating that the tryptophan environment resembles the native form of con A.

3.3.5 **ANS fluorescence:** Figure 18A shows ANS fluorescence emission spectra of con A at pH 7 in presence of PEG 200 and 400. As can be seen from the figure, maximum increase in ANS emission intensity with a blue shift in emission maximum in con A was at 30%(v/v) PEG 400 (curve 5) and 200 (curve 4) followed by 70% (v/v) PEG 200 (curve 3) and 400 (curve 2). Native con A in the absence of PEGs showed negligible ANS binding (curve 1). Since ANS is known to bind to hydrophobic patches on protein, binding of ANS to hydrophobic regions of protein has been widely used to study the folding intermediates formed during protein folding. As molecules of ANS bind to hydrophobic surfaces on the protein with greater affinity, it can be concluded that at
30%(v/v) PEGs a compact intermediate with remarkable increase in number of solvent accessible non-polar clusters of con A exists [124,125]. The emission maximum at 30% (v/v) PEGs blue shifts to 482nm relative to native (500nm), further suggesting that ANS binds to protein. Figure18B depicts the ANS fluorescence intensity at 480 nm as the function of % (v/v) PEG. The notch in figure 18B at 30%(v/v) PEGs indicates an intermediate appearing during the structural transition from native to polyol (PEG)-induced state. There was a further decrease in ANS fluorescence at 70%(v/v) PEGs because of disruption of hydrophobic patches.

3.3.6 Size exclusion chromatography: Since con A is a non-covalently associated tetramer at pH 7, one possibility is that the intermediate seen in spectroscopic studies in presence of PEGs must be compact (resembling native state) as compared to 6M Gdn-HCl. To clarify this issue, size exclusion chromatography was performed. The gel filtration analysis of native con A at pH 7 on seralose 6B column confirms the tetrameric nature of the protein (the elution volume corresponds to a molecular mass of 104 KDa). Gel filtration analysis in the presence of 30%(v/v) PEGs showed decrease in elution volume corresponding to less compact con A as compared to native (see Table VI). This indicates that the dimensions of the molecule have slightly increased in the presence of polyols. The elution volume in presence of 70% (v/v) approaches to 6 M GdnHCl that further confirms that the state at 70%(v/v) resembles the denatured state. At 30 %(v/v) PEGs an intermediate value of elution volume is obtained, suggesting that the protein is relatively compact at this PEG concentration and resembles to native state. The state obtained in presence of PEG 200 was found to be more compact (less increase in Ve) than that in PEG 400. Thus, the results indicate a slight increase in the hydrodynamic dimensions of the lectin in presence of PEGs, which could be due to loosening of tertiary contacts. However, native like tertiary structure is retained as compared to complete loss of structure at 6 M GdnHCl.

3.3.7 GdnHCl stability studies: The fluorescence intensity at 337nm as a function of GdnHCl is depicted in figure 19A. As can be seen from the figure, PEG 400 resists the loss of structure in presence of increasing concentration of GdnHCl than PEG 200 and native. The fraction of protein denatured (fD) of con A at pH 7 and in presence of
Fig. 17A. Tryptophan fluorescence emission spectra of con A in 60 mM sodium phosphate buffer, pH 7.0 (curve 1); curve 2 represents con A at 30%(v/v) PEG 200, curve 3 represents con A at 30%(v/v) PEG 400, curve 4 represents con A at 70%(v/v) PEG 200, curve 5 represents con A at 70%(v/v) PEG 400, curve 6 represents 6 M Gdn HCl-denatured con A. The protein concentration was 15 μM and the pathlength was 1 cm.
Fig. 17B. Effect of increasing concentrations of polyols $(v/v)$ i.e. PEG 400(♦) and PEG 200 (▲) on con A at pH 7 as followed by fluorescence intensity measurements at 337 nm.
Fig. 18A. ANS fluorescence emission spectra: native con A (curve 1); con A in the presence of 70%(v/v) PEG 400 (curve 2), 70%(v/v) PEG 200 (curve 3), 30%(v/v) PEG 200 (curve 4), 30%(v/v) PEG 400 (curve 5) in 60 mM sodium phosphate buffer, pH 7. The protein concentration was 15 μm and the pathlength was 1 cm.
Fig. 18B. Effect of increasing concentrations of polyols % (v/v) i.e. PEG 400 (●) and PEG 200 (▲) on con A at pH 7 as followed by ANS fluorescence at 480 nm.
<table>
<thead>
<tr>
<th>Properties</th>
<th>Conditions</th>
<th>Elution Volume (Ve) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>pH 7.0, 25°C</td>
<td>145</td>
</tr>
<tr>
<td>PEG 200</td>
<td>pH 7.0, 25°C, 30% (v/v)</td>
<td>130</td>
</tr>
<tr>
<td>PEG 200</td>
<td>pH 7.0, 25°C, 70% (v/v)</td>
<td>112</td>
</tr>
<tr>
<td>PEG 400</td>
<td>pH 7.0, 25°C, 30% (v/v)</td>
<td>118</td>
</tr>
<tr>
<td>PEG 400</td>
<td>pH 7.0, 25°C, 70% (v/v)</td>
<td>110</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>pH 7.0, 25°C, 6 M</td>
<td>109</td>
</tr>
</tbody>
</table>
30%(v/v) PEGs with increase in GdnHCl denaturation is shown in figure 19B. \( f_0 \) of con A at pH 7 and in the presence of 30% PEGs were calculated taking con A at pH 7, in the presence of 30%(v/v) PEGs at 0M GdnHCl as native point respectively. At pH 7, no intermediates were present in detectable amount, thus it showed a single-step, two-state cooperative transition. In the presence of PEGs too, a single-step, two-state weakly cooperative transition was obtained. The weakly cooperative unfolding of PEG-con A was indicative of its molten-globule like nature. There are similar examples in many globular proteins including equine, canine, cytochrome c and SNase [126-128]. Fraction denaturation was least in PEG 400 followed by PEG 200 and native. This shows GdnHCl denaturation was less in presence of PEG 400 than 200. In native con A, a sigmoid curve is obtained with large variation range in \( f_0 \) while in PEG-con A, the narrow range is obtained. Figure 19C shows the change in MRE values at 223nm. The transition of native was found to be cooperative while of PEG 200 and 400 was weakly cooperative. The transitions were consistent with the fluorescence results. Figure 19D shows the changes in extinction coefficient as the function of GdnHCl at 292nm by absorption spectroscopy. 30%(v/v) PEG 200 and PEG 400 exhibit a single-step, two-state transition similar to native. From GdnHCl data, we conclude that during unfolding transition of native, 30%(v/v) PEG 200 and PEG 400-con A, no detectable intermediates were obtained. The analysis of the denaturation profiles of native con A and in the presence of 30%(v/v) PEGs shows their unfolding was cooperative and weakly cooperative respectively. Further, in presence of PEG 400 the loss of structure is less as compared to PEG 200 as greater number of hydroxyl groups is present in PEG 400, which resists the GdnHCl denaturation.

Thus, it can be concluded that at 30%(v/v) PEGs, con A exists as molten globule state with the retention of a compact secondary and some tertiary structure, enhanced hydrophobic surface area and weakly cooperative transition curve of GdnHCl denaturation.
Effect of PEGs on the functional properties of Con A

3.3.8 Interaction of Con A with polysaccharide (dextran and glycogen) in the presence of PEGs: Effect of PEG 200 and 400 on the precipitin reaction of con A with polysaccharides was investigated by turbidity measurements at 350 nm by spectrophotometer (figure 20A) and at 410 nm by spectrofluorometer (figure 20A inset). The concentration of % (v/v) PEGs corresponding to 50% decrease in absorbance at 350 nm yields C_50 (per liter). C_50 is the concentration of polyol required to achieve 50% inhibition of the specific precipitin reaction. The C_50 for PEG 200 (34%) was greater than PEG 400 (24%). Approximately 60% and 82% activity was lost in dextran-con A precipitation reaction in the presence of PEG 200 and 400 respectively. Figure 20A (inset) shows the same trend at 410 nm on spectrofluorometer as for con A and dextran in the presence of PEGs. The activity loss at 30% (v/v) PEG 400 and 200 in dextran-con A was 53% and 37% respectively.

In figure 20B the decrease in turbidity was observed in the presence of PEG 400 and 200 on con A-glycogen precipitation as monitored by turbidity method at 350 nm. PEG 400 inhibited the precipitation more as compared to PEG 200. C_50 for PEG 400 and 200 glycogen-con A precipitation was 18% and 16% (v/v) respectively. The activity loss of con A for glycogen was 51% and 60% in presence of PEG 400 and 200 respectively. Fig 20B (inset) shows the inhibition of precipitation of glycogen-con A in presence of PEGs by spectrofluorometer at 410nm. Similar pattern was obtained as seen in figure 20B. The maximum activity loss in the presence of PEG 400 and 200 was 71% and 60% respectively.

3.3.9 Interaction of Con A and glycoproteins (ovalbumin and ovomucoid): Figure 21A shows con A-ovalbumin precipitin reaction in the presence of PEG 200 and 400 at 350 nm. The concentration of PEG 400 and 200 at which 50% inhibition occurred was 14% and 12% (v/v) respectively. The maximum % activity loss of con A with ovalbumin was 95% in PEG 400 and 91% in PEG 200. The activity loss at 30% (v/v) PEG 200 and PEG 400 was 75% and 77% respectively. Experiments on the inhibition of con A-ovalbumin precipitin reaction by PEG 200 and 400 were also performed by turbidity methods at 410 nm on spectrofluorometer (see figure 21A inset).
Fig. 19. GdnHCl denaturation of con A at pH 7 (■), 30% (v/v) PEG400 (♦), 30% (v/v) PEG 200 (▲): (A) Fluorescence intensity at 337 nm versus GdnHCl.
(B) Data is plotted between $f_d$ versus GdnHCl concentration, fraction of protein denatured by change in fluorescence emission maximum as a function of GdnHCl.
(C) GdnHCl induced transition as monitored by MRE measurements at 223nm.
(D) $\Delta \varepsilon$ versus GdnHCl: change in value of $\varepsilon_{292}$ ($\Delta \varepsilon$) as a function of GdnHCl.
Fig. 20A. Effect of PEG 400 (♦) and 200 (▲) on con A-dextran quantitative precipitin reaction at 350 nm on spectrophotometer in TM buffer, pH 7.

(Inset). Effect of PEG 400 (♦) and 200 (▲) on con A-dextran quantitative precipitin reaction at 410 nm on spectrofluorometer in TM buffer, pH 7.
Fig. 20B. Effect of PEG 400 (○) and 200 (▲) on con A-glycogen quantitative precipitin reaction at 350 nm on spectrophotometer in TM buffer, pH 7. (Inset) Effect of PEG 400 (○) and 200 (▲) on con A-glycogen quantitative precipitin reaction at 410 nm on spectrofluorometer in TM buffer, pH 7.
Fig. 21A. Effect of PEG 400 (♦) and 200 (▲) on con A-ovalbumin quantitative precipitin reaction at 350 nm on spectrophotometer in TM buffer, pH 7.

(Inset) Effect of PEG 400 (♦) and 200 (▲) on con A-ovalbumin quantitative precipitin reaction at 410 nm on spectrofluorometer in TM buffer, pH 7.
Fig. 21B. Effect of PEG 400 (★) and 200 (▲) on con A-ovomucoid quantitative precipitin reaction at 350 nm on spectrophotometer in TM buffer, pH 7. (Inset). Effect of PEG 400 (★) and 200 (▲) on con A-ovomucoid quantitative precipitin reaction at 410 nm on spectrofluorometer in TM buffer, pH 7.
Figure 21B shows con A-ovomucoid precipitation reaction in presence of PEG 200 and 400. PEG 200 or PEG 400 maximally inhibited the con A-ovalbumin precipitin reaction as compared to above-mentioned ligands. 93% decrease in presence of maximum concentration of PEG was obtained in the activity of con A towards ovomucoid. The loss in the activity of con A with ovomucoid was 75% and 77% in the presence of 30%(v/v) PEG 400 and PEG 200 respectively. C50 value obtained for con A and ovomucoid was 10%. Inset of figure 21B shows the same trend at 410 nm by spectrofluorometer as observed for figure 21B.

3.4 Discussion
The effect of PEGs on con A at pH 7 was carried out by spectroscopic techniques (fluorescence, ANS binding and CD), size exclusion chromatography and GdnHCl denaturation as well as carbohydrate binding specificity. PEG 200 and 400, being smaller molecules penetrate the protein structure and form contacts with the surface of protein. While the larger species of PEGs cannot penetrate the protein structure, a shell that is impenetrable to co-solvent is formed around the protein molecule. Higher molecular weight PEGs have larger excluded volume, leading to a larger protein preferential hydration. As a result, due to preferential exclusion higher PEGs lead to stabilization instead of denaturation. The following spectral information was obtained. (1) From far-UV CD spectrum, there was retention of secondary structure at 30%(v/v) PEGs, which was lost on increasing the concentration further. (2) From Near-UV CD band at 292 and 283, assigned to tryptophan and tyrosine respectively, some tryptophan and tyrosine side chains were inferred to be constrained by interhelix interactions. (3) From fluorescence studies we observed that 30%(v/v) PEGs alter the tryptophan environment of native con A. (4) ANS binding was maximum at 30%(v/v) PEG 400, followed by 30%(v/v) PEG 200. (5) From size exclusion chromatography, an intermediate state was obtained which was compact as compared to 6 M GdnHCl. (6) The GdnHCl unfolding of 30%(v/v) PEGs–induced state showed single-step weakly cooperative transition. In all the results obtained, the effect of PEG 400 is more pronounced as compared to PEG 200; this is because PEG 400 has a larger molecular weight and greater number of OH groups. The effect of polyols increases with increasing concentration and number of OH groups [129]. Destabilization of human serum albumin and bovine serum albumin by PEGs has also
been reported earlier [130,131]. Thus, intermediates obtained at 30%(v/v) PEGs retain secondary structure with enhanced hydrophobic surface area, as observed by their strong ability to bind ANS. GdnHCl denaturation studies showed the transition was a single-step, two-state process. These species may hence be described as compact molten globule-like intermediates, as similar properties have been observed for the molten globule states of a number of proteins. “Molten globule” (MGs) are partially structured protein folding intermediates that adopt a native-like over all back bone topology in the absence of extensive detectable tertiary interactions. It is important to determine extent of specific tertiary structure present in ‘molten globules’ and to understand the role of specific side-chain packing in stabilizing and specifying the MG structure. Moreover, the A-state of equine ferricytochrome c has native-like compactness, native-like helix content, a native helix-helix interactions and one native heme ligand [132,133]. Tertiary A-state having been reported for α-lactalbumin [113], RNase H [114], myoglobin [134,135] and ubiquitin [115]. For many proteins, a native-tertiary structure in the presence of organic solvents has been reported [136].

Yet, unusually, con A intermediate, despite the expanded tertiary structure, retains its carbohydrate binding activity to a considerable degree. The precipitin reaction involving con A and specific polysaccharide or glycoprotein resembles an antigen-antibody reaction. Accordingly con A–ligand reaction leading to specific precipitation reaction can be represented by

\[
\text{Con A} + \text{L} \leftrightarrow \text{complex (Soluble)} \leftrightarrow \text{complex (Precipitate)} \] (1)

where L is a multivalent ligand. It is the secondary aggregation of the soluble complex i.e. the second step that was monitored by turbidity method in this study. Accordingly, of the two organic solvents used the concentration required to achieve maximal sharp inhibition was highest for ovomucoid and minimum for dextran. This may be due to the slight disruption of tertiary structure of con A in the presence of high concentration of PEGs, which leads to decrease in precipitation as obtained in native-ligand combinations. Since hydroxyl groups are more in PEG 400 than PEG 200 more inhibition was obtained in all the combination of PEG 400 than PEG 200. A comparison of structural and functional properties of intermediate state of con A in presence of PEGs is summarized in
Table VII

Comparison of the structural and functional propensities of intermediate states of concanavalin A in presence of PEGs.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Native</th>
<th>PEG200 at 30%(v/v)</th>
<th>PEG400 at 30%(v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE</td>
<td>-5,100</td>
<td>-5,610</td>
<td>-7,200</td>
</tr>
<tr>
<td>ANS Fluorescence</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Activity</td>
<td>1.4 (a)</td>
<td>0.9 (a)</td>
<td>0.12 (a)</td>
</tr>
<tr>
<td></td>
<td>1.1 (b)</td>
<td>0.2 (b)</td>
<td>0.18 (b)</td>
</tr>
<tr>
<td></td>
<td>0.6 (c)</td>
<td>0.35 (c)</td>
<td>0.25 (c)</td>
</tr>
<tr>
<td></td>
<td>1.5 (d)</td>
<td>0.45 (d)</td>
<td>0.3 (d)</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>0%</td>
<td>51% (a)</td>
<td>60% (a)</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>79% (b)</td>
<td>80% (b)</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>37% (c)</td>
<td>53% (c)</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>75% (d)</td>
<td>77% (d)</td>
</tr>
<tr>
<td>(C_{50})</td>
<td>-</td>
<td>18% (a)</td>
<td>16% (a)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>14% (b)</td>
<td>12% (b)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>34% (c)</td>
<td>24% (c)</td>
</tr>
<tr>
<td></td>
<td>---</td>
<td>10% (d)</td>
<td>10% (d)</td>
</tr>
</tbody>
</table>

\(a\) denotes glycogen, \(b\) denotes ovalbumin, \(c\) denotes dextran, \(d\) denotes ovomucoid

\(C_{50}\): Concentration of organic solvent required to bring 50% decrease in absorbance.

Activity of native is taken as 100%. Activity: the concentration at which maximum turbidity was obtained with the lectin and ligand. +: less, ++: little, +++: denotes more ANS binding.
Table VII. Our studies extend on the characterization of compact molten globule states of con A in presence of PEGs i.e. having high hydrophobic residues and retention of secondary and tertiary structure. From these studies, it appears that lectin intermediates in presence of polyols may have all the necessary features compatible with carbohydrate binding. Thus, it can be concluded that molten globule is the minimum structural requirement of lectins in general, for the carbohydrate binding specificity. PEG–molten globule complex formation is analogous to the observed interaction between chaperonins and a molten globule intermediate [137].

3.5 Concanavalin A interaction with specific and non specific sugars:

3.5.1 Effect of pH on concanavalin A: CD measurements: Figure 22A summarizes the plot of far-UV CD spectra of concanavalin A at 217nm as the function of pH from 2 to 10. At pH 7, a negative trough at 217nm was obtained with a crossover at 209nm (data not shown). The position of the trough at 217nm corresponds with the trough position (217-219nm) obtained from proteins known to be in $\beta$ conformation. As can be seen from the figure on decreasing the pH from 7 to 2 or increasing from 7 to 10 there was change in MRE values. This shows the loss of native secondary structure. The pH sensitivity of concanavalin A indicates that electrostatic interactions are important in maintaining the unique $\beta$ like conformation of concanavalin A [138,139]. Figure 22B shows the trends found for MRE at 269 and 293nm as a function of different pH range from 2 to 10. On increasing the pH up to 10 or decreasing up to 2, there was loss of tertiary structure as can be seen by change in MRE values.

Fluorescence measurements: Figure 23 depicts the effect of pH range from 2 to 10 on the fluorescence intensity at 335 nm of concanavalin A (as described by CD data). At pH 7, $\lambda_{\text{max}}$ is obtained at 335nm in native state (data not shown for clarity). On decreasing the pH from 7 to 2 there was decrease in fluorescence intensity because the polarity of the regions to which the tryptophan is being exposed must be changing to more polar environment (indicating tryptophan was approaching to the surface of the protein). On increasing the pH, there was increase in fluorescence intensity as compared to native. This indicated that initially tryptophan was in a polar solvent, gets internalized and now in non-polar environment. The sensitivity of concanavalin A to alkali indicates that
electrostatic interactions are important in maintaining the unique conformation of concanavalin A. These results are consistent with our CD data.

3.5.2 **Effect of specific and non-specific sugars on Con A:** Figure 24 depicts the far-UV CD spectra of native glucopyranoside (curve 2), concanavalin A at pH 7 (curve 1), and in the presence of 50 mM methyl α-D-galactopyranoside (curve 3). A negative trough was obtained for native concanavalin A at 217nm, which was retained in the presence of methyl α-D-galactopyranoside with slight change in MRE. In the case of methyl α-D-galactopyranoside the negative CD intensity was increase by ~ 63%.

3.5.3 **Effect of pH in presence of sugars: CD studies:** For the formation of complex between concanavalin A and specific sugar, the presence of free amino and carboxyl groups are essential. CD studies have indicated that the binding of specific sugar to lectins causes the conformational changes in the protein resulting in the alteration of the tryptophan and tyrosine environment of the protein [140]. In our CD spectra of concanavalin A in the presence of 50mM methyl α-D-glucopyranoside, at all acidic pH the position of cotton effect at 217nm remains unchanged same as concanavalin A at pH 7 (data not shown). The far-UV CD data was plotted at 217nm as the function of pH [figure 25A]. Thus it can be concluded that with respect to MRE value at 217nm, throughout the acidic pH range from 5 to 2 the presence of methyl α-D-glucopyranoside provides the protection to concanavalin A on pH exposure. In the presence of non-specific sugar (methyl α-D-galactopyranoside) at acidic pH range, different MRE values were obtained. Methyl α-D-galactopyranoside induces α-helix in β-sheet concanavalin A as was evident by increase in MRE value at 222nm (data not shown for clarity). Figure 25B shows the MRE value at 293nm of concanavalin A as the function pH, in the presence of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside. In the presence of methyl α-D-glucopyranoside in pH range from 7 to 2 the structure does not appear to be disrupted at the carbohydrate binding site since carboxyl and amino groups of concanavalin A were not available for titration when this lectin was in contact with the glycoside. As the pka of α carboxylic acid is approx. 2.3, while that of aspartic acid side chain is 3.9 so loss of proton would have lead to the presence of negative charge on side chain of amino acid resulting in charge-charge repulsion. Since methyl α-D-glucopyranoside is sitting at the
carbohydrate-binding pocket and was in contact by hydrogen bonds, vander Waals and hydrophobic interactions with the side chain of amino residues, present in the pocket. So, it can be concluded that methyl α-D-glucopyranoside was responsible for the protection effect in acidic pH range while this effect was absent in methyl α-D-galactopyranoside.

Figure 26A shows the MRE value at 217 nm of concanavalin A at alkaline pH in the presence of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside. As can be seen from the figure, there was no significant change in MRE values at 217 nm in the presence of methyl α-D-glucopyranoside. In the presence of methyl α-D-galactopyranoside in this region of pH, a different state was obtained. Figure 26B shows the near-UV CD spectra in terms of the MRE values at 293 nm of concanavalin A at alkaline pH, in the presence of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside. In presence of methyl α-D-glucopyranoside, there was retention in MRE values at 293 nm almost same as native. At this pH the deprotonation of amino groups would have occurred resulting in net negative charge on protein main chain especially for the deprotonation of NH₂ group of amino acid present at the carbohydrate-binding site since pk for α NH₂ is 9.90. However, methyl αD-glucopyranoside binds to the carbohydrate binding pocket of concanavalin A which prevented titration of functional group especially α NH₂ group. Non-specific sugar, in our case, methyl αD-galactopyranoside altered the conformation of concanavalin A at different pH, hence resulting in a different stable state.

3.5.4 Intrinsic fluorescence: Figure 27 shows the fluorescence spectra of native concanavalin A at pH 7 (curve 3), in the presence of methyl α-D-glucopyranoside (curve 2), in the presence of methyl α-D-galactopyranoside (curve 1). At pH 7, when methyl α-D-glucopyranoside was at the binding site of native concanavalin A, λ-max was obtained at 335 nm with slight decrease in fluorescence intensity as compared to that of native protein (curve 2). While in the presence of methyl α-D-galactopyranoside at pH 7, a quenched fluorescence spectrum was obtained. The CD and fluorescence data together suggest that the conformational state of concanavalin A does not remains same as native in presence of methyl α-D-glucopyranoside as there is a change in the spectra. Thus, the
Fig. 22A. Far-UV CD values in the terms of MRE at 217nm as the function of pH from 2 to 10. Experiments were carried out in 0.02 M of the following buffers: pH 2, glycine HCl buffer; pH 3-5, sodium-acetate buffer; pH 7, sodium phosphate buffer; pH 8-10 gly-NaOH buffer. Protein concentration was 1μM.
Fig. 22B. Near-UV CD value in the terms of MRE at 293 and 269 nm as the function of pH from 2 to 10. Same buffer system was used as previously mentioned. Protein concentration was 10μM.
Fig. 23. Fluorescence emission values at 335nm of concanavalin A at different pH range from 2 to 10. Same buffer system was used as reported previously. Protein concentration was 5μM.
Fig. 24. Far UV CD spectra of concanavalin A at pH 7 (0.02 M sodium phosphate (curve 1), in the presence of 0.05 M methyl αD-glucopyranoside (curve 2), and in the presence of 0.05 M methyl α-D-galactopyranoside (curve 3) respectively. Protein concentration was 1μM.
Fig. 25A. MRE values at 217 nm at acidic pH from 7 to 2 (♦), in the presence of 0.05 M methyl α-D-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 1 μM.
Fig. 25B. MRE value at 293 nm at acidic pH (●) and in the presence of 0.05 M methyl α-D-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 10 μM.
Fig. 26A. MRE value at 217 at alkaline pH from 7 to 10 (•), in the presence of 0.05 M methyl α-D-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 1μM.
Fig. 26B. MRE value at 293nm at alkaline pH from 7 to 10 (○) and in the presence of 0.05 M methyl α-D-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 10μM.
Fig. 27. Fluorescence spectra of concanavalin A at pH 7 (0.02 M sodium phosphate) (curve 3), in the presence of 0.05 M methyl α-D-glucopyranoside (curve 2), in the presence of 0.05 M methyl α-D-galactopyranoside (curve 1) respectively. Protein concentration was 5 μM.
presence of specific sugar (methyl α-D-glucopyranoside) at pH 7 does not induce much conformational alterations. In the presence of methyl α-D-galactopyranoside a stable conformational state, different from native was obtained which involves the masking of tryptophan residue or causing some conformational changes in the lectin, which results in the internalization of tryptophan residue.

On titrating concanavalin A from pH 7 to 2, there was gradual decrease in fluorescence intensity at 335nm. While in the presence of methyl α-D-glucopyranoside the fluorescence intensity remains almost same as that of native (figure 28). Since methyl α-D-glucopyranoside is a highly polar molecule due to it there has been gross conformational change as a result of binding leads to internalization of tryptophan. Since blue shift is occurring (data not shown), this shift is associated with the binding indicates that water is excluded in the complex. Thus, protecting it from titration at all acidic pH as predicted from the plot of FI at 335 nm as a function of pH. In the presence of methyl α-D-glucopyranoside no major conformational alterations on the exposure at low pH were observed, indicating the protective role of specific sugar. Conformational changes in tertiary structure of protein as monitored by fluorescence is reflection to changes in the aromatic amino acid residues (tryptophan and tyrosine) and also change in protein environment due to deprotonation of aspartic acid present at the carbohydrate binding site and are chiefly involved in the interaction with specific sugar [141,142]. Glutamic acid and also other main chain aspartic residues are also responsible for this conformation of concanavalin A at pH 2. Moreover, X-ray crystallographic analysis has delineated the exposed nature of two of the four tryptophans of concanavalin A [143]. As can be seen from the plot of fluorescence intensity at 335nm vs. pH in the presence of methyl α-D-glucopyranoside (figure 29) the polar binding cavity of concanavalin A was protected, which prevents it from alkaline pH denaturation. While a decrease in fluorescence intensity at 335nm is obtained in the presence of methyl αD-galactopyranoside, which persists as obtained at pH 7. At alkaline pH the bound methyl αD-glucopyranoside (to concanavalin A) reduces the number of charges on protein thus, nullifying the effect of pH when sugar is present in the carbohydrate-binding site. The amino acid present in
Fig. 28. Fluorescence intensity at 335nm of concanavalin A at acidic pH range (●), from 7 to 2 in the presence of 0.05M-methyl α-D-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 5μM.
Fig. 29. Fluorescence intensity at 335nm of concanavalin A at, at alkaline pH range (•) from 7 to 10 and in the presence of 0.05 M methyl αD-glucopyranoside (▲) and in the presence of methyl α-D-galactopyranoside (■). Protein concentration was 5μM.
carbohydrate binding site are aspartic acid and tyrosine. They mainly tyrosine, were prevented from deprotonation of functional groups which otherwise would have been negatively charged at alkaline pH. The conformational changes between pH 7 and 10 are more likely due to either deprotonation of lysine residues or ionization of tyrosine. This factor dominates and act as protective over the conformational changes in concanavalin A as caused by alkaline pH denaturation. Hence, specific sugar acted as a protector on concanavalin A over pH denaturation

3.6 Discussion: Lectin-carbohydrate complex usually involves hydrogen bonding, vander Waals forces and hydrophobic interactions. These non-covalent forces must have been responsible for the existing stable conformation when carbohydrate is present at the binding site. Keeping this conformation in view, the protective role of specific and non-specific saccharide to concanavalin A at different pH by using the fluorescence and CD spectroscopic techniques. Concanavalin A is a metal-requiring lectin specific for αD-glucopyranosyl and αD-mannopyranosyl residues. At neutral pH concanavalin A exists as a tetramer. As the pH is lowered dissociation to a dimer occurs, which is completed by about pH 5.5. The results of the present study demonstrated that under the experimental conditions, the conformation of concanavalin A is pH dependent. Methyl αD-glucopyranoside and not methyl αD-galactopyranoside has a protecting effect on concanavalin A. Since the isoelectric point of concanavalin A has been reported at pH 7.0, when pH is lowered on going from the positively charged protein to a protein approaching zero net charge, the conformation of the protein changes. The large alteration at alkaline pH is observed as compared to that at acidic pH because more acidic amino acid residues are present in concanavalin, A which acquire a negative charge and lead to charge repulsion. Results obtained on binding of non-specific sugars showed a major conformational changes occurring in the tertiary structure of the lectin. Thus, it can be interpreted as an intermediate state, which is, obtained at all pH and further resist the conformational alterations at different pH. The conformational changes can be supported as one tryptophan is situated near the lip of the large polar cavity (residue 182) while the other is involved in monomer monomer contact (residue 88) [143]. It has also been reported that the fluorescence of fluorescent labeled 4-methylumbelliferyl α-D-
mannopyranoside is quenched on binding to concanavalin A [141]. In a simpler fashion,
lectins, which have a high degree of carbohydrate specificity, make a useful model for
protein carbohydrate interactions. An attempted has been made here to monitor the effect
of pH on concanavalin A conformational states, assuming that best fit ligand will prevent
the protein to undergo any major conformational alteration under any stress condition.