Chapter 4

Formation And Stabilization Of Acid Unfolded Intermediate States Of Some Lectins In Presence Of Fluoroalcohols

Work from this chapter published in:

4.1 Introduction

4.1.1 Lentil lectin

Lentil lectin belongs to the leguminosae family to the group of glucose/mannose specific lectins. It is a glycoprotein and requires Ca\(^{2+}\) and/or Mn\(^{2+}\) metal ions to be active. It has a composition of \(\alpha_2\beta_2\). The two \(\alpha\)-chains being the light chains and \(\beta\)-chains being the heavy chains, with a molecular mass of 49 KD [150]. Other lectins belonging to this specificity group are lectins from pea (Pisum sativum), fava bean (Vicia faba), common vetch (Vicia cracca) and forage legume sainfoin (Onobrychis vicifolia) [150]. Studies have been performed by Marcos et al [151] in which they have compared the thermal stability of lentil lectin at pH 2 with its stability at pH 7 by differential scanning calorimetry, intrinsic fluorescence and CD-studies and have reported a compact residual structure of lentil lectin at pH 2. In our work we have shown the existence of a molten globule like folding intermediate of lentil lectin at pH 1 with some amount of secondary structure and disrupted tertiary structure, this intermediate is different from the compact denatured state at pH 2. Effect of
fluorinated alcohols TFE and HFIP was also checked on this acid unfolded intermediate obtained at pH 1.

4.1.2 Pea lectin

Pea lectin exists as a dimer, has a molecular weight 49,000, requires Ca\(^{2+}\) and Mg\(^{2+}\) ions for carbohydrate binding, and has a single carbohydrate binding site per monomer. Each of the pea lectin monomers consists of an \(\alpha\) and a \(\beta\) chain. The crystal structure of pea lectin shows that the polypeptide chain takes a convoluted pathway to form two major and one minor ant parallel \(\beta\) sheet structures [110]. Extensive random coil structures are overlaid onto the front of the molecule. The two monomers join together to form the typical canonical dimer.

The Conformational stability of the pea lectin has been determined by both isothermal urea-induced and thermal denaturation in the absence and presence of urea by Ahmad et al [152]. We have characterized two different folding intermediates of the lectin under the influence of low pH and have also investigated the effect of fluoroalcohols on one of the intermediates characterized.
4.1.3 Concanavalin A (ConA)

ConA, first crystallized by Sumner and Howell, 1936 [153] has proven to be an interesting and useful lectin. The molecular structure of Con A has been reported by Becker et al. 1976 [154], Becker et al. 1975, [115] Cunningham et al. 1975, [155] Reeke et al. 1975, [156] and Wang et al. 1975, [157]. It is composed of identical subunits of 237 amino acid residues. At neutral and alkaline pH, Con A exists as a tetramer of four identical subunits of approximately 26,000 daltons each. Below pH 5.6, Con A dissociates into active dimers of 52,000 daltons. Con A binds two metal ions per monomer: a transition metal at site S1 and Ca$^{2+}$ at site S2. Both ions must be present for saccharide binding. Con A reacts with non-reducing α-D-Glucose and α-D-mannose. The molecular weight of Con-A is 104 kDa and the pI is 4.5-5.5. The detailed structure [158, 159] reveals several striking features, including a number of regular structures that allow for an elegantly simple set of subunit interactions. In particular, the molecule has a unique arrangement of β-structures, which is of considerable interest to the question of how proteins fold.
4.1.4 Fluoroalcohols (2,2,2 trifluoroethanol and 1,1,1,3,3,3-hexafluoro-isopropanol)

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 [160-162]. Non-native states are easily attained by solvent perturbation [69,163,164]. An extensively studied co-solvent that modifies the protein conformation is alcohol. Alcohol destabilizes hydrophobic cores of protein while it enhances secondary structure formation by minimizing exposure of peptide backbone. They exert thermodynamic force to minimize exposure of peptide backbone, this intrachain interaction acts not only among residues neighboring in the primary sequence to form α-helices but also among those at a distance in the sequence to induce a compact conformation. However due to non-polar character of alcohol a counteracting force is also exerted that expands the protein conformation [165-167]. A balance between these two opposing forces is responsible for the highly helical but expanded conformation of proteins in alcohol solutions [168, 169]. Among various alcohols, fluoroalcohols are often used because of their high potential for stabilizing the helical structure candidate alcohols of this type are 1,1,1,3,3,3-hexafluoro-isopropanol and 2,2,2 trifluoroethanol. The high helix
inducing capacity of TFE as compared to other non-fluorinated alcohols can be accounted to the fluorination of the alcohol but the enhanced activity of HFIP cannot be explained simply on the basis of the additive effects of the constituent parts (CF3 surface area, etc). It is suggested that the "cooperative formation of micelle like clusters of HFIP is important".

Studies on alcohol effects provide insights into biologically important events because the alcohol solution mimics the environment of biomembrane [170], modifies folding pathways of proteins [171, 172] and induces the assembly of biologically relevant peptides [173, 174]. TFE is a protein denaturant, which has also been shown to induce the molten globule state in many proteins [175, 176], TFE is often preferred in such studies because of its high potential in stabilizing the α-helical structure [177, 178]. The secondary structures stabilized by TFE are assumed to reflect conformations that prevail during early stages of protein folding [176, 178, 179]. HFIP has been used to unfold aggregates of the Alzheimer's amyloid peptide Aβ [180-182] or prion protein peptide [183,184]. The unfolding of amyloid aggregates by HFIP results in the formation of helical structure. HFIP is also used in the field of peptide chemical synthesis because it effectively dissolves peptide aggregates that are insoluble in both water and strong hydrophobic solvents.
4.2 Results and Discussion

4.2.1 Fluoroalcohol-induced stabilization of the α-helical intermediates of lentil lectin: Implication for non-hierarchical lectin folding:

4.2.1.1 Secondary and tertiary structure of lentil lectin at different pH

Far and near-UV CD studies of lentil lectin were carried out in the pH range 7-0.8. Figure-15 (a) shows the pH transition curve at 217 nm, with decrease in pH from 7 to 0.8 a continuous loss of secondary structure was observed. Figure 15(b) represents the far UV-CD spectrum of native lectin at pH7 with a trough between 220 nm-230 nm and a positive peak near 200 nm characteristic of a β-structure (curve-1), at pH 2 protein showed some residual β-structure (curve-2).

![Figure 15](image-url)

These features are in accordance with earlier data presented by Marcos et al [151]. On further lowering the pH to 1 it was found that the protein looses the residual β-structure which existed at pH 2 and acquires an unordered confirmation (curve-3) This acid unfolded state is different from the completely denatured state of the protein in presence...
of 6 M GndHCl (curve-4). Thus the spectrum of protein at pH 1 shows an intermediate state, which is different from the native state as well as from the completely denatured state.

Figure-16 (a) shows the pH transition curve at 270 nm. As observed for the secondary structure, in this case also a continuous loss of tertiary structure was observed with decreasing pH. Figure-16 (b) depicts the near-UV CD spectrum of lentil lectin at pH 7 (curve-1), pH 2 (curve-2), pH 1 (curve-3) and in presence of 6 M GndHCl (curve-4).

![Figure 16](image)

**Fig. 16** pH transition curve in the Near-UV region (a). Ellipticity measurements were carried out at 270 nm. Near-UV CD spectrum of lentil lectin at pH 7 (— — —), curve-1; pH 2 (-----), curve-2; pH 1 (— — ), curve-3 and in presence of 6M GndHCl (----------), curve-4 (b).

As can be seen from the figure the spectrum at pH 2 shows resemblance with that of the native protein indicating the presence of a compact tertiary structure (as reported by Marcos et al [151]), although the spectrum at pH 1 has spectral features resembling to those of curve-2 but the intensity of the signals decrease and approach to that of lentil lectin in the presence of 6M GndHCl, reflective of less ordered tertiary structure. This data shows that some tertiary contacts that were retained at pH 2 are disrupted at pH 1.
Figure 17 depicts the intrinsic fluorescence spectra of lentil lectin.

![Fluorescence Spectra Image]

**Fig. 17** Tryptophan fluorescence spectrum of lentil lectin. Lentil lectin at pH 7, in 20 mM sodium-phosphate buffer (---), curve-1; at pH 2, in 20 mM Glycine-HCl buffer (----), curve-2; at pH 1, in 20 mM Glycine-HCl buffer (---), curve-3 and in presence of 6M GndHCl (.....), curve-4.

The red shift from 330-340 nm obtained in case of acid denatured lectin at pH 2 (curve-2) and at pH 1 (curve3) as compared to the native at pH 7 (curve-1) can be accounted to the exposure of tyrosine and tryptophan residues, lowering of fluorescence intensity at pH 1 from pH 2 again indicates loss of structure at pH 1 state but the red shift of the acid denatured states is less and fluorescence intensity greater than the protein in presence of 6 M GndHCl with λ<sub>max</sub> at 350 nm thus at pH 1 although protein shows loss of tertiary structure as compared to pH 2 state but is not completely denatured.

To confirm the environment of tryptophan residues fluorescence-quenching experiment was performed using the uncharged molecules of acrylamide as described by Eftnik and Ghiron [127]. Figure-18 shows the Stern-Volmer plot of quenching of fluorescence by acrylamide in native, acid unfolded at pH 1 and 6 M GndHCl-denatured lentil lectin.
Results for the tryptophan analogue NATA are also included as a standard for complete accessibility to quencher.

![Stern-Volmer plot of acrylamide quenching. Native lentil lectin at pH 7 (—▲—); acid denatured lentil lectin at pH 1 (—●—) and NATA (—●—) and GnHCl (—■—). Values shown are the ratios of fluorescence in the absence of acrylamide (F₀) to the fluorescence at that concentration of quencher (F).](image)

Acrylamide quenching parameters of lentil lectin have been summarized in Table-VI. $K_{sv}$ for the acid denatured state was found to be higher (5.1) than that for the native state (1.3) accompanied by a red shift from 330-340 nm.
These results indicate that the tryptophan residues in the acid-unfolded state were more accessible to quenching by acrylamide than in the native state. The red shift in the emission maximum of tryptophan indicated that the residues became more accessible to the solvent in the acid denatured state. The $K_s$ values for the GndHCl-denatured and NATA were significantly higher than those for native and acid unfolded state suggesting that the tryptophan residues were not fully accessible to the quencher even at pH 1. These results are in agreement with the results deduced from near-UV CD and intrinsic fluorescence studies. ANS binding studies showed maximum binding at pH 1 and almost no binding at pH 7 and in presence of 6 M GndHCl (figure-19), which is again indicative of the presence of an intermediate state at pH 1. A considerable amount of ANS binding was also seen in the pH 2 state, which indicates that an intermediate exists at pH 2 in which hydrophobic patches are exposed but to some lower extent as in case of pH 1 state.
Thus, from CD and fluorescence studies taken together we can say that at pH 1 lentil lectin exists as an intermediate state with unordered secondary structure and disrupted tertiary structure. In order to further confirm the intermediate state at pH 1, GndHCl denaturation was performed and monitored by intrinsic fluorescence spectroscopy. The fluorescence intensity thus obtained was plotted against increasing GndHCl concentration (figure-20) the unfolding transition curves show that unfolding of native protein is a cooperative process (curve represented by filled circles), transition started at 1.4 M GndHCl and became constant after 3 M GndHCl. On the other hand acid unfolded protein showed lack of cooperativity where a sharp transition occurred at GndHCl concentration as low as 0.2 M and decreased continuously there after (curve represented by open circles). This lack of cooperativity associated with loose tertiary structure confirms the intermediate state of lentil lectin at pH1.
Fig. 20 Guanidine Hydrochloride-induced unfolding transition curve as measured by intrinsic tryptophan fluorescence at 332 nm. Protein concentration was 0.15 mg ml\(^{-1}\) in 20 m sodium phosphate buffer, pH-7 (—•—) and in 20 mM Glycine-HCl buffer, pH-1 (—o—).

4.2.1.2 Limited proteolysis

In order to further confirm the presence of acid unfolded intermediate limited proteolysis was performed. The proteolytic fragments were separated by SDS-PAGE, Protein: enzyme ratio was 50:1 (w/w). An acid-unfolded protein has a more open and extended conformation as compared to the native state and hence it is very likely that in the acid unfolded state the trypsin cleavage sites get exposed and when subjected to trypsic digestion result in greater number of fragments than the native state. Figure-21 shows the gel pattern of limited trypsic digestion of lentil lectin in native state at pH 7 (A) and in acid unfolded state at pH 1 (B), lane 1 and 4 represent undigested preparations of pH 7 and pH 1 while lanes 2,3 and 5,6 contain reaction mixtures in which digestion was allowed for 30 minutes and 60 minutes respectively. As can be seen in the figure the major bands prominent in all the pH 7 preparations diminished in lane-5 and further in lane-6 the bands almost disappeared. This decrease in band intensity and finally disappearance of the major bands signify the cleavage of the major fragment into smaller digestive fragments with increasing time interval. No such band pattern
was found with native preparation. These results are suggestive of the fact that at pH 1 protein exists as an extended intermediate.

Fig. 21 Limited tryptic digestion of A: Lentil lectin at pH 7; B: Lentil lectin at pH 1. The digestions were performed at protein: trypsin ratio of 50:1 (w/w). The samples were incubated for 30 mins (lane-2, 5) and 60 mins (lane3, 6), lanes 1 and 4 contain undigested preparations

4.2.1.3 Effect of TFE and HFP on acid unfolded lentil lectin

TFE and HFIP are known to stabilize the α-helical structure in proteins and their fragments. These fluoroalcohols are also known to effect protein structure. Studies conducted by Segawa et al [185] showed that the effect of TFE in inducing the helical conformation is not non-specific and the fragments arising from the helical region of the native structure have a high tendency to form a helix in the presence of TFE, whereas the β-region has a low tendency to do so. But the results described by Shiraki et al [179] suggest that the helical propensity of proteins in the presence of TFE varies significantly according to protein species, and are somewhat different from the expectation that the helical propensity in TFE is correlated with the helical propensity of the native structure. Effect of TFE (0-70% v/v) was checked at pH 1 preparation of lentil lectin, for clarity only 0%, 20% and 70% curves are depicted in figure-22 (a), curve-1, 2 and 3 respectively along with native protein at pH 7 (curve-4). In presence of 60% (v/v) 2,2,2 Trifluoroethanol (TFE) acid unfolded lentil lectin acquired some features of an α-helical structure with a trough near 208 nm and a slight shoulder around
217 nm. Effect of HFIP from 0-70% (v/v) was also observed in the far UV-region. This transition was significant up to 30% HFIP (curve-3), further increase in HFIP concentration up to 70% had little effect on the helical structure, so for the sake of clarity only) 0%, 10%, 20% and 30% curves are shown in figure-22 (b), curve-4 and 5 correspond to acid-unfolded protein (0% HFIP v/v) and native protein at pH 7 respectively. More evident and clear transition to α-helix in β-rich lectin was observed at much lower concentration of HFIP (20% v/v), curve-2 figure-8 (b). Interestingly it was observed that at 10% HFIP (curve-1) the acid unfolded structure showed a minimum at 217 nm, which is a feature of a β-structured protein while addition of HFIP at concentrations more than 10% (curve-2) changed the far-UV spectrum dramatically to that of an α-helical structure with a sharp minima at 208 nm and 222 nm.

![Figure 22](image) Far-UV CD spectrum of lentil lectin at pH 1 showing the effect of increasing concentrations of (a). TFE, 0% TFE; (—) curve-1, 30% TFE (-----) curve-2 and 60% TFE (—·—) curve-3. Curve-4 corresponds to native protein. (b). HFIP, 0% HFIP (——) curve-4, 10% HFIP (——) curve-1, 20% HFIP (—·—) curve-2, 30% HFIP (........) curve-3. Curve-5 corresponds to native protein taken for comparison.

The decreased helix-stabilizing effect of TFE Vs HFIP has been indicated by Cort & Anderson [186]. Alcohols exert thermodynamic force to minimize exposure of peptide backbone [165,167], this intrachain interaction acts not only among residues
neighboring in the primary sequence to form α-helices but also among those at a
distance in the sequence to induce a compact conformation. However due to non-polar
character of alcohol a counteracting force is also exerted that expands the protein
conformation [165-167]. A balance between these two opposing forces is responsible
for the highly helical but expanded conformation of proteins in alcohol solutions
[168,169]. In many previous studies [187,188] it has been observed that in a low
concentration range of HFIP, the force minimizing the exposure of main chain
components is probably more dominant than the force that expands the denatured chain.
From figure-23 it can be seen that with increasing concentration of TFE and HFIP
fluorescence intensity goes on increasing along with a blue shift in both the cases. This
blue shift along with increase in fluorescence intensity, as compared to the acid
unfolded state, could be attributed to the conformational changes in the vicinity of
tryptophan residues, presumably due to internalization in a more hydrophobic
environment with the formation of α-helical structure as is shown from the far UV-CD
studies.

Fig. 23 Effect of increasing concentrations of TFE (—•—) and HFIP (—■—) on acid-
unfolded state of lentil lectin as monitored by changes in fluorescence intensity at
332 nm. Changes in λ max (inset).
Thus, addition of fluoroalcohols introduce non-native ordered secondary structure in the unordered state at pH 1, and the induction of helicity, as already discussed is greater in case of HFIP while figure-24 shows that no significant effect of these fluoroalcohols was found on the overall pH-disrupted tertiary structure of the lectin (as discussed in figure 16b).

Fig. 24 Near-UV CD spectrum of lentil lectin at pH 7 (——) curve-1; acid unfolded lentil lectin in presence of 70% TFE (—·—), curve-2; in presence of 30% HFIP (———), curve-3 and in presence of 6M GndHCl (........), curve-4.

Fig.25 depicts extrinsic fluorescence studies. ANS binding decreased with increasing TFE concentration and showed maximum binding at 30% HFIP concentration. Maximum ANS binding in presence of 30% HFIP, substantial amount of secondary structure and absence of tertiary structure clearly indicates the presence of HFIP induced molten globule like intermediate. No such intermediate was detected with TFE although some amount α-helix formation was seen at 70% TFE (v/v). Thus HFIP and TFE stabilize the acid unfolded state differently.
Fig. 25 ANS fluorescence of lentil lectin at pH 1 as a function of increasing concentrations of fluoroalcohols. In presence of TFE (—○—) and HFIP (—■—).

Some published data suggest that HFIP/water is a distinctly different solvent system from TFE/water, in particular at 4-10%, Denda et al [189]. Also Hirota et al [188] in their studies on β-lactoglobulin show that HFIP causes the transition of a native (β-sheet) to helical state at a concentration approximately one third the concentration required for a comparable effect by TFE. The high helix inducing capacity of TFE as compared to other non-fluorinated alcohols can be accounted to the fluorination of the alcohol but the enhanced activity of HFIP cannot be explained simply on the basis of the additive effects of the constituent parts (CF3 surface area, etc). It is suggested that the “cooperative formation of micelle like clusters of HFIP is important”. 

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4.2.1.4 Determination of protein activity

The activity of pH 7 preparation was taken as 100% and thus % activity of the other preparations of the protein was determined. At pH 1 loss in activity was found with both dextran and bromelain while the reported intermediate at the given pH was found to regain activity with bromelain in presence of HFIP and TFE. Activity with dextran in presence of fluoroalcohols could not be determined as turbidity developed in the corresponding blank preparation i.e. TFE and HFIP formed complex with dextran, (the results have been summarized in Table–VII). The observed changes in activity of different lectin preparations could be accounted for by our previous results. The acid denatured protein showed unordered structure and hence considerable decrease in activity was obtained as compared to the native preparation. Addition of fluoroalcohols induced ordered structure in the acid unfolded state, although non-native but the induction of secondary structure resulted in the regain of activity of the protein to some extent.

Table-VII. % activity measured for lentil lectin under different conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bromelain (% activity)</th>
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<tbody>
<tr>
<td>pH 7</td>
<td>100</td>
</tr>
<tr>
<td>pH 1</td>
<td>25</td>
</tr>
<tr>
<td>70% TFE</td>
<td>40</td>
</tr>
<tr>
<td>30% HFIP</td>
<td>56</td>
</tr>
</tbody>
</table>

Regain of activity in presence of HFIP and TFE was also found to be in accordance to the amount of secondary structure induced by the two. HFIP induced more structure as compared to TFE and hence a greater regain in activity of about 30% was observed with HFIP as compared to a 15% regain with TFE. Our results show that the fluoroalcohols (TFE and HFIP) stabilize the acid unfolded state of lentil lectin, which is mainly a β-
sheet protein, by inducing α-helical contacts. Interestingly, it was observed that induction of the non-native structure resulted in regain of protein activity to some extent. It has been shown by Shiraki et al [179] that β-lactoglobulin which consists predominantly of β-sheets, instead of following hierarchical model proceeds through non-hierarchical model of protein folding according to which the native structure is not necessarily formed sequentially as established hierarchy of the native structure. Although the hierarchical model of protein folding is popular, our results presented here point out the possibility of the formation of a helical structure preceding the formation of the native β-sheet structure as already shown for β-lactoglobulin [179], and support the non-hierarchical model of protein folding for lentil lectin. As already discussed alcohol-induced stabilization of the helical structure is known for some β-sheet proteins, including β-lactoglobulin, nevertheless no comprehensive work has been carried out with lectins and this is for the first time we report here a non-hierarchical model of protein folding pathway for a lectin. A scheme describing a model for folding has been given below. An attempt has been made to cover both the structural and functional aspects of the lectin under the influence of fluoroalcohols.
4.2.2 Characterization of a common intermediate of pea lectin in the folding pathway induced by TFE and HFIP:

4.2.2.1 Secondary and tertiary structure of pea lectin at different pH as observed by CD-studies.

Fig. 26 (a) shows the far-UV CD spectra of pea lectin at pH 7 (curve-1), pH 2.4 (curve-2), pH 1 (curve-3) and in presence of 6 M GndHCl (curve-4), it can be seen that the conformation of the protein at pH 2.4 and pH 1 is different from that of native as well as completely denatured state in presence of 6M GndHCl. From the transition curve plotted at 217 nm, Fig.26 (b) it can be stated that secondary structure of pea lectin is quite resistant to low pH. Upto pH 2.4 there is only a little change in structure. Below this pH, at pH 1 further loss in structure is seen.

The known pea lectin amino acid sequence and knowledge of the secondary structure shows that it has 27 acidic amino acid residues and most of them are approximately 50% exposed and few of these residues like Glu-119 and 223 and Asp-81, Asp-121, Asp-140 and Asp-195 are buried in the native form. It is probably the titration of the side chains of these acidic amino acid residues below pH 2.4 which is responsible for the sharp transition observed. Fig.27 (a) shows the near-UV CD spectra of the lectin at pH 7 (curve-1), pH 2.4 (curve-2), pH 1 (curve-3) and completely denatured in presence of 6 M
GndHCl. Curves 2 and 3 seem to resemble the spectrum of the native protein at pH 7. Transition curve plotted at 285 nm as a function of pH (Fig.27b) show that there is no considerable change in MRE values on going from pH 7 to pH 2.4, then MRE values decrease up to pH 1.4. At pH 1 slight gain of structure was observed, regain in tertiary structure has been shown for glucose oxidase in an earlier communication from our lab [190].

![Fig. 27 Near-UV CD spectrum of pea lectin at pH 7 (—), curve-1; pH 2.4 (— — —), curve-2; pH 1 (———), curve-3 and in presence of 6M GndHCl (········), curve-4. (a). pH transition curve in the Near-UV region Ellipticity measurements were carried out at 285 nm (b).](image)

### 4.2.2.2 Tryptophan environment of pea lectin observed by intrinsic fluorescence.

The fluorescence data reported also supports the compact conformation of the protein at pH 2.4. The transition curve, Fig. 28, shows that as pH is decreased from pH 7 it causes enhancement in fluorescence intensity up to pH 2.4 accompanied by a blue shift of about 4 nm. A continuous decrease in fluorescence intensity along with a red shift of 4-5 nm is observed on going from pH 2.4 to pH 1, which is indicative of the exposure of tryptophan residues. The fluorescence spectrum of pea lectin at pH 7 (curve-1), pH 2.4 (curve-2), pH 1 (curve-3) and completely denatured preparation in presence of 6 M GndHCl is also shown (inset, Fig.28).
Fig. 28 Fluorescence intensity of pea lectin at 340 nm as a function of pH. Inset, Tryptophan fluorescence spectrum of pea lectin. Pea lectin at pH 7, in 20 mM sodium-phosphate buffer (---), at pH 2.4, in 20 mM Glycine-HCl buffer (-----), at pH 1, in 20 mM Glycine-HCl buffer (-----), and in presence of 6M GndHCl (-----).

Pea lectin has been shown to possess a total of 29 aromatic amino acid residues out of which 5 are tryptophan residues. Trp-53, Trp-128 and Trp-206 are a little exposed while Trp-52 and Trp-227 are buried in the native form. Rest of the aromatic residues are contributed by 10 tyrosine and 14 phenylalanine amino acids. All the phenylalanine residues are highly buried while in case of tyrosine Tyr-46, Tyr-100, Tyr-179 and Tyr-191 are more buried than the rest.

4.2.2.3 ANS binding studies.

Fig. 29 depicts the binding of the hydrophobic dye (ANS) to different structural forms of the lectin under varying pH conditions.
Fig. 29 ANS Fluorescence of pea lectin as a function of pH. Inset, Fluorescence emission spectra of ANS bound to native pea lectin at pH 7 (---), curve-3; acid unfolded pea lectin at pH 2.4 (— — —), curve-2; acid unfolded pea lectin at pH 1 (— * —), curve-1 and completely denatured pea lectin in 6M GndHCl (………), curve-4.

The transition curve shows maximum binding of ANS to pea lectin at pH 1 and considerable binding to the 2.4 preparation as compared to that of native state at pH 7. The spectra for protein at pH 1, 2.4, 7 and in presence of denaturing concentration of GndHCl are shown in inset, Fig.29, curves-1, 2, 3 and 4 respectively. The intrinsic fluorescence and ANS data show that at pH 2.4 the overall conformation of the protein is compact. The tryptophan residues are buried but the hydrophobic patches are somewhat more exposed than they are in the native state. At pH 1 unfolding occurs and the tryptophan residues as well as the hydrophobic patches get exposed allowing the binding of ANS, which results in the enhanced fluorescence intensity.

4.2.2.4 Acrylamide Quenching studies.

The observation that at pH 2.4 protein has a compact conformation is supported by acrylamide quenching experiment.
Acrylamide [M]

Fig. 30 Stern-Volmer plot of acrylamide quenching. Native pea lectin at pH 7 (—△—); acid denatured pea lectin at pH 1 (—×—); pea lectin at pH 2.4 (—•—) and NATA (—○—) and GndHCl denatured pea lectin (—■—). Values shown are the ratios of fluorescence in the absence of acrylamide (F₀) to the fluorescence at that concentration of quencher (F).

Figure-30 shows the Stern-Volmer plot of quenching of fluorescence by acrylamide in native, acid unfolded at pH 1 and pH 2.4 and 6 M GndHCl-denatured pea lectin. Results for the tryptophan analogue NATA are also included as a standard for complete accessibility to quencher.

Acrylamide quenching parameters of pea lectin have been summarized in Table-VIII. Ksv for the acid denatured state at pH 1 was found to be higher (2.7) than that for the native state (1.2) and that for the lectin preparation at pH 2.4 (1.5) accompanied by a red shift from 330-340 nm.
Table-VIII. $K_s$ values obtained from Stern-Volmer plot

<table>
<thead>
<tr>
<th>Subject</th>
<th>$K_s$ (M$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>Native Pea lectin</td>
<td>1.2</td>
</tr>
<tr>
<td>Acid-unfolded Pea lectin at pH 1</td>
<td>2.7</td>
</tr>
<tr>
<td>Acid-unfolded Pea lectin at pH 2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>GndHCl-denatured Pea lectin</td>
<td>5.2</td>
</tr>
<tr>
<td>NATA</td>
<td>14.9</td>
</tr>
</tbody>
</table>

These results indicate that the tryptophan residues in the acid-unfolded at pH 1 were more accessible to quenching by acrylamide than in the native state as well as the protein at pH 2.4. The $K_s$ values for the GndHCl-denatured and NATA were significantly higher than those for native and acid unfolded state suggesting that the tryptophan residues were not fully accessible to the quencher even at pH 1. These results are in agreement with the results deduced from near-UV CD and intrinsic fluorescence studies. Near and far-UV CD and fluorescence studies taken together indicate that secondary and tertiary structure of pea lectin is resistant to acidic pH down to 2.4. At pH 1 there is slight loss in secondary structure and regain in tertiary structure.

4.2.2.5 GndHCl induced transitions in the lectin at different pH.

In order to further confirm the intermediate states obtained at pH 2.4 and pH 1, GndHCl denaturation was performed and monitored by intrinsic fluorescence spectroscopy. The fluorescence intensity thus obtained was plotted against increasing GndHCl concentration (Fig-31).
The unfolding transition curves show that unfolding of native protein is a cooperative process (curve represented by filled circles), transition started at 1.4 M GndHCl and became constant after 3 M GndHCl. Some amount of cooperativity was also shown by protein at pH 2.4 supporting the CD and fluorescence data that pea lectin at pH 2.4 is quite stable and shows resistance to GndHCl denaturation. On the other hand acid unfolded protein at pH 1 showed lack of cooperativity where a sharp transition occurred at GndHCl concentration as low as 0.2 M and decreased continuously there after (curve represented by open circles). This lack of cooperativity associated with loose tertiary structure confirms the intermediate state of pea lectin at pH 1.

4.2.2.6 Effect of fluoroalcohols on the intermediate obtained at pH 2.4

Fig-32 shows the far-UV CD spectra of pea lectin at pH 2.4 in the presence of different concentrations of TFE.
Fig. 32 Far-UV CD spectrum of pea lectin at pH 2.4 showing the effect of increasing concentrations of TFE, 0% TFE (—), curve-2; 40% TFE (—), curve-3; 80% TFE (—), curve-4 and 90% TFE (-----), curve-5. Curve-1 and 6 correspond to native and GndHCl denatured protein respectively.

Curve-1 represents native pea lectin at pH 7, the spectrum as shown earlier is typical of a β-structured protein, curve-2 corresponds to the protein at pH 2.4 which represents an intermediate state different from native as well as completely denatured state in presence of 6 M GndHCl (curve-6). Increasing concentrations of TFE stabilize this state upto 80% TFE (v/v), which is evident from the increasing MRE values at 217 nm, only curve 3 and 4 corresponding to 40% and 80% TFE respectively, have been shown for the sake of clarity. Schonbrunner et al in their studies on all-β-sheet protein Tendamistat have shown that TFE induces a partially folded state in tendamistat that has lost most of its specific side chain interactions but that retains nearly all of the native β-sheet structure [191]. It is also clear from the figure that further at 90% TFE (v/v), curve-5, the stabilizing effect on the secondary structure is lost and the MRE value at 217 nm decreases.
Fig. 33 Near-UV CD spectrum of pea lectin at pH 7 (----) curve-1; acid unfolded pea lectin at pH 2.4 (---), curve-2; pea lectin at pH 2.4 in presence of 80% TFE (-----), curve-3; in presence of 4% HFIP (----), curve-4 and in presence of 6M GndHCl (.........), curve-5.

Loss in tertiary structure at 80% TFE concentration is seen from near-UV CD data (Fig-33, curve-3), curve-1, 2 and 5 correspond to native lectin, lectin at pH 2.4 and in presence of 6 M GndHCl.

Fig. 34 Effect of increasing concentrations of TFE on acid-unfolded state of pea lectin as monitored by fluorescence spectroscopy. Native pea lectin at pH 7 (---), curve-1; at pH 2.4 (---), curve-2; acid unfolded pea lectin at pH 2.4 in presence of 5%, curve-3; 80%, curve-4 and 90% TFE, curve-5. Curve-6 (.........) represents lectin in presence of 6 M GndHCl.
Intrinsic fluorescence spectra Fig-34 shows that the protein at pH 2.4 in presence of 5% TFE (v/v), curve-3 has fluorescence intensity more than that of native form, curve-1 which is again in accordance to our previous results indicating a compact tertiary structure at pH 2.4, curve-2, which is not affected at low concentration of TFE. With increasing TFE concentrations fluorescence intensity goes on decreasing, along with a red shift of the λ_{max}. Only 80%TFE, curve-4 and 90% TFE, curve-5 have been shown for clarity.

![Intrinsic fluorescence spectra](image)

**Fig. 35** ANS fluorescence of pea lectin at pH 2.4 in presence of fluoroalcohols. In presence of 80% TFE, curve-1 and 4% HFIP, curve-2. Native pea lectin at pH 7 (---), curve-4; at pH 2.4 (----), curve-3. Curve-5 (.....) represents lectin in presence of 6 M GdnHCl.

ANS studies conducted in presence of varying TFE concentrations show maximum binding of ANS at 80% TFE, figure-35 (curve-1), curve-3 corresponds to the protein preparation at pH 2.4 and curve-4 and 5 to native and completely denatured states. Thus the conformation in which the protein existed at pH 2.4 is so changed in presence of TFE.
such that the secondary structure is stabilized up to 80% (v/v) of TFE while the tertiary structure which was retained by the protein at the above said pH is lost and the folding intermediate of pea lectin at pH 2.4 changes to another intermediate state in presence of 80% TFE (v/v).

Effect of hexafluorisopropanol was also studied on the intermediate state, which has been shown to exist at pH 2.4. Figure-36 represents the far-UV CD spectra of the 2.4 preparation of the protein in presence of varying HFIP concentrations.

![Far-UV CD spectrum of pea lectin at pH 2.4 showing the effect of increasing concentrations of HFIP](image)

Fig. 36 Far-UV CD spectrum of pea lectin at pH 2.4 showing the effect of increasing concentrations of HFIP, 0% HFIP (---), curve-2; 4% HFIP (--), curve-3; 12% HFIP (---), curve-4; 16% HFIP (-----) curve-5; 30% HFIP (- - -), curve-6. Curve-1 corresponds to native protein taken for comparison.

Curve-1 shows the native state at pH 7 while curve-2 is indicative of the protein preparation at pH 2.4. Pea lectin in presence of 4% HFIP is represented by curve-3. In low concentration range of HFIP the pH 2.4 intermediate shows an increase in MRE at 217 nm retaining residual β-structure up to 12% HFIP (curve-4). At 16% HFIP (v/v) a sharp transition to typical α-helix is observed with a minima at 208 nm and 222 nm (curve-5). Beyond 30% HFIP, curve-6, no change in the spectra was observed. Loss in
tertiary structure at 4% HFIP is shown in Fig-33, curve-2. In presence of 4% HFIP a conformation is obtained similar to the one in presence of 80% TFE.

Fig. 37 Effect of increasing concentrations of HFIP on acid-unfolded state of pea lectin as monitored by fluorescence spectroscopy. Native pea lectin at pH 7 (—), curve-1; at pH 2.4 (---), curve-2; acid unfolded pea lectin at pH-2.4 in presence of 4%, curve-4; 8%, curve-5; 12%, curve-6; 16%, curve-7 and 30% HFIP, curve-8. Curve-3 (......) represents lectin in presence of 6 M GndHCl.

Protein at 4% HFIP, curve-4, Fig-37, shows maximum intrinsic fluorescence intensity which goes on decreasing accompanied by a red shift in $\lambda_{\text{max}}$ with increasing HFIP concentrations suggesting the exposure of aromatic residues (only curves-4-8 corresponding to 4%, 12%, 16%, 20% and 40% have been shown for the sake of clarity). Native protein is represented by curve-1, curve-2 and 3 represent protein at pH 2.4 and in presence of 6 M GndHCl respectively. A similar trend was observed in case of TFE. The observation deducted from far-UV CD spectrum that conformation of the protein at 4%HFIP and 80% TFE resemble each other is supported by maximum binding of ANS to the protein in presence of 4% HFIP, figure-35(curve-2) and 80% TFE, figure-35 (curve-
In many proteins there is clear evidence for conversion of β-sheet to α-helix upon addition of TFE and HFIP, as judged by CD measurements [179]. It has also been reported that HFIP is a more effective helix inducer than TFE [186]. The greater ability of HFIP in inducing α-helical structure cannot be explained by a simple additive contribution of abundant F atoms and bulky alkyl groups, suggesting that the cooperative formation of the micelle-like assemblies is involved [188].

Here we show that although HFIP and TFE differ in their helix inducing capacity and have different modes of action on a particular protein leading to different types of structure at respective concentrations but in case of pea lectin an intermediate structure is obtained that is common to both TFE and HFIP. As discussed earlier, an intermediate is obtained at 80% TFE which lacks α-helical structure, a similar conformation is acquired by the protein in presence of 4% HFIP, further when HFIP concentration is increased structural rearrangements occur leading to the increased α-helical contacts. This transformation from β-structure to α-helical structure starts from 16% HFIP and is completed at around 30%HFIP, further increase in HFIP concentration was found to have no affect on protein structure. Although the hierarchical model of protein folding is popular, our results presented here also point out the possibility of the formation of a helical structure preceding the formation of the native β-sheet structure as already shown for β-lactoglobulin [179], and support the non-hierarchical model of protein folding.
4.2.3 Structural Intermediates of acid unfolded Con-A in presence of fluoroalcohols.

4.2.3.1 Acid denaturation of Con-A

Con-A was subjected to acid unfolding in the pH range of 7-0.6. Circular dichroism and fluorescence studies showed that Con-A exists as molten globule like intermediate at pH 2. Fig. 38(a) shows the secondary structure of Con-A under different conditions, as can be seen from the figure, curve-2 that represents the lectin at pH 2 closely resembles the native state, which is represented by curve-1. Lectin at pH 2 is entirely different from the completely denatured state in presence of 6M GdnHCl, curve-3.

![Fig. 38 (a) Far-UV CD spectra of Con-A at different pH. Native Con-A; curve-1, Con-A at pH 2; curve-2, Denatured Con-A in presence of 6M GdnHCl; curve-3.](image)

Fig 38(b) shows the tertiary structure of the protein in native state, at pH 2 and in presence of 6M GdnHCl represented by curve 1, 2 and 3 respectively. Curve-2 shows loss of signals prominent in that of the native state and approaches towards the guanidine hydrochloride state indicating the loss of tertiary contacts at pH 2.
Fig. 38 (b) Near-UV CD spectra of Con-A at different pH. Native Con-A; curve-1, Con-A at pH 2; curve-2, Denatured Con-A in presence of 6M GndHCl; curve-3.

Fig 38 (c) shows maximum binding of ANS to Con-A at pH 2, Thus figure 38 as a whole clearly indicates the existence of Con-A as molten globule like intermediate at pH 2 with native like secondary structure, disrupted tertiary structure, and maximum binding of ANS.

Fig. 38 (c) ANS Fluorescence transition curve of Con-A as a function of pH.
4.2.3.2 MG-like state of Con-A under the influence of Fluoroalcohols.

The secondary structures stabilized by TFE are assumed to reflect conformations that prevail during early stages of protein folding [176,178,179]. HFIP is reported to have a much higher potential than TFE to induce α-helical conformation. The marked potential of HFIP cannot be simply explained by simple additive contribution of F-atoms. It is related to a high preference of HFIP to form the micelle like assembly [188]. It is known that addition of alcohols to already unfolded proteins and peptides induces secondary structures, in particular, the helical conformation [192,193,178,179].

Effect of 2,2,2 trifluoroethanol on acid unfolded Con-A.

Fig.39(a) and (b) show that on addition of TFE to the MG-like intermediate of Con-A at pH 2 there was a sudden change in the conformation of the protein which is evident from the figure by a considerable decrease in CD value at 223 nm, of acid unfolded Con-A in presence of 2% TFE. On further increasing the concentration of TFE, CD values went on increasing and it was observed 10% TFE marked the beginning of the structural transition from beta to helix.

![Graph A](image)

![Graph B](image)

Fig. 39 (a) Far-UV CD spectrum of pH 2 intermediate of Con-A in presence of varying TFE concentrations. Native Con-A; curve-1, Con-A at pH 2; curve-2, 8%TFE; curve-3, 10%TFE; curve-4, 20%TFE; curve5, 30%TFE; curve-6, denatured Con-A in presence of 6M GndHCl; curve-7.

(b) Transition plot showing the changes in far-UV CD in pH 2 intermediate of Con-A with increasing concentrations of TFE at 223 nm.
As shown in figure 39(a) the spectrum representing the protein in presence of 10% TFE (spectrum-4) undergoes a shift of few nm indicating a possible change in conformation. At 30% TFE (spectrum-6) Con-A acquires the typical α-helical conformation with minima at 208 nm and 222 nm, further increase in TFE concentrations only resulted in intensifying the signals and no change in structure was observed. Although near-UV CD analysis showed disruption of the residual tertiary structure of the acid unfolded Con-A (figure-38 b), intrinsic fluorescence studies (figure-39(c)) showed that fluorescence intensity goes on increasing upto 10% TFE and then decreases.

![Transition curve showing the fluorescence intensity of pH 2 intermediate of Con-A](image)

**Fig. 39 (c)** Transition curve showing the fluorescence intensity of pH 2 intermediate of Con-A at 340 nm as a function of varying concentrations of TFE.

Low concentrations of TFE caused opening up of the beta structure and changed the conformation such that the chromophoric groups tyrosine and tryptophan were separated from the vicinity of some quencher such as glutamate or aspartate. This could be a possible reason for the increase of fluorescence intensity up to 10% TFE.

As mentioned above at 10% TFE a change in secondary structure was observed and the protein started to acquire an α-helix conformation, hence due to the hydrogen bonding induced by TFE and formation of a new structure the chromophoric groups might have again gained the proximity of some quencher groups which resulted in the decrease of
fluorescence intensity. Alcohols are known to weaken non-local hydrophobic interactions while promoting local polar interactions (i.e., hydrogen bonds) in proteins [167,194].

Extrinsic fluorescence studies showed maximum binding of ANS to the conformation induced by 10% TFE, figure-39 (d).

![Fluorescence transition curve of pH2 intermediate of Con-A as a function of varying concentrations of TFE.](image)

**Fig. 39 (d) ANS Fluorescence transition curve of pH2 intermediate of Con-A as a function of varying concentrations of TFE.**

From these observations we can say that addition of TFE caused beta to alpha transition as expected because TFE has the propensity to induce helical structure in proteins. In the pathway from acid unfolded structure having residual beta structure to a one showing typical helical features, a MG like intermediate was formed in presence of 10% TFE having non-native secondary structure lost tertiary structure and showed maximum binding of ANS.
Effect of hexafluoro-isopropanol on acid unfolded Con-A:

Fig. 40 (a) Far-UV CD spectrum of pH 2 intermediate of Con-A in presence of varying HFIP concentrations. Native Con-A; curve-1, Con-A at pH 2; curve-2, 2%HFIP; curve-3, 6%HFIP; curve-4, 8%HFIP; curve-5, 16%HFIP; curve-6, 60%HFIP; curve-7 denatured Con-A in presence of 6M guanidium hydrochloride; curve-8. (b) Transition plot showing the changes in far-UV CD in pH 2 intermediate of Con-A with increasing concentrations of HFIP at 223 nm.

Figure-40 (a) shows the spectra of Con-A under different conditions, spectrum-1 and 2 depict Con-A in the native state and at pH 2 respectively. Spectra from 3-7 correspond to Con-A at pH 2 in presence of 2%, 6%, 8%, 16% and 60% HFIP respectively. In this case also it was seen that HFIP induced α-helix in Con-A, which showed β-sheet structure at pH 2. A sudden decrease in CD value was observed when HFIP was added and then CD values increased with increase in concentration of the fluoroalcohol (Fig-40 (b)), a slight shoulder at 208 nm generated at 8% HFIP while a typical helical structure with shoulders at 208 and 222 nm was formed at 16% HFIP, which in case of TFE was formed at 30%. HFIP has been found to have much higher propensity to induce α-helical structure as compared to TFE.

Intrinsic fluorescence first increased up to 6% and then went on decreasing with increase in HFIP concentration, figure-40 (c).
Fig. 40 (c) Transition curve showing the fluorescence intensity of pH 2 intermediate Con-A at 340 nm as a function of varying concentrations of HFIP.

Maximum ANS binding was found with acid unfolded Con-A in presence of 6% HFIP, pointing out the possibility of existence of a MG-like intermediate at this particular HFIP concentration (figure-40 (d)).

Fig. 40 (d) ANS Fluorescence transition curve of pH 2 intermediate Con-A as a function of varying concentrations of HFIP.

Thus we can say the protein in presence of HFIP showed a somewhat similar pattern of conformational changes as was shown in presence of TFE. Here again, in presence of
HFIP, loss of tertiary structure was observed as the protein underwent transition in structure from beta to alpha (figure-41).

![Near-UV CD spectrum of Con-A under various conditions. Native Con-A; curve-1, Con-A at pH 2; curve-2, 10% TFE; curve-3, 6% HFIP; curve-4, denatured Con-A in presence of 6M GndHCl; curve-5.](image)

In presence of fluoroalcohols the pH 2 intermediate was transformed to a state with predominant \(\alpha\)-helical secondary and lost tertiary structures. In the pathway of these transformations MG-like intermediates were formed at 10% TFE (v/v) and 6% HFIP (v/v) respectively.