Monomeric Banana Lectin at Acidic pH Overrules
Conformational Stability of Its Native Dimeric Form

5.1. Introduction:
Understanding the factors that decide how a linear polypeptide chain folds into its
unique three-dimensional structure relic one of the fundamental questions in biology.
Several models for the mechanism of protein folding have been proposed and there is
ample argument concerning competing hypothesis. Current sources of content consist
of whether there are on-pathway intermediates in the folding and if so, what the
nature of such partially folded intermediates is. There are substantial evidences which
indicate that proteins of smaller molecular weight under appropriate conditions fold to
the native state within a few milliseconds with no detectable intermediate (Privalov,
1996, Dill and Chan, 1997). Such folding is consistent with smooth-funnel energy
landscape models. On the other hand, for many proteins there are extensive
experimental data for transient intermediate, including the molten globule (Ptitsyn,
1987, Radford and Dobson, 1995). The molten globule is an intermediate state of
protein stabilized by acidic/alkaline pH, moderate concentration of strong chemical
denaturants at a certain temperature. The general properties of this intermediate state
are the presence of marked secondary structures, massive or significant loss of tertiary
structures together with exposed hydrophobic clusters (Kuwajima, 1989, Christensen
and Pain, 1991, Ptitsyn, 1995). The available literatures support that the molten
globule state (and other nonnative states of protein molecule) can stay in living cells
and can be mixed up in a number of physiological processes owing to the fact that the
negative electrostatic potential of the cell surface may attract protons from the
immensity of solution leading to a local drop in pH, thus resulting in partial
denaturation of proteins (Prats, 1986, Bychkova et al., 1988). There is now abundant
data available regarding molten globule state of monomeric protein, which has greatly
improved our knowledge about protein folding problem and folding intermediates
(Griko, 2000, Park, 2004, Kelkar et al., 2010).

Lectins are (glyco) proteins which specifically bind to mono or
oligosaccharide reversibly. Lectins are found in every kingdom of life. They perform
various biological functions ranging from host-pathogenic interaction, cancer
metastasis, cell–cell communication, embryogenesis, mitogenic stimulation as well as
the tissue development (Lis and Sharon, 1998, Drickamer, 1999, Chandra et al., 1999, Loris, 2002). Their high affinity and specificity for glycoconjugates have found many applications in biological and biomedical research (Sharon and Lis, 1990). Banana lectin (BL) is a member of jacalin-related superfamilly of lectins and explicitly binds to mannose and glucose containing oligosaccharides (Peumans et al., 2000, Meagher et al., 2005). It is a homodimeric protein where each subunit has a molecular weight of 14.5 kDa and consists of 141 amino acid residues with a single Trp at position 10 (Figure 5.1). Also each subunit has twelve β-strands arranged in a β-prism-I fold. BL is known to act as a potent inhibitor of HIV replication which is main perpetrator in the list of fatal diseases (Swanson et al., 2010). In this work, we investigated the unfolding of BL at acidic pH using several spectroscopic as well as hydrodynamic techniques. The stability of the protein was compared under different conditions using temperature and chemical denaturants. In several instances, the individual subunits of dimeric/oligomeric proteins are reported to be less stable than native and biologically functional state of protein as it is believed that interchain interactions that bind the subunits impart additional stability to the dimer/oligomer. However, to the best of our knowledge, this is the first report on the monomeric state of protein being more stable than its native dimeric state.

Figure 5.1. Ribbon structure of banana lectin (2BMY) generated from PyMol. Trp residues are shown in both subunits.
5.2. Material and Methods:

5.2.1. Materials:
Bananas were purchased from local shops. Urea, 1-analino-8-naphthalene sulfonate (ANS) Sephadex-75 was purchased from Sigma Chemicals Co. USA. All other chemicals used were of analytical grade.

5.2.2. Protein purification:
BL was purified by the procedure of Koshte et al. (Koshte et al., 1990). 250 g of banana pulp was soaked in 1 L distilled water containing 250.0 mM NaCl, 5.0 mM MgCl2 and 5.0 mM CaCl2. The mixture was subjected to homogenization followed by drop wise addition of 4.0 M NaOH to maintain pH at 7.4. The homogenate was incubated for 15.0 min after that 0.1M Glucose was added to the final volume and stirred for 2.0 h at room temperature. The solution was centrifuged at 18000 rpm for 30 mins. The pellet was discarded, 85% ammonium sulphate was added to the supernatant and kept overnight for precipitation followed by centrifugation at 18000 rpm for 45 mins. The pellet was collected and redissolved in Tris-FICI buffer of pH 7.4 and dialyzed for overnight. The clear suspension was loaded onto Sephadex-G75 affinity column. The elution was carried out using 0.5 M glucose dissolved in buffer pH 7.4. The eluent was again dialyzed to remove the glucose and lyophilized for further use. The purity of lectin was checked by SDS-PAGE and the concentration was determined by Lowry and BCA methods.

5.2.3. Buffer Preparation:
pH measurements were carried out on Mettler Toledo pH meter (Seven Easy S20–K) using Expert “Pro3 in 1” type electrode having the least count of the pH meter of 0.01 pH unit. The acid denaturation of BL was carried out in 20.0 mM of following buffers: Tris-HCl (pH 7.0-7.4), sodium phosphate (pH 6.0), sodium acetate (pH 3.5–5.0), glycine-HCl (pH1.6–3.0) and KCl-HCl (pH 0.8–1.4). All the buffers were filtered through 0.45µm syringe filter. Before all of the spectrophotometric measurements the protein samples were incubated for overnight at room temperature.

5.2.4. Circular Dichroism:
CD measurements were performed with a Jasco spectropolarimeter (J-815), equipped with a Jasco Peltier-type temperature controller (PTC–424S/15). The instrument was
calibrated with D-10-camphorsulfonic acid. The measurements were carried out at 25 °C. Spectra were collected with a protein concentration of 0.15 mg ml⁻¹ with 0.1 cm path length and 1.0 mg ml⁻¹ with 1 cm path length for far-UV and near-UV CD respectively. Each spectrum was the average of 2.0 scans. The results were expressed as mean residual ellipticity (MRE) in deg cm² dmol⁻¹, which is defined as:

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

where \( \theta_{\text{obs}} \) is the CD in millidegree, \( n \) is the number of amino acid residues in one subunit (141 for BL), \( l \) is the path length of the cell in centimeters and \( C_p \) is the molar fraction of proteins.

5.2.5. Thermal and Chemical Denaturations:

The thermal unfolding of BL was carried out by heating the samples and measuring the temperature-dependent CD response at 218 nm from 25 °C to 95 °C using a temperature rise of 1 °C/min in a water-jacketed cell attached to the Multitec M-H-03 water circulator. For chemical denaturation experiments, protein samples (0.15 mg ml⁻¹) were allowed to equilibrate overnight with 0–10 M urea at desired pH.

5.2.6. Fluorescence measurements:

Fluorescence measurements were performed on Hitachi spectrofluorometer (F-4500). For acid and chemical denaturation the fluorescence spectra were measured with a protein concentration of 0.15 mg ml⁻¹. The intrinsic spectra were recorded between 300 to 400 nm with excitation wavelength of 295 nm. For light scattering measurements, the excitation and emission wavelength was set at 350 nm as we have used this method in our previous report (Ahmad et al., 2009b). Both the excitation and emission slit widths were set at 5 nm. For extrinsic fluorescence measurement, the protein samples were incubated with 50-fold molar excess of ANS at room temperature in dark. The samples were excited at 380 nm and emission spectra were recorded between 400–600 nm.

5.2.7. Acrylamide quenching experiments:

For acrylamide quenching experiments, aliquots of 5 M acrylamide (quencher) stock solution were added to the protein samples (0.15 mg ml⁻¹) to achieve the desired range of quencher concentration (0–0.12 M). Excitation wavelength was set at 295 nm in order to excite Trp residues only and the emission spectrum was recorded in the range
300–400 nm. The decrease in fluorescence intensity at $\lambda_{\text{max}}$ was analyzed according to the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv} [Q]$$  \hspace{1cm} (2)

where $F_0$ and $F$ are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher respectively, $K_{sv}$ is the Stern–Volmer constant and $[Q]$ is concentration of the quencher.

5.2.8. Dynamic Light Scattering (DLS):

DLS measurements were performed with protein concentration of 1.0 mg ml$^{-1}$ using DynaPro–TC–04 dynamic light scattering equipment (Protein Solutions, Wyatt Technology, Santa Barbara, CA) equipped with temperature-controlled microsampler. Before the measurements, all the samples were kept for overnight (12 hrs) incubation. Prior to scanning, all the solutions were spun at 10,000 rpm for 15 min and filtered through microfilter (Millipore Millex-HV hydrophilic PVDF) having a pore size of 0.45 μm followed by further filtration using 0.22 μm pore sized filter (Whatman International, Maidstone, UK). Measured size was presented as the average value of 50 runs. Data were analyzed by using Dynamics 6.10.0.10 software at optimized resolution. The mean hydrodynamic radius ($R_h$) and polydispersity ($P_d$) were estimated on the basis of an autocorrelation analysis of scattered light intensity based on the translational diffusion coefficient, by Stokes–Einstein equation:

$$R_h = \frac{kT}{6\pi\eta D_w^{25^\circ C}}$$  \hspace{1cm} (3)

where $R_h$ is the hydrodynamic radius, $k$ is the Boltzman’s constant, $T$ is the absolute temperature, $\eta$ is the viscosity of water and $D_w^{25^\circ C}$ is the translational diffusion coefficient.

5.2.9. Size Exclusion Chromatography (SEC):

Size exclusion chromatography of BL was performed at pH 7.4 and pH 2.0 on the 120 ml column from the Äktura Purifier (Amersham Bioscience AB, Uppsala, Sweden) using a Sephadex G-75 matrix. The column was equilibrated with desired buffers containing 0.8 M Glucose and 0.15 N NaCl. The elution was carried out at a flow rate of 60 ml h$^{-1}$ and the absorbance of eluted fractions was read at 280 nm. Blue dextran was used to determine the void volume of column.
5.2.10. Data Analysis:

Chemical and thermal denaturation data from CD and fluorescence spectroscopy were analyzed on the basis of two-state unfolding model. For a single step unfolding process, N = U, where N is the native state and U is the unfolded state, the equilibrium constant $K_u$ is defined as

$$K_u = \frac{f_u}{f_n}$$ (4)

With $f_u$ and $f_n$ being the molar fraction of U and N, respectively.

$$f_\delta = \frac{(Y_{\text{obs}} - Y_n)}{(Y_u - Y_{\text{obs}})}$$ (5)

Where $Y_{\text{obs}}$, $Y_n$ and $Y_u$ represent the observed property, property of the native state and that of unfolded state respectively while $f_\delta$ corresponds to the fraction of denatured protein. The change in free energy of unfolding in water $\Delta G_u^o$ is obtained by the linear extrapolation method (Shirley et al., 1989). The relationship between the temperature and concentration of denaturant $\Delta G_u^o$ is approximated by the following equation:

$$\Delta G_u = -RT \ln K_u$$ (6)

and

$$\Delta G_u = \Delta G_u^o + m(D)$$ (7)

where $m$ is the experimental measure of the dependence of $\Delta G_u$ on temperature, $R$ is the gas constant (1.987 cal K$^{-1}$ mol$^{-1}$) and $T$ is 298 K. The concentration of denaturant at which the protein is half denatured (when $\Delta G_U = 0$) is given by $D_{1/2}$ and derived from equation 7 as follows:

$$\Delta G_u^o = -m D_{1/2}$$ (8)
5.3. Results:

5.3.1. Acid induced unfolding of BL:

5.3.2. Far-UV CD measurements:

Far-UV CD is one of the most sensitive spectroscopic techniques for analyzing secondary structure of the protein (Yang et al., 1986, Fast et al., 2009). A comparative change in far-UV CD spectra of BL at different pH is shown in Figure 5.2A. Native state of BL (pH 7.4) exhibited a single negative peak at 218 nm giving an indication for the presence of β-conformation, a feature typical for several lectins (Ahmad et al., 2007). The spectra of BL at pH 2.0 were quite similar to that of the native state (pH 7.4) a sign for considerable secondary structures being retained under acidic environment but in case of pH 0.8 a significant decrease in ellipticity was found while the denatured state (6 M GdnHCl) appeared to have lost all the conformational elements. pH induced alteration in secondary structure of BL were further evaluated through changes in MRE at 218 nm as shown in Figure 5.2B. No change in ellipticity values was noticed in the pH range 1.4 to 7.4 indicating pronounced stability of BL against acidification. Further lowering of pH resulted in a noticeable decrease in ellipticity which suggested some loss of secondary structure driven possibly by the charge-charge repulsions occurring at extremely low pH.

5.3.3. Near UV-CD measurements:

The CD spectra in near-UV (250- 310 nm) range provides valuable information regarding changes in the environment of aromatic residues. The near-UV CD spectra of BL (Figure 5.2C) in native state revealed minima around 279 nm and 285 nm, a characteristic of buried aromatic chromophores particularly Trp (Naseem and Khan, 2005). The intensity of the signals was considerably diminished following incubation at pH 2.0. These data taken together with far-UV CD results suggested that BL retained significant secondary structure content with loose tertiary contacts at pH 2.0 and thus indicated the existence of molten globule state at this pH.
Figure 5.2. pH induced secondary structural change of BL. (A) Far-UV CD spectra of BL at pH 7.4 (■), pH 2.0 (▲), pH 0.8 (▼) and 6 M GdnHCl (●). (B) The change in mean residual ellipticity (MRE) of BL at 218 nm was plotted with respect to pH. (C) Near-UV CD spectrum of BL at pH 7.4 (■), and at pH 2.0 (▲). For far-UV CD and Near-UV CD measurements protein concentration was taken 0.15 and 1.0 mg ml⁻¹. BL was incubated at different pH overnight prior to measurements.

5.3.4. Intrinsic fluorescence:

Intrinsic fluorescence is an important method to study protein conformation because it reveals the environment-dependent solvent exposure of the Trp indole ring and the tyrosine aromatic side chains (Eftink, 1994, Eftink and Shastry, 1997). The main intrinsic fluorescence probes of protein conformation, dynamics and intermolecular interactions are Trp, Tyr, and Phe. Out of these three, Trp is the most important probe because the indole ring is highly sensitive to its neighboring environment making it an ideal choice for reporting protein conformational changes and interactions with other molecules. The emission spectrum with excitation wavelength of 295 nm is mainly
dominated by Trp fluorescence. As shown in Figure 3A, the native state of BL exhibited low fluorescence intensity (FI) and maximum fluorescence emission ($\lambda_{\text{max}}$) at 326 nm indicating that the Trp residues are buried in the core of the protein. One possible explanation for the lower FI value of native pH is that the Trp fluorescence could be quenched by neighboring amino acid side chains such as Met76 of BL, which is in close proximity (~4 Å, PDB: 2BMY) with the only Trp10 side chain (Ballew et al., 1996, Yuan et al., 1998). At pH 2.0, the emission maxima ($\lambda_{\text{max}}$) of protein was red shifted by 3 nm along with significant increases in FI similar change were also reported for diphtheria toxin (Blewitt et al., 1985). For denatured state (6 M GdnHCl), $\lambda_{\text{max}}$ was shifted to 353 nm, indicating that Trp were maximally exposed to the solvent (Ahmad et al., 2010, Shukla et al., 2005). The pH dependant changes in FI of BL at 326 nm are shown in Figure 5.3B. A gradual increase the FI was noticed following acidification up to pH 2.0 which together with significant red shift (from 326 to 329 nm) indicates that Trp residues were in a non polar environment (Sedlak and Antalik, 1999, Boscolo et al., 2009). Due to the partial loosening of structure, Met76 and Trp10 residues might have displaced far apart to inhibit the quenching. The decrease in FI below pH 2.0 could be intrinsic quenching of Trp residues caused by conformational changes upon extreme acidification.

5.3.5. Rayleigh Scattering measurements:

To check the possibility of pH dependent aggregation, we performed light scattering measurements. It is reported that if extent of light scattering is fivefold more in comparison to the native state so the solution is having aggregates (Santiago et al., 2010). The results are shown in Figure 5.3C. As evident, the extent of light scattering at pH 2.0 was insignificant to that of native state and hence the chances of aggregation can be safely ruled out. As in our previous report we have found 12 times more scattering in the BL when it is incubated with SDS pH below two units of pI (Khan et al., 2012).
Figure 5.3. Effect of pH on the tertiary structure of BL was investigated by intrinsic fluorescence spectroscopy. (A) Intrinsic fluorescence spectra of BL at pH 7.4 (- - ), pH 2.0 (- • - ) and 6 M GdnHCl (- o - ) after excitation at 295 nm. (B) Change in fluorescence intensity at 326 nm of BL was plotted against pH. (C) Rayleigh Scattering measurements of BL at pH 7.4 (- - ) and pH 2.0 (- • - ) after excitation at 350 nm and spectra was measured in the range of 300-400 nm. All the measurements were performed after 12 hrs incubation in 20.0 mM of respective buffer and final protein concentration was taken 0.15 mg ml⁻¹.

5.3.6. Extrinsic fluorescence (ANS binding) measurements:

ANS is a widely used hydrophobic dye for detecting the non-native states such as molten globule (MG) states in proteins (Ramboarina and Redfield, 2003, Safarian et al., 2006). Fluorescence spectra of BL with ANS at pH 7.4 and 2.0 (Figure 5.4.A) revealed that binding of ANS to hydrophobic patches resulted in a prominent blue shift along with a significant increase in fluorescence intensity at pH 2.0 while at pH 7.4 the ANS is unable to bind BL because hydrophobic residues are buried in the core.
of the proteins. A plot of ANS fluorescence intensity at 480 nm as a function of pH is also shown in Figure 5.4B. ANS fluorescence was maximum at pH 2.0 suggesting considerable exposure of hydrophobic clusters that either remain inaccessible in native state (pH 7.4) or were minutely accessible in the denatured state of protein (6M GdnHCl). Native state of BL exhibited maximum emission of ANS fluorescence at 519 nm which decreased to 501 nm at pH 2.0 Figure 5.4C. Taken together, the above results confirm the existence of the MG - state of BL at pH 2.0 having pronounced secondary structure along with significantly disrupted tertiary contacts and hydrophobic clusters considerably exposed to the solvent.

![Figure 5.4](image_url)

**Figure 5.4.** Change in Trp position was monitored by ANS dye binding. (A) Extrinsic fluorescence spectra of BL at pH 7.4 (-●-), pH 2.0 (-▲-) and 6 M GdnHCl (-☆-). (B) pH dependant changes in ANS fluorescence intensity of BL at 480 nm. (C) Change in wavelength maximum of BL was plotted verses pH. Prior to measurements all the samples were incubated for 12 hrs in a 20.0 mM respective buffer with 0.15 mg ml⁻¹ protein concentration and 50 times more ANS ( 265.0 μM) were added in all samples.
5.3.7. Dynamic Light Scattering (DLS):

The pH dependent changes in hydrodynamic radii ($R_h$) were studied by DLS measurements. The $R_h$ of folded BL (pH 7.4) was obtained 2.9 ± 1 nm (Figure 5.5A) with 7.8% polydispersity (Table 5.1), which is less than 30%, thus confirm that solution has a homogenous molecules. At pH 2.0, $R_h$ values were obtained 1.7 nm and 3.7 nm is pointing towards incomplete monomerization (Figure 5.5B). Besides, the increase in $R_h$ of the two species at pH 2.0 as compared to at pH 7.4 can be attributed to the perturbation of tertiary structure, thus supporting the existence of MG-state at this pH.

![Figure 5.5](image)

*Figure 5.5. Change in hydrodynamic radii of BL at different pH were monitored by DLS. Hydrodynamic radii ($R_h$) of BL at (A) pH 7.4 and (B) pH 2.0 and autocorrelation graph of BL (C) at pH 7.4 and (D) at pH 2.0. BL (1.0 mg ml$^{-1}$) were incubated at pH 7.4 and 2.0 for 12 hrs incubation.*
Table 5.1. pH induced changes in hydrodynamic radii ($R_h$) of BL.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$R_h$ (nm)</th>
<th>[%] PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>2.9</td>
<td>7.8</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>1.7, 3.6</td>
<td>10.08, 12.05</td>
</tr>
</tbody>
</table>

5.3.8. Size Exclusion Chromatography (SEC):

The elution profile of BL was obtained at pH 7.4 and pH 2.0 is depicted in (Figure 5.6) from size exclusion chromatography. A single peak around 61.45 ml was observed at pH 7.4 while the elution profile of protein at pH 2.0 revealed two peaks that were centered around 59.65 ml and 87.98 ml, although the later peak was not so sharp. The progressive monomerization of protein at pH 2.0 might have caused this peak to diminish resulting in only two peaks corresponding to the dimeric (59.65 ml) and relatively greater number of monomeric (87.98 ml) species. The distorted shape and lower elution volume of the later peak at pH 2.0 compared to at pH 7.4 can be attributed to the loss of tertiary structure (Bose and Clark, 2005). The results establish that monomer of BL was found at pH 2.0 although complete monomerization was not found.
Figure 5.6. pH dependent monomerisation of BL. Size exclusion profile of BL at pH 7.4 and pH 2.0. Before performing the experiments the BL (1.0 mg ml⁻¹) was incubated at respective pH for overnight.

5.3.9. Acrylamide quenching experiments:

To verify the environment of Trp residues, a fluorescence-quenching experiment was performed using the uncharged molecules of acrylamide as explained (Eftink and Ghiron, 1981). Figure 5.7, shows Stern-Volmer plot of BL at pH 7.4, 2.0 and in the presence of 6 M GdnHCl while Table 5.2, summarizes the $K_{sv}$ obtained under respective conditions. The values for $K_{sv}$ were in the order $4.48 \text{ M}^{-1}$ (6 M GdnHCl) $>$ $2.01 \text{ M}^{-1}$ (pH 2.0) $>$ $1.95 \text{ M}^{-1}$ (pH 7.4). The data implied that Trp residues were maximally exposed in the presence of 6 M GdnHCl, relatively less exposed at pH 2.0, even lesser in case of pH 7.4, indicating that exposure of hydrophobic residues at pH
2.0 and in turn authenticated the destabilization of tertiary structure at pH 2.0, which validate the presence of a molten globule like state (Khan et al., 2007).

![Graph showing acrylamide quenching](image)

Figure 5.7. Exposure of Trp was monitored by acrylamide quenching. Stern-Volmer plot obtained from acrylamide quenching of BL at pH 7.4 (- - ), pH 2.0 ( - △ - ) and in the presence of 6.0 M GdnHCl (− → ).

Table 5.2. Acrylamide quenching constant ($K_qM$) values of BL obtained at different pH values.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$K_q[M^{-1}]$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>1.95</td>
<td>0.98</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>2.01</td>
<td>0.98</td>
</tr>
<tr>
<td>6 M GdnHCl</td>
<td>4.48</td>
<td>0.99</td>
</tr>
</tbody>
</table>

5.3.10. Chemical and thermal denaturation of BL:

The interesting results obtained from spectroscopic and hydrodynamic studies prompted us to compare the stability status of BL under native condition (pH 7.4) where protein existed as a homodimer and at molten globule state (pH 2.0) where
monomeric BL predominated. The stability was checked using temperature unfolding in addition to urea as denaturants.

5.3.11. Urea induced Conformational Changes of BL at Native and Molten globule State:

Urea induced structural transitions of BL were monitored by far-UV CD and intrinsic fluorescence spectroscopic techniques.

5.3.12. Secondary structure alterations:

Far-UV CD studies were carried out to study the effect of urea on the secondary structure of the BL at two different pH i.e 7.4 and 2.0 respectively. Figure 5.8A, recapitulates the effect of increasing urea concentration on loss of ellipticity at 218 nm of BL. At pH 7.4, no significant change in ellipticity was observed up to 5 M. However, a large sigmoidal change was observed from 5 to 8 M urea. In case of pH 2.0, BL showed relatively more resistance and no secondary structure change was observed up to 6 M urea, followed by a large sigmoidal change that reached a plateau around 10 M Urea. The midpoint of sigmoidal denaturation (C_m), an index to determine the stability of protein, was found to be 6.2 M for BL at pH 7.4. In case of pH 2.0, the C_m value increased to 7.4 M which indicated that the secondary structure of BL acquired more stability after minomerization. The transition midpoint and free energy change (ΔG) of urea induced unfolding of BL at two pH values were determined by far-UV CD and fluorescence data are summarized in Table 5.3.

6.3.13. Tertiary structure alterations:

The urea induced tertiary conformational changes in BL at pH 7.4 and 2.0 were also studied through changes in FI at 326 and 329 nm as a function of increasing concentration of urea. As shown in Figure 5.8B, urea denaturation profile of BL at both condition followed a two-state transition. At pH 7.4, the tertiary structure of protein remained unaffected by 2 M urea while complete loss of structure was observed with 6 M urea resulting in a midpoint of transition at 4.1 M. At pH 2.0, the molecular unfolding initiated beyond 3.0 M urea and complete unfolding was achieved with 9.0 M of chaotrop. The C_m value shoots up to 6.1 M which clearly pointed towards a more stable state of BL being attained at pH 2.0 with respect to at native state. The wavelength maximum of BL at pH 7.4 in the absence of urea was found around 326 NM. The wavelength maximum of BL at pH 7.4 was started red
shifting above 3 M urea concentration and maximum red shift were observed in the presence of 9 M urea with a difference of 22 nm. However at pH 2.0, the wavelength shift started at higher urea concentration but a shift in wavelength is not much more significant even at 9 M urea. The fluorescence spectra of BL at both pH 7.4 and 2.0 is shown in Figure 5.8C and 5.8D. The above results clearly suggested that monomeric BL (pH 2.0) is chemically more stable than its dimeric form (pH 7.4).

Figure 5.8. Urea-induced denaturation of BL was monitored by changes in secondary and tertiary structure. (A) Urea induced unfolding transition curves of BL at pH 7.4 (— ■ —) and pH 2.0 (— ▲ —) monitored through ellipticity measurement at 218 nm by far-UV CD. (B) Urea induced unfolding of BL at pH 7.4 (— ■ —) and pH 2.0 (— ▲ —) monitored by a change in fluorescence emission intensity at 326 nm plotted as a function of urea concentration after excitation at 295 nm. (C) Fluorescence emission spectra of BL at pH 7.4 (8 C) and (D) at pH 2.0, at 0 M urea (— ■ —), 5 M urea (— ▲ —), 7 M urea (— △ —) and 9 M urea (— Θ —).
Table 5.3. Urea-induced unfolding parameters of BL at different conditions.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Methods</th>
<th>Transition midpoint ($C_m$)</th>
<th>$\Delta G_u^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4, 25 °C</td>
<td>FI at 326nm</td>
<td>4.1</td>
<td>3.13±0.20</td>
</tr>
<tr>
<td></td>
<td>MRE at 218nm</td>
<td>6.2</td>
<td>3.85±0.085</td>
</tr>
<tr>
<td>pH 2.0, 25 °C</td>
<td>FI at 329nm</td>
<td>6.1</td>
<td>3.43±0.088</td>
</tr>
<tr>
<td></td>
<td>MRE at 218nm</td>
<td>7.4</td>
<td>5.44±0.133</td>
</tr>
</tbody>
</table>

5.3.14. Thermal Denaturation:

The chemical stability of BL was also complimented by thermal stability. Far-UV CD spectra of BL at pH 7.4 were taken at different temperatures (Figure 5.9A). No changes in spectra could be noticed up to 75.0 °C beyond which a regular decrease in ellipticity was observed and spectra shifted towards higher wavelength due unfolding. Interestingly at pH 2.0 the spectra peak shifted towards shorter wavelength with increase in temperature (Figure 5.9B). Besides, no significant loss of ellipticity was observed even at 95.0 °C. Further, the temperature dependant changes in ellipticity at 218 nm of BL were monitored and shown in Figure 5.9C. The thermal denaturation profile of BL at pH 7.4 revealed sigmoidal transitions with midpoint of transition ($T_m$) at 77 °C. However at pH 2.0, no sigmoidal change was noticed over the entire temperature range(20-90 °C) although a considerable increase in ellipticity was recorded up to 70 °C suggesting induction rather than loss of secondary structure (Figure 5.9D). Thus, induction of secondary structure at pH 2.0 indicated uninterrupted gain of stability following monomerization. The results obtained from these experiments further strengthened the fact that acid-induced monomeric state, and not native dimeric form, of BL has more stability against temperature. Taken together, all these data further confirmed that BL acquires more chemical as well as thermal stability in its monomeric form than its native dimeric form. The overall results obtained in the present study are summarized in Figure 5.10.
Figure 5.9. Effect of temperature on monomeric as well as dimeric form of BL. 
(A) temperature dependent change in far-UV CD spectra of BL at (A) pH 7.4, 20 °C (-■-) and (B) pH 2.0, 20 °C (- ■-), 75 °C (- ▲-), 80 °C (- ▲-), and 90 °C (- ○-). (C) Thermal denaturation profile of BL at pH 7.4 and (-■-) (D) pH 2.0 (- ■-) obtained from changes in ellipticity at 218 nm.
5.4. Discussion:

Newly synthesized proteins are unfolded and do not have any structure. Over a short span of time, they attain various typical structures including secondary, tertiary and quaternary. The conformational elements acquired by polypeptides are stabilized by various non-covalent and covalent interactions. Breaking these interactions result in denaturation of the protein with either partial or complete loss of structure and its biological activity, that facilitates understanding the protein folding mechanism. The present study was divided into three parts: (i) pH denaturation (ii) chemical denaturation and (iii) thermal denaturation. The first part covers the pH dependent conformational change of BL to trace out any intermediate state that might encompass the folding pathway of protein. An intermediate state with characteristic features of a molten globule state was found to be populated at pH 2.0 as analyzed by CD, intrinsic as well extrinsic fluorescence methods. Size exclusion chromatography as well as DLS data confirmed that acid pH the dimeric BL was accompanied by monomerization. A single peak corresponding to native BL was observed at pH 7.4.
The signal intensity as well as elution volume was more for monomeric species of protein at pH 2.0. Besides, the $R_v$ value of both monomeric as well as dimeric species increased to 1.7 nm and 3.7 nm respectively owing to the conformation transition induced by extreme low pH as observed during the CD and fluorescence analysis. The intramolecular charge-charge repulsion at extreme pH might have resulted in relatively extended conformation at low pH and similar explanation was also found in case beta-lactamase, cytochrome c, and apomyoglobin (Goto et al., 1990). Besides, the polydispersity value indicated that the number of monomeric species were considerably high with respect to dimeric species at pH 2.0 suggested that acid-induced MG state of BL exist possibly in the former state. The pH dependant change in subunit status has been reported earlier for several multimeric proteins. The several noncovalent interactions that held the subunit of multimeric proteins are likely to be dissociated with change in pH (Sinha and Surolia, 2005). The second part of this study was to compare the chemical stability of of BL at two different pH. Chemical stability of BL determined at neutral pH using guanidine hydrochloride has already been reported showing that BL is quite stable (Gupta et al., 2008). In the present study, chemical stability of BL was examined at both monomeric (pH 2.0) as well as dimeric (pH 7.4) state using urea as a denaturant. Urea is widely exploited as chaotrops for checking the stability of proteins at neutral as well as acidic pH (Rosner and Redfield, 2009, Dar et al., 2007, Pace et al., 1992). The equilibrium unfolding of BL in the presence of urea was found to be a cooperative process in which the protein molecule undergoes unfolding without a stabilization of intermediate at both pH values (7.4 and 2.0) similarly no intermediates were found as in the case of GdnHCl denaturation of same lectin at pH 7.4 (Gupta et al., 2008). Oligomeric proteins have usually more chemical stability than its monomer form because of additional noncovalent and covalent interactions (Mitra et al., 2002). In case of BL, however, it was noticed that monomeric state (pH 2.0) of protein was more stable in comparison to dimeric native state which was evident from $C_m$ values as well as free energy changes (Table 5.3). $C_m$ values for BL at pH 7.4 as determined by far-UV CD and intrinsic fluorescence analysis were found to be 4.1 M and 6.2 M respectively. The difference in the two $C_m$ value reflects the perturbation of two different conformational elements (secondary and tertiary structure). In case of monomeric BL, the $C_m$ values increased to 6.1 M and 7.4 M respectively. Similarly, free energy change for unfolding of monomeric BL was more than dimeric protein which is quite an unusual finding this
may be due to the development of positive charge on the protein and exposure of hydrophobic patches, urea is probably not able to interact at its lower concentration because urea is a neutral molecule with negligible hydrophobic moiety. However, at the higher concentration of urea might have disturbed the overall charge on BL, consequently resulting in unfolding of the molecule. Previously it is reported that urea interact with proteins nonspecifically at sub-global level by hydrogen bonding and Van der Waals interaction with main chain and side chain groups of protein (Bhuyan, 2002, Kumar et al., 2004). Concanavalin A, Cramoll I and SBA are legume lectins which monomerize and form MG states up to 3.0 M urea (Varejao et al., 2011, Chatterjee and Mandal, 2003, Sinha and Surolia, 2007) but this non-leguminous BL shows the tertiary structural changes from 4.0M and secondary structural changes from 5 M urea which might be due to monomerization of native dimeric state of BL. This signifies that BL is more stable than other leguminous lectins. Urea concentrations up to 3-4 M affect BL in similar fashion at pH 2.0 or 7.4, after that the species formed on-pathway (possible monomers) are more stable in acidic conditions than at pH 7.4. Third part of this study was to check the stability of BL by employing temperature scan in the range of 20-95 °C. Thermal denaturation of BL was monitored by far-UV CD spectroscopic techniques aimed to follow secondary structural changes of the protein. Thermal denaturation of BL was a cooperative process without any intermediate at pH 7.4 but an intermediate state was found at pH 2.0. Tm value of BL was quite high at pH 7.4 (77 °C) indicating considerable stability against temperature change. Interestingly, the thermal denaturation profile was quite different at pH 2.0 where an intermediate state was acquired near 70 °C which did not denature completely even when heated beyond 95 °C, again confirming the significant increase in stability of BL in monomeric conditions.

5.5. Conclusions:

Acid denaturation of BL yielded an intermediate state at pH 2.0 and the monomerization of homodimeric BL was also found. Besides, the monomeric state of BL had more chemistry and thermal stability which, to the best of our knowledge, is the first report on significant stability exhibited by an individual subunit of an otherwise dimeric protein than the native state. An increase in intrasubunit interactions in monomeric level than in dimeric condition may be the possible reason for such finding.