NJC

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/njc

Journal Name

ARTICLE

A study of conformational changes of β-lactoglobulin in the vicinity of critical point of binary mixed solvents

R. Umapathi and P. Venkatesu*

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

In this study, we present the influence of bovine milk β-lactoglobulin (β-LG) as an impurity on the critical mixture of isobutyric acid (IB) + water (W) by using the density (ρ) coexistence curve, fluorescence spectroscopy and dynamic light scattering (DLS) measurements. Further, the coexistence curve density data used to obtain the critical exponents (β), which depicts the shape of coexistence curve. Our experimental results explicitly elucidate that the critical region of IBW decreases with increasing the concentration of β-LG. The critical temperature (T_c) of IBW can be modulated by the addition of different concentrations of β-LG. The modulations in T_c values can be attributed to the β-LG induced interactions of solvent molecules with the residues of β-LG. The observed deviations in the T_c with temperature predict unambiguously the formation of the solvation structure at T_c for the critical mixture in absence as well as in the presence of β-LG. Our results show that β-LG can significantly affect the critical binary mixture of IBW. On the experimental side and to the best of our knowledge, this is the first experimental evidence that most of the protein entangles in the upper, IB-rich phase.

Introduction

Globular proteins have received massive attention and popularity because of their unique solution behavior and wide usage in biological, biophysical and biochemical processes.[1-5] β-lactoglobulin (β-LG) is a water soluble globular protein found in bovine milk of most mammals. The β-LG has a molecular mass of about 18.3 kDa and its structure consists of 162 amino acid residues.[2,4] 51% β sheet and 7% α-helices are present in the native state, and exists as a dimer under physiological conditions.[6] β-LG contains five cysteine (Cys) residues located at positions 66, 106, 119, 121 and 160 and two tryptophan (Trp) residues. Clearly these Cys residues form two disulfide bonds between Cys 66 and Cys 160 and between Cys 106 and Cys 119.[6,7] In other words, a free Cys 121 lies buried in the middle of β-LG structure and participates to the protein stability interactions.[8-10] The conformational changes in the monomer, leading to an increased reactivity of the free thiol group which is Cys 121.[10] It consists of β-sheet structure with nine stranded anti-parallel β barrels and one small helix.[11,12] Schematic diagram of the crystal structure of β-LG which is downloaded from the protein data bank and processed with the PyMOL viewer software and is shown in Fig. 1. Folding and unfolding studies of β-LG in different co-solvents have been extensively studied over the past few decades by many researchers.[12-16] The native structure of β-LG has been shown to bind numerous hydrophobic ligands, polycycles, aromatic compounds, catchein and sodium dodecyl sulfate.[17-21] The structural arrangements of β-LG modifications are of great interest, because its functional and biological properties are greatly affected by additives and obviously influenced on the protein conformation and association state.[22] In this context, it is interesting to see the effect of β-LG on the critical binary mixture. However, the details of the β-LG-induced phase separation mechanism and interactions are apparently absent in the open literature.

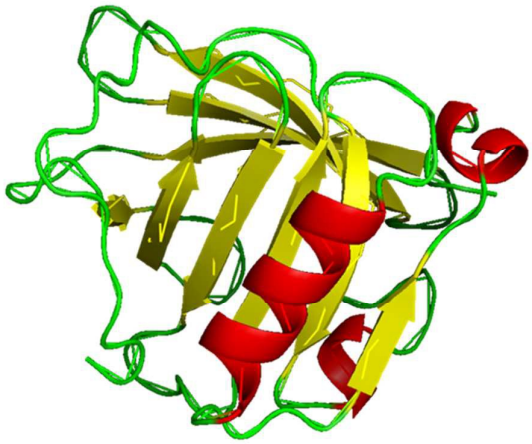


Fig. 1. Schematic diagram of the crystal structure of β-LG, which was downloaded from the protein data bank and processed with the PyMOL viewer software.

The phenomenon of fluid phase equilibria has been very interesting and attractive area for physical chemists and chemical physicists. The coexisting liquid phases are formed when two mutually incompatible solvents are mixed. At particular temperature, the volumes of two phases are equal, then these phases are called coexisting liquid phases. Obviously, isobutyric acid (IB) and water (W), solvent mixture (IBW) is a well known coexisting liquid phases and is particularly attractive in science and technology in the recent years because of its wide applications in extraction

Department of Chemistry, University of Delhi, Delhi – 110007, India
e-mail*: venkatesup@hotmail.com; pvenkatesu@chemistry.du.ac.in;
Tel: +91-11-27666646-142; Fax: +91-11-2766 6605.
Electronic Supplementary Information (ESI) available:See DOI: 10.1039/x0xx00000x

and separation as well as chemical reactions.[23-26] IBW critical mixture has been considered as promising solvents to upper critical solution temperature (UCST) behavior.

Different detailed experimental methods[27-37] of critical mixtures have been reported by several researchers to understand the conformational behavior of third component in the vicinity of the critical point of the binary solvent, and these results have received surprisingly much attention. The addition of third component, which is considered as an impurity may couple to the composition and may affect the critical behavior of the binary liquid mixtures.[38-42] The influence of the third component as an impurity on IBW has been of continuing interest, of fundamental practical importance. Several studies have been attempted on the critical mixture of IBW in the presence of various impurities including ionic salts,[26,43-48] polymers,[27,49-52] ionic liquid,[53] oxides,[24,25,54,55] charcoal,[56] acetone[57] and silica gel.[58] To the best of our knowledge, very little is known about influence of protein[27] on critical mixture of IBW. Nonetheless, the influence of protein on the IBW critical binary component systems has been investigated scarcely. The detailed information about the influence of the protein on critical binary mixture is essentially required to delineate the structural features and intermolecular interactions that are critical for raft association.

The main goal of this study is to acquire a clear understanding on the conformational changes of *β*-LG near the critical point of coexisting liquid phases of IBW. We have simultaneously performed precise measurements of the coexistence curve of IBW mixture near its critical point in the presence of various concentrations of *β*-LG using density (ρ) measurements. Additionally, we combined several approaches such as fluorescence spectroscopy and hydrodynamic diameter (d_H), diffusion coefficient (D), correlation function and scattered intensity by dynamic light scattering (DLS) measurements in order to elucidate how *β*-LG affect on IBW near its critical region.

Materials and methods

β-lactoglobulin (*β*-LG) from Bovine milk essentially salt-free lyophilized powder (molecular weight: 18.3 kDa) and isobutyric acid (99.9% purity) were purchased from Sigma-Aldrich and were used without further purification. Ultra pure water at 18.3 Ω cm resistivity (from Ultra 370 Series, Rions, New Delhi, India) was used for sample preparation. The solvents were filtered using a 0.45 μm disposal filter (Millipore, Millex-GS) before performing the experiment.

Sample preparation of IBW and *β*-LG in IBW

Clear solutions of IBW and *β*-LG in IBW were prepared gravimetrically using a Mettler Toledo balance with a precision of ±0.0001 g. Special care was taken in order to avoid the evaporation of IB and water during the weighing process. The critical mixture IBW prepared from 2.5080 g of water and 1.5505 g of IB with a UCST of 26.924 ±0.002 ^{o}C using an equal volume criterion. The critical composition of the IBW was 0.3826 mass fraction (x_c) of IB which is in good agreement with the literature values.[7,34,51-53,59-62.] It has been already noted that the binary mixture IBW has an UCST 26.92 ^{o}C, therefore we placed the samples in a temperature-regulated bath at 30 ^{o}C for 24 h to achieve the equilibrium. Later, the temperature was decreased slowly (by a step of 0.001 ^{o}C) until a phase boundary was observed in the centre of the vial. Clearly, the phases form at the meniscus between two layers. The cell containing the solution mixture was immersed in the isolated water bath where it could be observed through a window of the water bath.

Experimental procedure

Density measurements for the coexisting liquid phases of IBW and *β*-LG in IBW

The values of the density (ρ) of coexisting phases of IBW as well as *β*-LG in IBW were determined as a function of temperature using a vibrating tube densitometer (Anton Paar DMA 4500 M, Austria) with some modifications. The detailed procedure and apparatus used in this work had been elucidated elsewhere.[44,51,53,63] A bubble-free sample was introduced by means of a medical syringe into a homebuilt sample cell containing small Teflon coated magnetic bar. The cell was then sealed air tight, connected to two HPLC tubes and secured in a sample holder with a water submersible magnetic stirrer. Later, the tubing was connected to a many-way chromatographic valve, which was also connected to the vibrating tube of the densitometer. The sample holder and the complete experimental setup were placed in a water bath, made of 2 cm thick transparent plexi glass. The temperature of the water bath was controlled with an accuracy of ±0.002 ^{o}C using a high precession RTD probe thermometer connected to a Eurotherm temperature controller (3500 series) which was further controlled by a personal computer. The Eurotherm read the temperature and sent back the appropriate heat to the water bath through a proper controlled power supply and an electric heater. The densitometer consists of a stainless steel U-tube which is placed in a metal block, with an accuracy of ±0.00005 g/cm^3. Initially, the stirrer was turned on for at least 30 min to thoroughly mix the two coexisting phases inside the sample cell. After that the stirrer was turned off and the sample was left undisturbed for at least 30 min, in order to separate phases completely under gravity. After each measurement, special care was taken to clean the walls of the stainless steel U-tube with water and then with acetone to remove any contamination present in the densitometer.[51] For each measurement, we have checked carefully the T_c value of the sample, which gives the solution is in the coexisting liquid phase. Moreover, no shift of the T_c was observed during the measurements which were ensured by checking the T_c values before and after ρ measurements. In, this way we ensured that both coexisting phases are in equilibrium while we measured the densities. Precautions were taken during sample injection into the U-tube to avoid air bubbles.

Fluorescence measurements for the coexisting liquid phases of IBW and *β*-LG in IBW

To ascertain the effect of *β*-LG on the coexisting phases of IBW, we employed steady-state fluorescence measurements with a Cary Eclipse spectrofluorimeter (Varianoptical spectroscopy instruments, Mulgrave, Victoria, Australia). We have chosen Nile Red as an extrinsic fluorescent probe in the current study because it is easily soluble in proteins.[53] Nile Red was dissolved in the bulk solution of IB (10 mL) so that its absorbance was less than 0.1, and then this solution was again used for preparing the coexisting phases of IBW. The excitation wavelength was set at 555 nm in order to calculate

the contribution of Nile Red to the overall fluorescence emission. The experiments were performed in the range of 21–32 °C by using a 1 cm sealed cell and both the excitation and emission slit widths were set at 5 nm and corrected for background signal.[53] The temperature of the fluorescence cuvette chamber was first raised above the critical temperature to 32 °C, at which a homogeneous mixture of IBW was formed. The steady-state scan was taken for the mixture at that temperature. Then, the temperature was lowered by 0.5 °C and the scan was taken after a time interval of 1 h. Since, in the IBW system, the diffusion of the components was very slow, we allowed a significant time interval to achieve complete equilibrium of the molecules for each of the components in each phase. [53] A similar procedure was applied to the remaining samples which containing *β*-LG in IBW.

Dynamic light scattering measurements for the coexisting liquid phases of IBW and *β*-LG in IBW

To explore the influence of *β*-LG on IBW regarding the complete phenomenal changes, critical composition, fluctuations in the scattered intensity and hydrodynamic diameter (d_H), we have performed the dynamic light scattering (DLS) measurements by using the Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., UK), equipped with He-Ne laser (4 mW, 632.8 nm) and fitted with an automatic laser attenuator with a transmission of 100 to 0.0003%. This instrument measures the movement of particles under Brownian motion and converts this motion into size by using the Stokes-Einstein equation. All data were obtained from the instrumental software. The time-averaged intensities were measured at a scattering angle of 90°. An advanced avalanche photodiode, Q.E. > 50% at 632.8 nm was used as a detector. The temperature accuracy of the instrument was ± 0.1 °C. The temperature was lowered stepwise from 29 to 22 °C in steps of approximately 0.2 °C and at every level kept constant until thermal equilibrium was attained. The measurements for each temperature were performed and recorded repeatedly for 50 runs to improve the signal-to-noise ratio of the experiment. The reported d_H values are an average of three measurements. The instrument has an in-built automated correlator for the detection of the scattered intensity and the necessary autocorrelation function calculations. Furthermore, the mutual diffusion coefficient (D) is obtained from analysis of the time scale for the decay of fluctuations in the intensity of light scattered by the solution. The detailed procedure of the determination of autocorrelation function and D was elucidated in our previous paper.[53]

Results and discussion

Influence of *β*-LG on the coexisting liquid phases of IBW monitored by density measurements

The influence of protein on coexisting liquid phases has remained a cornerstone of fundamental protein chemistry and phase separation technology. To explore the protein behavior with varying concentration of *β*-LG in IBW, we have opted the density (ρ) measurements of the coexisting phases of IBW with and without *β*-LG as a function of temperature (T) upon approaching very close to T_c. The experimental densities ρ_1 and ρ_2 of two phase regions of the IBW and with five different *β*-LG concentrations (0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL) were reproducible and are displayed in Fig. 2 as a function of temperature and also provided in the supporting information (Table 1S). Each coexistence curve consists of 20 pairs of measured ρ values at temperatures ranging from 21.975 to 26.919 °C. It can be seen from Fig. 2 that the entire shape of the coexistence curves for *β*-LG in the IBW system is similar from those for pure IBW critical mixture.

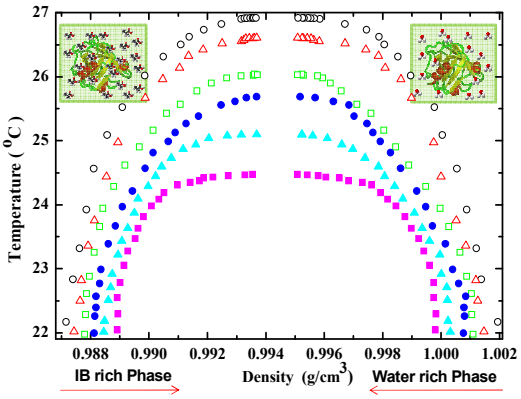


Fig. 2. Coexistence curves of the temperature dependence of the density measurements for *β*-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

The effect of *β*-LG, at various concentrations on the T_c is very significant. As shown in Fig. 2, coexistence curves noticeably shift down with increasing *β*-LG concentration in the IBW critical mixture. It is evident that T_c of *β*-LG in IBW exhibits a strong dependence on the amount of *β*-LG and the nature of the functional groups of protein that are preferentially absorbed by the solvent molecules. The pure IBW system has critical density (ρ_c) of 0.99365 g cm^{-3} at 26.923 °C from our experimental coexistence curve which is consistent with the literature values.[45,51,64,65] Table 1 lists the measured values of the T_c, and ρ_c of IBW mixtures and *β*-LG in IBW.

Table 1. Observed critical temperature (T_c), critical density (ρ_c) critical exponent (β) and hydrodynamic diameter (d_H) as a function of concentrations of *β*-LG in the IBW mixture.

Concentration of *β*-LG. (mg/mL)	T_c ± 0.002 (°C)	ρ_c±0.00005 (g/cm^3)	β±0.007	d_H (nm)*
0	26.923	0.99366	0.325	135.14
0.1	26.614	0.99367	0.363	150.97
0.25	26.059	0.99369	0.428	195.12
0.5	25.702	0.99368	0.381	219.91
1.0	25.112	0.99372	0.358	247.53
1.5	24.481	0.99358	0.330	297.65

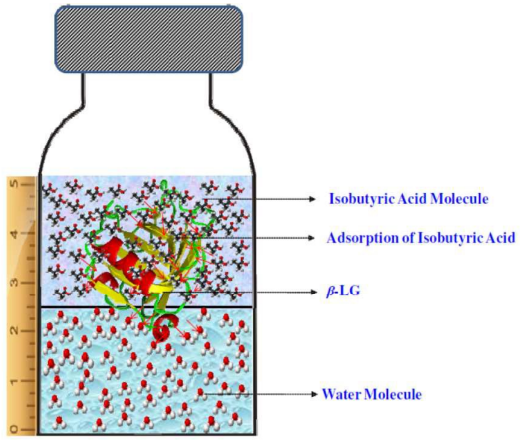
Fig. 2 depicts that the coexistence curves obviously diminish with increasing concentrations of *β*-LG in IBW mixture at all five investigated concentrations, since a minute amount of *β*-LG impurity affects the critical region of IBW. This variation induces a modification of the phase transition region affected with the addition of *β*-LG to IBW mixture, and presumably the protein preferentially absorbs by solvent molecules. We have firstly

measured the ρ values of β-LG (1 mg/mL) in water and the observed values are 0.99680 and 0.99743 g/cm^3 at 26.923 and 24.481 ^{o}C, respectively. Similarly, the ρ experimental values of β-LG (1 mg/mL) in IB are 0.94560 and 0.94799 g/cm^3 at 26.923 and 24.481 ^{o}C, respectively. Observed experimental ρ values of 1 mg/mL of β-LG in IB and water were listed in Table 2. The results show that β-LG

Table 2. Observed experimental ρ and d_H values of β-LG (1 mg/mL) in W and in IB at their respective T_c values.

T_c (^{o}C)	ρ (g/cm^3)		d_H (nm)*	
	β-LG in W	β-LG in IB	β-LG in W	β-LG in IB
25	0.99727	0.94735	15	569.7
26.923	0.99680	0.94560	15.9	471.6
26.614	0.99691	0.94598	16.5	499.5
26.059	0.99703	0.94642	20.3	506.1
25.702	0.99713	0.94677	17.5	541.2
25.112	0.99730	0.94746	15	573.3
24.481	0.99743	0.94799	13.9	601.1

increases ρ values of the water molecules as compared to ρ values of β-LG in IB molecules. Obviously, we would expect that the β-LG would be reside dominatingly in water-rich phase. Interestingly, our ρ results of β-LG in IBW show that β-LG entirely migrated into IB rich phase (Fig. 2 and Table 1S), since the observed the ρ values increased in IB rich phase as compared to W-rich phase. Careful assessment of Fig. 2 reveals that ρ values slightly increase in IB phase where as the ρ values decrease in W-rich phase with increasing the concentration of β-LG. This is because β-LG is more absorbing IB molecules, which makes both coexisting phases mutually less soluble and therefore decrease in UCST of IBW with addition of β-LG. The probable mechanism has been schematically shown in scheme 1.



Scheme1. Schematic illustration of β-LG behavior in IBW coexisting liquid phases.

On the other hand, to quantify the behavior of β-LG in IBW mixture in the vicinity of the critical point, we obtained shape of the potential in the critical region i.e., β from the ρ measurements of both coexisting phases with the relative distance from the T_c, by the relation $(\rho_1 - \rho_2) \alpha (T_c - T)^{\beta}$, were ρ_1 and ρ_2 are the individual ρ values for each co-existing phase, and the results are demonstrated in Fig. 3. The detailed analysis for obtaining the β values from experimental ρ values of both coexisting phases is depicted in our previous papers.[51-53] The obtained value of β = 0.325 ± (0.007) for the IBW binary mixture is in consistent with the three-dimensional Ising value of β = 0.325 and with the results found in different studies by a number of researchers.[51-53,61,64,66,67]

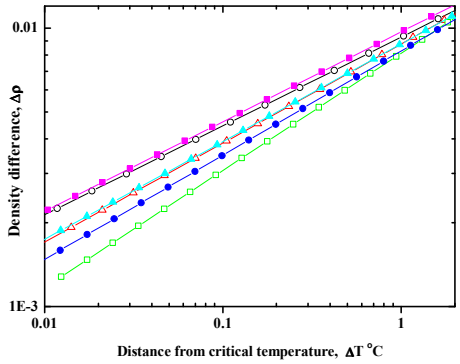


Fig. 3. Logarithmic representation of the density difference (Δ_ρ) between the coexisting phases with respect to the distance from the critical temperature (ΔT).

The obtained β values for β-LG in IBW mixtures are graphically displayed in Fig. 4 and also included in Table 1. Fig. 4 illustrates the change of β with various concentrations of β-LG (0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL) in IBW mixture. The results in Fig. 4 clearly show

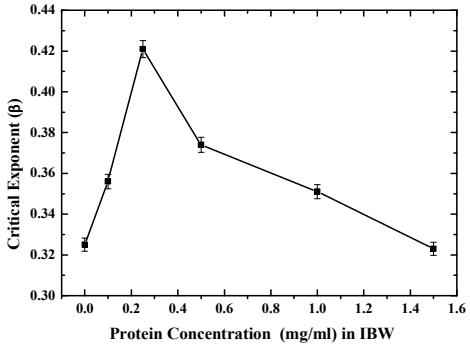


Fig. 4. Influence of β-LG on β as a function of β-LG concentration in IBW mixture. Error bars indicate the standard deviation.

that β values suddenly increased from 0.325±0.007 to 0.428±0.007 with increasing the concentration of β-LG from 0.0 to 0.25 mg/mL, respectively. Later, these values decrease moderately with increasing the concentration of β-LG from 0.5 to 1.5 mg/mL. Interestingly, the obtained β values of β-LG in IBW are fully renormalized β which is consistent with the theory of Broseta and Leibler.[68] The values obtained for β-LG in IBW are 0.363±0.007, 0.428±0.007, 0.381±0.007, 0.358±0.007 and 0.330±0.007 for the five different concentrations of 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL respectively. As shown in Fig. 4 the results explicitly elucidate that the addition of β-LG into IBW, β values are varying because β-LG produces changes in intrachain interactions between the functional groups of the protein and an indirect interactions between the β-LG

residues and the solvent molecules. This phenomenon is consistent with our earlier results in which polymer reduces the β in IBW mixture, in which the polymer produces changes in intrachain interactions between the segments of PEO and an indirect attraction between the polymer chain and the solvent molecules.[51] When *β*-LG is added in small concentration to IBW, *β*-LG preferably adsorbs to molecules of solvents, and thus changes occur in vicinity of critical point. Moreover, the addition of *β*-LG as an impurity induces an important change at the location of the critical region of the binary system.

Fig. 5 provides an overview of the values of T_c for IBW critical mixture as a function of *β*-LG concentration. As can be seen in this Figure, T_c values decrease with increasing the concentration of *β*-LG. The T_c values of *β*-LG in IBW at five different concentrations of 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL were experimentally located at 26.614±0.002, 26.059±0.002, 25.702±0.002, 25.112±0.002 and 24.481±0.002 ^{o}C respectively. The decrease in the T_c of IBW indicates that the *β*-LG biomolecule favors mixing of the solvent molecules and the interactions between the *β*-LG and solvent molecules. Presumably, the decrease in the temperature is the evidence of conformational changes in the *β*-LG which indicates that the *β*-LG functional groups interact unequally with the solvent molecules.

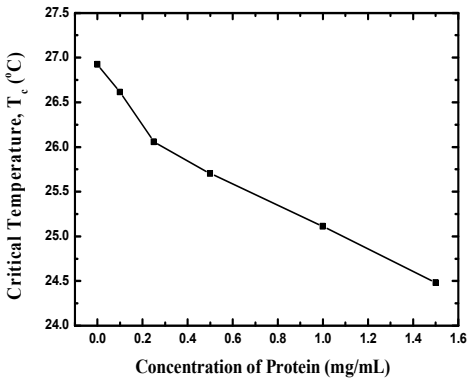


Fig. 5. Effect of *β*-LG on T_c, as a function of *β*-LG concentration in IBW mixture.

Influence of *β*-LG on the coexisting liquid phases of IBW monitored by fluorescence spectroscopy measurements

To evaluate the effect of *β*-LG on the critical behavior of IBW, we have further performed fluorescence measurements. Our previous study[53] made us to take Nile red once again as a suitable probe to examine the conformational changes of *β*-LG in the IBW. Nile red is poorly soluble in water, therefore, an insignificant overall solvation capacity of pure water exists for Nile red in aqueous media.[69] On the other hand, the probe exhibits high solubility in pure IB and in IBW mixtures at high temperatures where a single homogeneous phase is obtained. Therefore, we slowly decreased the temperature of IBW towards its UCST value, so that the changes in the probe emission intensities could be monitored with a decrease in temperature.

Fig. 6 displays steady state fluorescence emission spectra measurements of *β*-LG at various concentrations in IBW at 25 ^{o}C. The results in Fig. 6 reveal that protein free IBW binary liquid mixture exhibits low intensity at a wavelength of 642.5 nm and even the intensity enhancement is not significant in the liquid mixture. However, the IBW emission intensity is increased and eventually reached a maximum with the addition of *β*-LG concentration. This indicates that *β*-LG plays a significant role in inducing phenomenal changes in the IBW binary liquid mixture solution. Further, this enhancement in intensity is more pronounced at the higher concentration than that of lower concentration of *β*-LG. The intensity is increased with increasing the concentration of *β*-LG which is a direct result for the increasing solvent molecular interactions among the amino acid residues. The intensity gradually increased, which is showing the protein becomes aggregated on approaching closer to critical point. The amino acid and carbonyl groups of the solvatochromic dye should also specifically interact strongly with solvent molecules, and due to the change in polarity. We observe a solvatochromism effect/shift with the gradual addition of protein in the homogeneous region. However, the degree of extent of change in emission intensity was strongly influenced at the higher concentrations of the protein. The maximum intensity with a hypsochromic shift (642.5 to 610 nm) was observed with the addition of 1.5 mg/mL concentrations of the protein. The steady state fluorescence emission intensity results elucidated the protein induced structural changes of IBW from the changes in Nile Red fluorescence emission intensities.

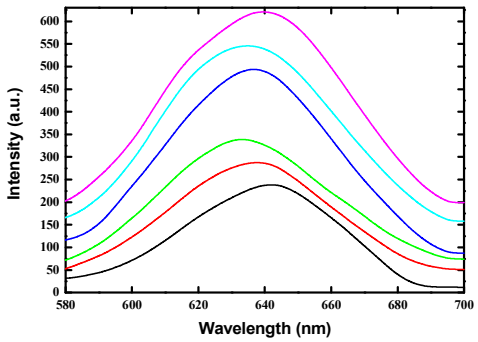


Fig. 6. Steady state fluorescence emission spectra for *β*-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

The fluorescence intensity *vs* temperature dependence of Nile red in IBW binary liquid mixture in the absence and presence of different *β*-LG concentrations (0.0, 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL) has shown in Fig. 7. The T_c of IBW in *β*-LG was obtained from the changes in the Nile red intensities with variations in the temperature. To see the intensity discrepancy of the probe in IBW, we slightly increased the photomultiplier tube (PMT) voltage of the fluorimeter.[53] Simultaneously, we found that, as T_c is approached, the intensity of the fluorescent probe is decreased. This seems to be the declining effect of the water interactions around the Nile red molecule at T_c. However, the sharp enhancement in intensity at UCST is resulted from the coupling of probe molecules onto the

surface of hydrophobic portion of IBW. In IBW solution the hydrophobic collapse process of solvent molecules is induced by thermal effect, solely. Interestingly, the intensity enhancement shifts towards lower temperature, from 27 to 24.3 ^{o}C, upon addition of increasing concentrations of *β*-LG.

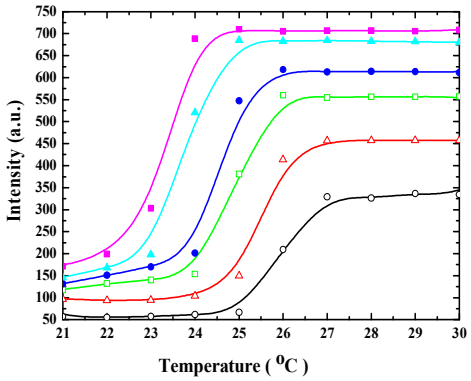


Fig. 7. Intensity *vs* temperature graph for *β*-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

As can be seen from Fig. 7, the fluorescence intensity results are interesting and coincide well with the observed T_c values obtained by *ρ* measurements. However, the T_c values for *β*-LG in IBW as measured by intensity measurements, were experimentally located at 27, 26.4, 25.9, 25.4, 24.8 and 24.0 ^{0}C for 0.0, 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL, respectively. The results clearly indicate that *β*-LG strongly influences on the T_c of the IBW critical mixture. Moreover, on comparing the intensities in Fig.7, one can observe that the emission intensities of the probe increase sharply in the presence of *β*-LG. The shift of higher intensities towards lower temperatures indicates that the diminishment of *β*-LG in IBW. As a result, the influence of *β*-LG on the critical region of IBW mainly depends on the molecular interactions between amino acid residues of protein and solvent molecules. This enhancement is observed only when the local environment around the probe is changed; probably due to the solvation effects in the critical mixture while approaching the T_c.[69] From the experimental results, it is obvious that the solvophobic interactions in the neighboring solvent molecules in the presence of *β*-LG are stronger than those in the absence of *β*-LG. Thus, the formation of a new solvation surface disturbs the environment of the probe in IBW, thereby, enhancing the emission intensities from lower temperatures. Evidently, the fluorescence results are in good agreement with the *ρ* measurements.

Influence of *β*-LG on the coexisting liquid phases of IBW monitored by dynamic light scattering measurements

DLS is a powerful tool to probe the protein behavior in critical mixture.[53,71] To ascertain the *β*-LG behavior on IBW, we further conducted DLS measurements for obtaining d_H and diffusion coefficient (*D*) for *β*-LG in IBW mixture as a function of temperature. An automatic temperature scan of the sample chamber allows observation of size, correlation function and the scattering intensity. DLS experiments allow us to predict the existence of the exponential decay function in a near-critical fluid mixture.[70,71] By scattering light from solvent molecules, their geometrical size and their state of Brownian motion can be measured. Fluctuations in both the local concentration and the local *ρ* contribute to the fluctuations in the scattered intensity for a binary solution.[72] In the present study, we focus our attention on the statistical fluctuations of the light scattered by a critical liquid mixture of IBW while approaching its T_c. The scattered intensity distinction between metastable and unstable states can also be made in the terms of the fate of concentration fluctuations. The autocorrelation function technique was employed in the analysis of photon-count data to obtain the scattering intensity and critical phenomena for observing thermal diffusivity of the binary liquid mixtures.[53] Maximum scattering intensity and minimum *D* was defined as the T_c for the critical mixtures. The DLS signals for a scattering angle of 90^{o} were examined from 22 to 28 ^{o}C for the pure critical mixture. While approaching the T_c, we noticed a large increase in the scattered intensity of the sample. For the sake of brevity and presentation, fluctuations in the scattering intensity with d_H (size) of IBW or *β*-LG in IBW at T_c is shown in Fig. 1S. The results in Fig. 1S clearly show that there is a formation of large clusters of molecules at their respective T_c of critical mixtures by the variation of size with the addition of *β*-LG in different concentrations. Additionally, Fig. 8 exemplarily depicts the resulting autocorrelation functions at different temperatures for the critical mixture of IBW and *β*-LG in IBW. The *D* was calculated from the best fit of Fig. 8 to an exponential decay of the time correlation function. Fig. 9 illustrates the temperature dependency of the *D* of IBW and *β*-LG in IBW. The temperature at which the minimum *D* value is referred to as T_c.[53]

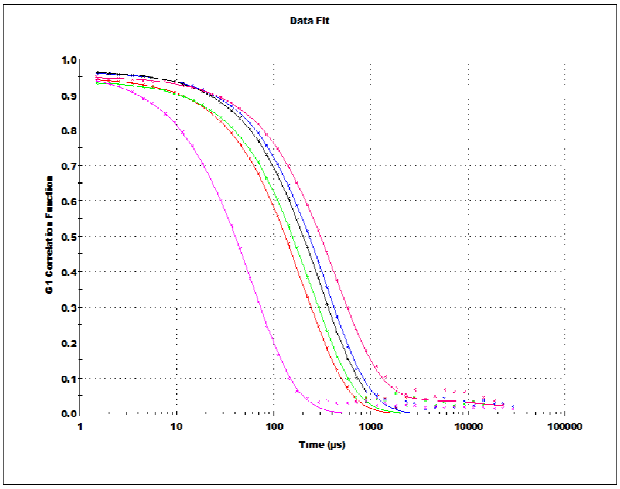


Fig. 8. Autocorrelation function (ACF) analysis for *β*-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

The results in Fig. 9 show a *D* of 21.6 μ^2 s^{-1} at 28 ^{o}C for IBW which gradually decreases to a lower value of 4.42 μ^2 s^{-1} at 26.7 ^{o}C. Upon moving to a lower temperature, the value of the *D* again starts increasing from 26.2 ^{o}C towards higher values. The minimum *D* value is 4.42 μ^2 s^{-1} at 26.7 ^{o}C which correlates well with the observed T_c values of 26.924 and 27.0 ^{o}C by *ρ* and fluorescence measurements for the critical mixture of IBW, respectively.

Additionally, the variations in the D in the presence of β-LG correlate well with the observed experimental values of ρ and fluorescence measurements. From the Fig. 9, we have observed minimum D of 4.42 μ^2 s^{-1} at 26.7 °C, 4.42 μ^2 s^{-1} at 26.5 °C, 2.80 μ^2 s^{-1} at 25.9 °C, 2.33 μ^2 s^{-1} at 25.1 °C, 3.28 μ^2 s^{-1} at 24.4 °C and 3.48 μ^2 s^{-1} at 24.1 °C for 0.0, 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL of β-LG in IBW respectively. The results explicitly explain that the T_c values of IBW decrease with increasing the β-LG concentrations in IBW mixture.

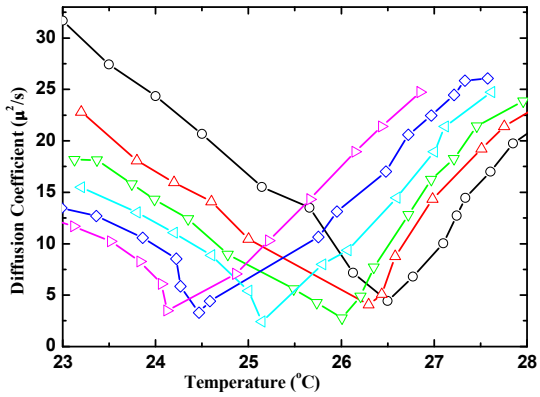


Fig. 9. Diffusion coefficient vs temperature graph for β-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

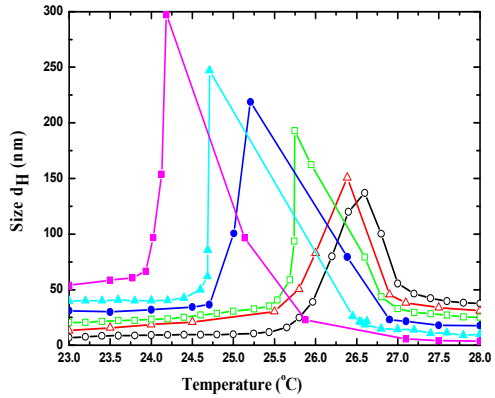
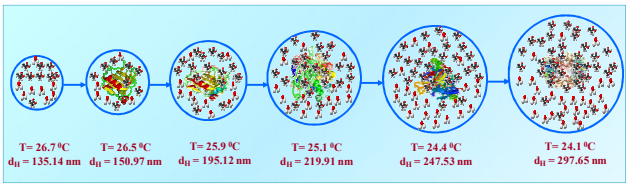


Fig. 10. Size *vs* temperature graph for β-LG, 0.0 (black), 0.1 (red), 0.25 (green), 0.5 (blue), 1.0 (cyan) and 1.5 mg/mL (pink) in IBW mixture.

Fig. 10 compares the representative effects of β-LG (with different concentrations) in IBW. The results in Fig. 10 clearly show that β-LG significantly increases the d_H values of IBW with increasing the protein concentration, from 135.14 nm (free β-LG) to 150.97, 195.12, 219.91, 247.53 and 297.65 nm for 0.1, 0.25, 0.5, 1.0 and 1.5 mg/mL of β-LG in IBW. The d_H values of IBW mixture and β-LG in IBW at critical points are also included in Table 1. The proposed mechanism has been schematically shown in scheme 2. It is interesting to note that the d_H values rapidly increase in the vicinity of the critical point, which indicates protein is completely aggregated at the critical region. Further, the β-LG obviously decreases the T_c values of IBW as the concentration of β-LG increases in IBW. It is evident that the D, d_H and T_c of β-LG in IBW exhibit a strong dependence on the concentration of β-LG.



Scheme 2. The influence of β-LG on IBW critical mixture. The schematic description of protein aggregation in IBW mixture as per DLS measurements (d_H values of β-LG in IBW at their respective critical temperatures).

We have initially measured the d_H values of β-LG (1 mg/mL) in water and the values are 15.9 and 13.9 nm at 26.7 and 24.1 °C (Table 2), respectively. Analogously, the observed d_H values of β-LG (1mg/mL) in IB are 471.6 and 601.1 nm at 26.7 and 24.1 °C (Table 2), respectively. Accordingly, we would expect that the β-LG would be resided predominantly in W-rich phase. However, our DLS results show that β-LG entirely migrated into IB-rich phase, since we observed the d_H values of β-LG in IBW are very high (Fig. 10) that are close to β-LG in IB values. These observations are quite consistent with the ρ measurements of β-LG in IB, water and IBW.

All our results clearly show that the critical region of IBW decreases with the addition of β-LG. Furthermore, the results elucidate that β-LG preferentially absorbed by IB molecules. Consequently, in this process some of the water molecules migrate into IB rich phase along with β-LG molecules. As evidence from ρ measurements the ρ values of β-LG in IB rich phases are higher than those of β-LG in water rich phase. This indicates that β-LG deeply penetrates into the IB-rich phases and then the functional groups of β-LG entangles with IB molecules. Such an entangled system may causes interactions between functional groups of the protein and attraction between the protein functional groups and solvent molecules, which significantly causes alteration of the critical region of IBW.

Conclusions

We have used novel experimental methods to investigate the conformational change of a protein in the vicinity of the critical point of IBW. To the best of our knowledge, these experiments confirm for the first time that the β-LG has aggregated on approaching closer to the critical point of the IBW mixture. On approaching close to the critical region, we observed a dramatic increase in the size of the β-LG to a value which is larger than the size far away from the critical region. This clearly indicates that the protein is aggregated at the critical region of the IBW mixture. Our results conclude that most of the protein entangles in the upper IB-rich phase. Our observations distinctly demonstrate that the existence of the critical region of IBW is modified with addition of β-LG. The results show that the T_c and β values are known to be very sensitive to the addition of impurities to the binary liquid critical mixtures. Apparently, our results show that the critical region of IBW shifts down with increasing the concentration of β-LG. We

assume that the quantitative knowledge of biomolecule-solvent interactions will help in understanding the behavior of third component in the coexisting liquid phases.

Acknowledgements

We acknowledge the financial support from the Department of Science and Technology (DST), New Delhi, India (Grant No. SB/SI/PC-109/2012).

References

1. A. Kumar and P. Venkatesu, *Chem. Rev.,* 2012, **112**, 4283-4307.
2. S. L. Maux, S. Bouhallab, L. Giblin, A. Brodkorb and T. Croguennec, *Dairy Sci. Technol.*, 2014, **94**, 409-426.
3. H. Wang and A. Ben-Naim, *J. Phys. Chem. B,* 1997**, 101**, 1077-1086.
4. Y. Dror, T. Ziv, V. Makarov, H. Wolf, A. Admon and E. Zussman, *Biomacromolecules*, 2008, **9**, 2749–2754.
5. G. Kontopidis, C. Holt and L. Sawyer, *J. Dairy Sci.*, 2004, **87**, 785–796.
6. S. Brownlow, J. H. M. Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. North and L. Sawyer. *Structure,* 1997, **5,** 481-495.
7. M. Z. Papiz, L. Sawyer, E. E. Eliopoulos, A. C. North, J. B. C. Findlay, R. Sivaprasadrao, T. A. Jones, M. E. Newcomer and P.J. Kraulis, *Nature,* 1986, **324**, 383-385.
8. T. V. Burova, Y. Choiset, V. Tran and T. Haertle, *Protein Engineering,* 1998**, 11**, 1065–1073.
9. T. Croguennec, S. Bouhallab, D. Molle. B.T. Kennedy and R. Mehra, *Biochem and Biophys Res Comm,* 2003, **301**, 465-471.
10. D. Jayat, J. C. Gaudin, J. M. Chobert, T. V. Burova, C. Holt, I. McNae, L. Sawyer and T. Haertle, *Biochemistry,* 2004, **43***,* 6312-6321.
11. H. L. Monaco, G. Zanotti, P. Spadon, M. Bolognesi, L. Sawyer and E. E. Eliopoulos, *J. Mol. Biol.,* 1987, **197**, 695-706.
12. R. Wada, Y. Fujita and N. Kitabatake, *Biochim. Biophys. Acta*, 2006, **1760**, 841–847.
13. L. Sawyer and G. Kontopidis, *Biochim. Biophys. Acta,* 2000, **1482**, 136-148.
14. P. Busti, S. Scarpeci, C. Gatti and N. Delorenzi, *Food Research International,* 2002, **35**, 871-877.
15. C. Bhattacharjee and K. P. Das, *Eur. J. Biochem.,* 2000, **267**, 3957-3964.
16. T. Takekiyo, Y. Koyama, K. Yamazaki, H. Abe and Y. Yoshimura, *J. Phys. Chem. B,* 2013, **117**, 10142−10148.
17. K. Liskova, M. A. E. Auty, V. Chaurin, S. Min, K. Hun Mok, N. Brien, A. L. Kelly and A. Brodkorb, *Eur. J. Lipid Sci. Technol.,* 2011, **113**, 1207–1218.
18. S. L. Maux, L. Giblin, T. Croguennec, S. Bouhallab and A. Brodkorb, *J. Agric. Food Chem.,* 2012, **60**, 9476−9483.
19. A. H. Sneharani, J. V. Karakkat, S. A. Singh and A. G. Appu Rao, *J. Agric. Food Chem.,* 2010, **58**, 11130–11139.
20. C. D. Kanakis, I. Hasni , P. Bourassa, P. A. Tarantilis, M. G. Polissiou and H. Tajmir-Riahi, *Food Chem.,* 2011, **127,** 1046–1055.
21. L. Liu, E. N. Kitova and J. S. Klassen, *J. Am. Soc. Mass Spectrom.,* 2011, **22**, 310-318.
22. F. Morgan, A. Venien, S. Bouhallab, D. Molle, J. Leonil, G. Peltre, and D. Levieux. *J. Agric. Food Chem.,* 1999, **47**, 4543−454.
23. M. S. P. Stevar and A. Vorobev, *Transp Porous Med*., 2013, **100**, 407–424.
24. Y. W. Kim and J. K. Baird, *J. Phys. Chem. B*, 2005, **109**, 17262–17266.
25. J. Kohanoff, C. Pinilla, T. G. A. Youngs, E. Artacho and J. M. Soler, *J. Chem. Phys.,* 2011, **135**, 154505-154515.
26. M. Bouanz and D. Beysens, *Chem Phys Lett.,* 1994, **231**, 105-110.
27. A. I. Norman, B. A. Manvilla, E. L. Frank, J. N. Niamke, G. D. Smith and S. C. Greer, *Macromolecules,* 2008, **41**, 997-1008.
28. K. M. Dean, C. B. Babayco, D. R. B. Sluss and J. C. Williamson, *J. Chem. Phys.,* 2010, **133**, 074506-1.
29. H. L. Bianchi and M. L. Japas, *J. Chem. Phys.,* 2001, **115**, 10472-10478.
30. S. Morita, F. Tsunomori and H. Ushiki, *Eur. Polym. J.*, 2002, **38**, 1863-1870.
31. G. Staikos and A. Dondos, *Phys. Rev. A,* 1986, **33**, 4479-4481.
32. K. To, C. K. Chan and H. J. Choi*, Physica A,* 1995, **221**, 223-232.
33. I. H. Park and M. J. Kim, *Macromolecules*, 1997, **30**, 3849-3854.
34. R. S. Shresth, R. C. Mc Donald and S. C. Greer, *J. Chem. Phys.,* 2002, **117**, 9037-9049.
35. K. To and H. Choi, *J. Phys. Rev. Lett.,* 1998, **80**, 536-539.
36. B. J. de Gans, R. Kita, S. Wiegand and L. Strathmann, *J. Phys. Rev. Lett.,* 2003, **91**, 245501-25504.
37. A. Kumar, P. M. Reddy and P. Venkatesu, *RSC Adv.*, 2012, **2**, 6939–6947.
38. A. Toumia, N. Hafaiedha and M. Bouanza, *Fluid Phase Equilibria,* 2011, **305**, 185–191.
39. A. Kumar, H. R. Krishnamurthy and E. S. R. Gopal, *Phys. Rep.,* 1983, **98**, 57–143.
40. D. T. Jacobs, *J. Chem. Phys.,* 1989, **91**, 560–563.
41. A. Toumi and M. Bouanz, *Eur. Phys. J. E,* 2000, **2**, 211–216.
42. R. Okamoto and A. Onuki, *Phys. Rev. E: Stat., Nonlinear Soft Matter Phys.,* 2010, **82**, 051501.

43. N. Hadded and M. Bouanz, *Fluid Phase Equilibria,* 2008, **266**, 47-53.

44. H. L. Bianchi and M. L. Japas, *J. Chem. Phys.,* 2001, 115, **22**, 10472-10478.

45. A. Toumi and M. Bouanz, *J. Mol. Liqs.,* 2005, **122**, 74 - 83.

46. A. Toumi, M. Bouanz and A. Gharbi. *Chem. Phys. Lett.,* 2002, **362,** 567–573.

47. M. Wagner, O. Stanga and W. Schroer, *Phys. Chem. Chem. Phys.,* 2003, **5**, 1225-1234.

48. K. I. Gutkowski, H. L. Bianchi and M. L. Japas, *J. Phys. Chem. B,* 2007**, 111***,* 2554-2564.

49. M. L. Alessi, A. I. Norman, S. E. Knowlton, D. L. Ho and S. C. Greer. *Macromolecules,* 2005**, 38**, 9333-9340.

50. A. I. Norman, D. L. Ho and S. C. Greer. *Macromolecules,* 2007**, 40**, 9628-9639.

51. P. M. Reddy, P. Venkatesu and H. B. Bohidar. *J. Phys. Chem. B,* 2011, **115**, 12065-12075.

52. P. Venkatesu, *J. Phys. Chem. B***,** 2006, **110**, 17339-17346.

53. A. Kumar, P. M. Reddy and P. Venkatesu, *New J. Chem.,* 2012, **36**, 2266-2279.

54. B. Hu, J. K. Baird, P. K. Alvarez, K. C. Melton, D. A. Barlow and R. D. Richey. *Int J Thermophys.*, 2014, **35,** 841-852.

55. J. K. Baird, J. D. Baker, B. Hu, J. R. Lang, K. E. Joyce, A. K. Sides and R. D. Richey. *J. Phys. Chem. B,* 2015, **119**, 4041-4047.

56. T. J. Giesy, A. S. Chou, R. L. Mc Feeters, J. K. Baird and D. A. Barlow, *Phys. Rev. E,* 2011, **83**, 061201-0601208.

57. B. Hu and J. K. Baird. *J. Phys. Chem. A,* 2010, **114**, 355-359.

58. Y. W. Kim and J. K. Baird. *J. Phys. Chem. B,* 2005, **109,** 17262-17266.

59. R. F. Berg and M. R. Moldover, *J. Chem. Phys.* 1988, **89**, 3694-3704.

60. Y. Seki, T. Okabe, Y. Oshida, M. Kobayashi, T. Tsukada, M. Hozawa and M. Kubo, *Chem. Eng. Sci.*, 2004, **59**, 2685 - 2693.

61. M. Bouanz and D. Beysens, *Chem. Phys. Letts.*, 1994, **231**, 105-110.

62. M. Bouanz, *Phys. Rev. A,* 1992, **46**, 4888- 4893.

63. A. Toumi and M. Bouanz, *Eur Phys. J. E:softmatter Biol. Phys*., 2000, **2**, 211-216.

64. S. C. Greer, *Phys. Rev. A,* 1976, **14**, 1770-1780.

65. D. T. Jacobs and S. C. Greer, *Phys. Rev. E,* 1996, **54**, 5358-5363.

66. D. Beysens, A. Bourgou and P. Calmettes, *Phys. Rev. A,* 1982, **26**, 3589-3609.

67. W. V. Andrew, T. B. K. Khoo and D. T. Jacobs, *J. Chem. Phys.*, 1986, **85**, 3985-3991.

68. D. Broseta and L. Leibler, *J. Chem. Phys.,* 1989, **90**, 6652-6655.

69. K. I. Gutkowski, R. F. Prini, P. F. Aramendia and M. L. Japas, *J. Phys. Chem. B,* 2011, **115**, 15303-15313.

70. S. Limberg, L. Belkoura and D. Woermann, *J. Mol. Liqs.,* 1997, **74**, 223-237.

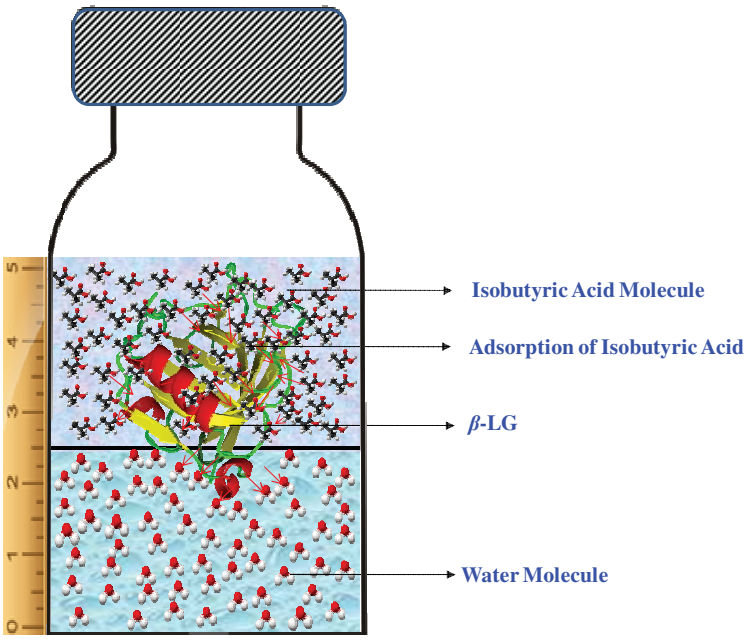
71. B. Kruppa and J. Straub, *Exp. Therm. Fluid Sci.,* 1993, **6**, 28-38.

72. D. A. Ivanov and J. Winkelmann, *Phys. Chem. Chem. Phys.,* 2004, **6**, 3490-3499.

A study of conformational changes of *β*-lactoglobulin in the vicinity of critical point of binary mixed solvents

R. Umapathi and P. Venkatesu*

Department of Chemistry, University of Delhi, Delhi – 110007, India



Most of the protein is entangled in the upper IB rich phase