FTIR AND ULTRASONIC STUDIES ON THE STABILIZATION OF A-LACTALBUMIN

Kathiroli, Kavitha[1], **Lakshmanan,** Palaniappan[1]

[1]*Department of Physics, Annamalai University – 608001, Tamilnadu, India*
E-mail address: **lp_dde_au@yahoo.com**

Abstract
Main aim of this work is to understand how the protein α-lactalbumin is affected by the variations in pH of the medium and presence of cosolvent. pH is one of the main denaturant of proteins and the addition of cosolvent in many cases is found to control the extent of denaturation. In this work fructose solution is considered as cosolvent and the extent of denaturation is analysed by ultrasonic methods and are further confirmed by FTIR amide-I second derivative spectra. Ultrasonic techniques along with the related parameters were applied for samples with various pH at 303 K. Obtained results shows that denaturation is sensitive to pH, however, acidic and alkaline behave totally in a different way. It was found that the impact of alkaline pH produces lesser denaturation and is slower whereas the impact of acidic pH is specific and instantaneous. Ultrasonic analysis shows that pH variation can denature the protein whereas the addition of cosolvent supports renaturation. FTIR spectra were recorded for the experimental samples from which the second derivative curve fitted spectra were constructed using Origin program. Quantitative assignment of peaks and the variations in cumulative areas calculated for the structures like α-helix, β-sheets etc confirms the observations of ultrasonic analysis that the pH variations aid in denaturation whereas the cosolvent supports the renaturation of protein.

Keywords: ultrasonic velocity, fructose, α-lactalbumin, FTIR, viscosity, pH, non-covalent interactions

Received: 09.06.2020 Reviewed: 15.06.2020 Accepted: 29.06.2020

INTRODUCTION

Proteins belong to macro molecules that commonly found in bio systems and are sole responsible for the structural maintenance of the systems. Structural stability of a protein is very important as regards its applications, especially as food allergens, diagnostics, therapeutics, etc. This structural stability largely depends on many factors, of which the pH, the temperature, and the salt concentration are relatively important. Any small variations in these factors drastically affect this native state and made the protein to be denatured. Folding or unfolding of proteins is an indication of their denaturation or renaturation. Additives made in any form or purpose may further denature or re-nature the original protein structure.

Dissolved sugars and polyhydric alcohols are in general used as a stabilizing agent for proteins (Gekko and Morikawa,1981; Gerlsma and Stuur, 2002). It is a fact that if these proteins are globular in nature, then they invariably undergo functional and conformational changes. Such changes are always associated with packing nature and are found to affect the intra and inter interactions and so can be revealed easily by physical means. Ultrasonic methods are one such techniques that involves their propagation characters, density, etc and computation of related parameters.

Protein study using ultrasonic techniques is an established attempt for resourceful information about the functional and structural features (Lin et al. 2002; Velusamy and Palaniappan, 2016). Various investigators (Hakin *et al* 1994, Zhang *et al*. 2015; Kanjilal *et al 2003*) have used such techniques at different pH environments. This is an extension of our earlier work (Velusamy and Palaniappan, 2016) in which glucose is attempted as cosolvent. Intermolecular free length and acoustic impedance were used by Aashees Awasthi and Shukla (2003) to explore the inertial and elastic properties of the proteins. Waris (2003) had made viscometric studies regarding protein stabilization by means of co-solvents. All these resources are used in the

present work to analyze the importance of pH variations (from 2 to 12) and use of fructose as cosolvent in the structural pattern of α-lactalbumin (α-LA) at 303 K using ultrasonic techniques. Further, the outcomes of the analyses are confirmed by FTIR studies in the same systems.

BACKGROUND INFORMATION

D-fructose, simply mentioned as fructose in this work, is a popular monosaccharide for which ample information are available in literature (Satyanarayana 2000)

Protein can be denatured by many ways. Based on the type or agent of denaturation, either the internal disulfide bonds or the amide bonds between peptide linkages will be ruptured and cause denaturation (Tatsumi et al. 1998). In the present study, pH and the cosolvent are the main agents of protein stability parameters and both have nothing to do with internal bonds. Further pH is closely associated with the zwitterions (pI) and to maintain it, phosphate buffers were used, as followed by Waris et al (2001) and Green (1993). Care should be taken so as to avoid protein aggregation for which Belyakova et al., (2003) have chosen an ionic strength of 0.005 mol dm^{-3} for the experiments. Same is adopted in this work also by using monobasic and dibasic sodium phosphates in buffer, as done by Elving et al (1956).

Parameters measured in this work comprises of ultrasound velocity (u), density (ρ), viscosity (η) and surface tension (τ). Four absolute parameters that are calculated from the measured values are adiabatic compressibility (β), intermolecular free length (L_f), acoustic impedance (z), relaxation time (t). Additionally three relative parameters were also evaluated namely relative association constant ((R_A), the partial apparent specific volume (φ_v) and the partial apparent specific adiabatic compressibility (φ_k).

As regards the stability of protein structure and functions, the surrounding environment and the associated components play a crucial role. Such entities always affects the static and dynamic properties the system. Of the four measured parameters here, the two static properties *viz.*, density and surface tension, are important in the sense that they can reveal the structural / conformational changes in the system. The other two measured parameters are dynamic properties and they are able to reveal the associated functional changes.

The validity of any measurement is checked by their uniqueness in various calculations that involves these parameters. This is attempted by considering different thermo acoustical parameters of significant meaning. In literature so many thermo acoustical parameters are available to define well about a material or medium. All these parameters basically define either the static tendencies or the dynamic tendencies and even the inter dependence of static and dynamic nature.

Keeping these concepts in mind, all the three groups of calculated parameters are considered in this work. The adiabatic compressibility and the intermolecular free length are the two for structural studies, the acoustic impedance and the acoustic relaxation time are the two for the functional studies and the relative association is the interdependence parameter of static and dynamic properties.

Further, there are quite possibilities for the temporary changes or shifts in the system and they have been studied using the two apparent parameters viz., apparent adiabatic compressibility and the apparent volume. For the purpose of generalizing and standardising the parameters as per the definitions (Robert et al., 1997), the last two parameters are taken as partial apparent specific volume and partial apparent specific adiabatic compressibility. These two are macroscopic entities and are sensitive to protein stability and dynamics (Nolting and Sligar 1993; Kharakoz and Sarvazya 1993).

Fructose quantity is chosen to be 1 M as adopted by Campbell and Farrell (2006) as this concentration doesn't affect amino acid residues in the chain. Further this concentration is apt for the term 'specific' in the last two parameters. Same way, protein concentration taken in the present work (5 mg/ml), though extremely quite high in terms of physiological values, it is worth to note that

this is needed to have good reliable ultrasonic values and the same can be extended for biosensor application studies (Malhotra et al., 2017). Thus the present study is well planned to analyze the situation in all possible dimensions with the available ultrasonic data.

Fourier transform IR spectroscopy is one of the recognized powerful tool in protein studies. (Noguchi 2007; Schultz 2000; Ramajo et al. 2005). Secondary structural components (α-helix, β-sheet etc) of proteins are used in the present study to confirm the observed influences of pH and cosolvent on the protein stability. It is better to remind that the X-ray structure of bovine native α-LA (Acharya 1991) has α-domain, β-domain, Trp residues, S-S bridges and residues of coordination of Zn^{2+} ions and they are shown in blue, green, blue, yellow and red colour respectively in Fig 1. More details of these domains are also provided by Permyakov and Berliner (2000).

MATERIALS AND METHODS

Materials

Chemicals of purity more than 99% are used in this work. Lactalbumin solution (5 mg/ml) was prepared using the powdered α-lactalbumin purchased from Sigma Aldrich. Buffers in acidic and alkaline range (pH 2,5,7,9 and 12) using 0.2 M aqueous sodium phosphates (NICE chemicals) were prepared. Protein (L) dissolved in these buffers (B) forms System I (B+L) whereas the same with the addition of 1 M fructose (F) solution as cosolvent forms the system II (B+F+L).

Measurements were made using the properly preserved stock solution after ensuring the attainment of thermal equilibration (303.00 ± 0.01 K). Digital pH meter (Model-HI-98107, HANNA Instruments) is used for pH measurement.

Measurements

Four basic properties viz., density, sound velocity, viscosity and surface tension were attempted in this work. Repeated closely matching trials were made for each measurement that generates a satisfactory standard deviation (not shown here) and finally the average of the observations is reported. Temperature control (± 0.01 K) is maintained by a water thermostatic bath (Ragaa Industries, Chennai, India

Specific gravity bottle (5 ml) method was used for density measurement, accurate to ±0.0001 kgm^{-3}. Mittal's model F-81 ultrasonic interferometer, 2 MHz was used for velocity measurement, accurate to ±0.1 ms^{-1}.

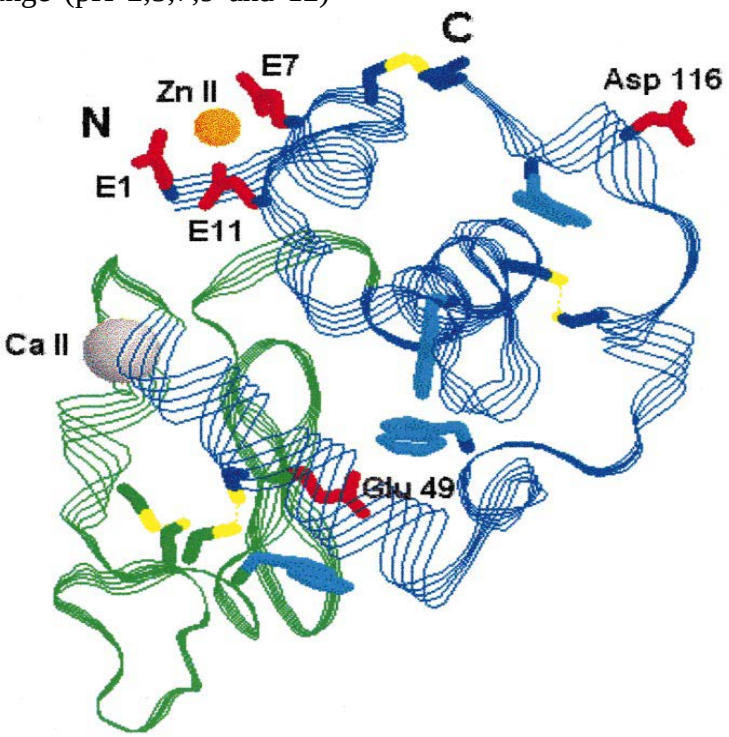

Fig 1. X-ray structure of bovine α-LA [24]

Ostwald's 10 ml viscometer for viscosity measurement (accurate to ± 0.001mNsm^{-2}) and drop (by platinum-iridium Du Nouy ring) weight method for surface tension, accurate to ±0.0001 kg were also carried out. All measurements were made at 303 K. Details of measurements, instruments and the procedures adopted are available in one of our earlier work (Palaniappan and Velusamy 2004).

Transmission mode FTIR Spectra (resolution of 1.0 cm^{-1}) of proteins were recorded in the region 4000-450 cm^{-1} by a FTIR (PERKIN ELMER) spectrometer at Sophisticated Analytical Instrument Facility Lab, Indian Institute of Technology, Madras, India. Appearance of amide I bands at 1659, 1650, 1640 and 1624 cm^{-1} of α-LA clearly confirms its purity as Boye et al., (1997) have noticed same peaks in their work.

Calculation of thermo-acoustical parameters

As stated earlier, the chosen thermo acoustical parameters are calculated using the following standard relations (Swain and Priyadarshini 2010; Velusamy et al., 2007; Walter et al., 1995; Ravichandran and Ramanathan 2010; Kadi et al., 2006).

$$\beta = [\rho u^2]^{-1} \quad (1)$$

$$L_f = K_T \beta^{1/2} \quad (2)$$

$$Z = u\rho \quad (3)$$

$$t = [4\eta/3u^2\rho] \quad (4)$$

$$R_A = [\rho/\rho_o]\,[u_o/u]^{1/3} \quad (5)$$

$$\varphi_v = 1/\rho_o + [\rho_o - \rho]/[C_p\,\rho_o] \quad (6)$$

$$\varphi_k = \beta_o\,[2\varphi_v - 2[u] - 1/\rho_o] \quad (7)$$

where K_T is 199.53 x 10^{-8} in S.I system at 303 K, ρ_o, u_o and ρ, u are respectively the density and sound velocity of the solvent and solution, concentration of protein is C_p, adiabatic compressibility of the solvent is β_o and [u] is the relative specific sound velocity increment given as.

$$[u] = [u-u_o]/u_oC_p \quad (8)$$

Further, the three relative parameters viz, R_A, φ_v and φ_k are calculated for the system of B+F+L by treating B+F as solvent. The necessary observations for this solvent were also made separately.

RESULTS AND DISCUSSION

Various measured parameters for system I and system II 303 K are in Table 1, whereas Table 2 shows the calculated values of first four thermo-acoustical parameters. Other calculated parameters against various pH values are depicted in Figs 2 to 4 respectively. Fig 5 is the recorded primary spectra of system I and II measured in pH 2, 5, 7, 9 and 12 at 303 K. The second derivative curve fitting spectra were sequentially depicted in Fig 6. Various assigned amide I frequencies for the secondary structure of protein are listed in Table 3 (Carbonaro and Nucara 2010; Dong et al., 1990). The assignment of peaks and their areas for each pHs were listed in Table 4. The consolidated quantitative measurement of these secondary structures in α-LA system with and without cosolvent is given in Table 5.

Measured parameters

The amount of mass accumulated in unit volume is referred as density and the accumulation of hydrogen ions is termed as pH. Density variation with respect to increase in pH reveals the influence of more number of hydrogen ions on the other components present in the system.

Table 1: Measured Parameters at various pH

pH	Density (ρ) kgm^{-3}		Sound velocity (u) m		Viscosity (η) mNsm^{-2}		Surface tension (τ) Nm^{-1}	
	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)
2	1018.4	1092.6	1552.2	1585.8	0.9121	1.2011	0.2343	0.2465
5	1020.2	1076.9	1538.7	1579.6	0.7527	1.0971	0.2477	0.2543
7	1019.6	1070.5	1533.0	1568.4	0.8231	1.2832	0.2492	0.2421
9	1021.4	1075.4	1539.8	1599.3	0.8228	1.3122	0.2426	0.2311
12	1033.2	1082.3	1561.2	1598.2	0.8991	1.3029	0.2014	0.2225

In all experimental solutions, either in system I or in system II, the amount of other component(s) protein and/or fructose is same whereas variation exists only in hydrogen ion concentration. Invariably, whether the fructose is present or absent, the ρ value is minimum at pH 7 and increases with acidic or alkaline pH is evident as per Table 1. A neutral pH cannot be attributed to structure breaking or structure making effects and hence the structure existing at this pH is taken as the native or primary helical structure of lactalbumin.

The planar and rigid peptide bonds are the major part of the primary structure. N terminal is having free amino group and the C terminal is having acid group. Acidic pH leads to positively charged amino acid with less mobility and coils of larger radius. The alkaline pH leads to negatively charged amino acid with high mobility but coils of smaller radius. It is good to note that the presence of fructose reduces the denaturation effect especially at extreme pH. This is the reason for the density reduction at pH 7 and is the most compact state.

On observing the sound velocity values (in Table 1) least value is found at pH 7 and higher at extreme pH. Moreover pH 2 and pH 7 records the almost same u value. Velocity being a dynamic character largely depends on the cohesion and mobility factors. In addition to the coiling or uncoiling mobility factors, the asymmetric α-helix of lactalbumin, suggests the mobility difference of C and N-terminal. The N-terminal with slight positive charge and C-terminal with slight negative charge (Walter et al., 1995) forms a dipole and it promotes the stability of α-helix. This also enhances the cohesion between the components. Literature reveals that amides can be converted in to their corresponding acids by pH variations (Walter et al., 1995; Ravichandran and Ramanathan 2010). As acids are in general cohesive and interactive, a higher sound velocity is observed at pH 12.

The perusal of Table 1, sound velocity for system II, reveals that the addition of fructose largely enhances the magnitude of sound velocity, though the trend remains unaltered. This large association is revealed by the increasing ultrasonic velocity (Kadi et al., 2006; Carbonaro and Nucara 2010; Dong et al., 1990; Cabilio et al., 2000). This trend of velocity may be taken to the indication of formation of intermolecular hydrogen bonding between solute-cosolvent molecules. This may either be weak types such as the van der Waals interactions or electrostatic interactions or the strong types that includes ion-dipole interaction (Miecznik and Golebiewski 2004). The net effect of all these non-covalent interactions forms the basis for the sound velocity increase.

Viscosity is another dynamic parameter measured here. This property can be explained by considering the basic features responsible for viscosity. In other words, the separation between the layers and their area are the main parameters. Lactalbumin at pI, which is around pH 5 (Palaniappan and Velusamy 2004), possess single chain, has no layers. This suggests less viscosity. But in other pH values, multi chain with many layers will be formed that leads to higher viscosity. Also in acidic pH, larger area coils and hence large viscosity can be expected. Thus viscosity will be least, larger and largest at pI, alkaline and acidic extremities respectively. Formation of more coils indicates the more denaturation of protein. Presence of fructose further increases the surface area and hence viscosity increases irrespective of pH range.

Cosolvent addition leads to increase the magnitude of η. This increase in η is reflected in the build-up of intermolecular interactions. In the Rayleigh scattering studies, Paolantoni *et al.* (2007) revealed that the glucose addition in aqueous reduced the water dynamics. It is a tacit assumption here that fructose may also behave so. A decrease in dynamics is reflected by an increase in viscosity as found in the present study. Thus it is evident that the cosolvent, fructose, is found to play a significant role in the system.

Surface tension is again a static property and in the case of solution, it characterizes the nature of solute rather than the solvent. For the system I in the present work, higher τ value is at pH 5 that reflects the significance of iso-electric

point of proteins at which a protein is in the undisturbed state. As the protein is in unstrained condition, the value of τ observed is solely due to the solvent (pure buffer) for system I. This is verified experimentally by measuring the surface tension of pure buffer (not shown here). The τ at other pH values show the influence of protein in the chosen solvent and it is interesting to notice the higher surface activity of proteins near their iso-electric point (pI). Thus their surface tension reduces as they are away from their pI. The primary helical structure is linear than a coiled structure. Coiled patterns are always with least radius and lower surface area that can have minimum surface tension. Thus surface tension is found to be maximum at the native state. Observed values of surface tension reassure the suggestions made earlier. This maximum τ value for lactalbumin at its iso-electric point is also confirmed by Belton and Twidle (1940) in their work.

For the stability of a dissolved solute in a solvent, the solute (protein) should not form further tiny cavities, or pockets, within the solvent or in solution. If the surface tension is more, then it is the protein may not form any cavity and the stability will be enhanced. The influence of cosolvent on the energy requirement conditions for forming a cavity in the solvent was demonstrated by Breslow and Guo (1990) and shown that it is reflected in the increased magnitude of surface tension. In this study, the cosolvent addition increases the surface tension and reduces the cavity formation lactalbumin and hence their stability got increased.

Calculated parameters

The perusal of Table 2 shows that it contains the calculated values of standard thermo acoustical parameters such as the adiabatic compressibility, intermolecular free length, acoustic impedance and the relaxation time.

Compressibility is the reciprocal of bulk modulus and it reveals the space that is available freely in the medium. An orderly structure is always tightly packed and has minimum compressibility. As the changes in the structural patterns are so fast, it is aptly called as adiabatic compressibility. Adiabatic compressibility, free length etc always best reveals the arrangements in the structure. Gekko *et al*. (1986) have established that a positive compressibility can be noticed for the globular proteins while a negative is for their constitutive amino acids due to the hydration effect. Thus it is evident that the protein interior will have very high compressibility.

In the present case, system I of Table 2 indicates that β and L_f forms one similar trend whereas the z and τ shows an exactly reverse trend with pH variations as well as with addition of cosolvent. β and L_f are positive for the considered system. It means that large number of cavities is available in the protein molecules and the solvation effect is overruled by these cavities. Higher β (pH 5) is indicative of native state whereas lower β (pH extremities) indicates denatured state. The addition of fructose occupies the available cavities or voids, as said in an X-ray crystal structure study (Acharya et al., 1991) and hence the magnitude of compressibility as well as free length decreases with the presence of fructose.

Table 2: Calculated Values of Adiabatic compressibility (β), Intermolecular free length (L_f), Acoustic impedance (Z) and Relaxation time (t)

pH	$\beta \times 10^{10}$ Pa^{-1}		$L_f \times 10^{11}$m		$Z \times 10^{-6}$ kgm^{-2}s^{-1}		$t \times 10^{10}$ s	
	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)	System (B+L)	System (B+F+L)
2	4.0755	3.6395	4.0280	3.8065	1.5807	1.7323	4.9564	5.8285
5	4.1400	3.7216	4.0598	3.8492	1.5697	1.7010	4.1549	5.4439
7	4.1733	3.7975	4.0761	3.8882	1.5630	1.6789	4.5801	6.4972
9	4.1292	3.6355	4.0545	3.8044	1.5727	1.7198	4.5301	6.3607
12	3.9709	3.6173	39760	3.7949	1.6130	1.7297	4.7604	6.2840

The observed trend is also in line with the observations of Chanasattru *et al.* (2007) wherein the effect of cosolvent on the variations of molecular characters of globular proteins was discussed. All these studies indicate that inclusion of cosolvent decreases the effective volume and hence the adiabatic compressibility. This may be due to the increased intramolecular bonding and the expulsion of water-containing voids by the added cosolvent (Sarat and Pragnya 2010). This view is supported by Timasheff (1992) wherein he observed that the cosolvent increases the osmotic pressure acting on the proteins. This clearly explains the observed minimum value of β and L_f at pH extremities of system II.

Table 2 (System I) shows that, cosolvent is there or not, the z of lactalbumin at pH 7 and pH 5 is almost same, minimum being at pH 7. This factor is governed by the elastic and inertial nature of the medium, thus there is a fluctuation at these pH values. Again this represents the reduction in non-covalent interactions and lends a support to the previous view of native state at pH 5 and denatured at pH extremities. Though the trend is same, addition of fructose is reflected by an increased magnitude of z, thereby indicates the constraints imposed on the system. This specifically establishes that fructose has a significant role on the structural variations of lactalbumin. Since the medium is basically aqueous and is having large number of ions fructose addition increases the electrostatic, hydrophobic and hydrogen bonding interactions and leads to a more compact form. Thus, the protein stabilization got improved.

The relaxation time (t) provides better insight in to the molecular interactions. As observed in Table 2, t values are having specific variations with change in pH from iso-electric point but the increasing pH towards alkaline is less pronounced. The mobility variations caused in this pH can again be attributed to this observed trend. As found in other parameters, the variations in t shows that the inclusion of fructose supports the compactness of the medium thereby improve the stability of proteins.

The comparative strength of static to dynamic nature of concerned solute (protein) is often termed as relative association (R_A). The discussions made so far shows that the primary helical structure exists either at pH 5 or at pH 7. The consideration of R_A clearly confirms the same view. The perusal of Fig 2 shows that for both systems, R_A is very close to 1.0000 and it means that static and dynamic nature are equal to each other and this is the highly compact state. The deviation from unity at other pHs, represent the denaturation.

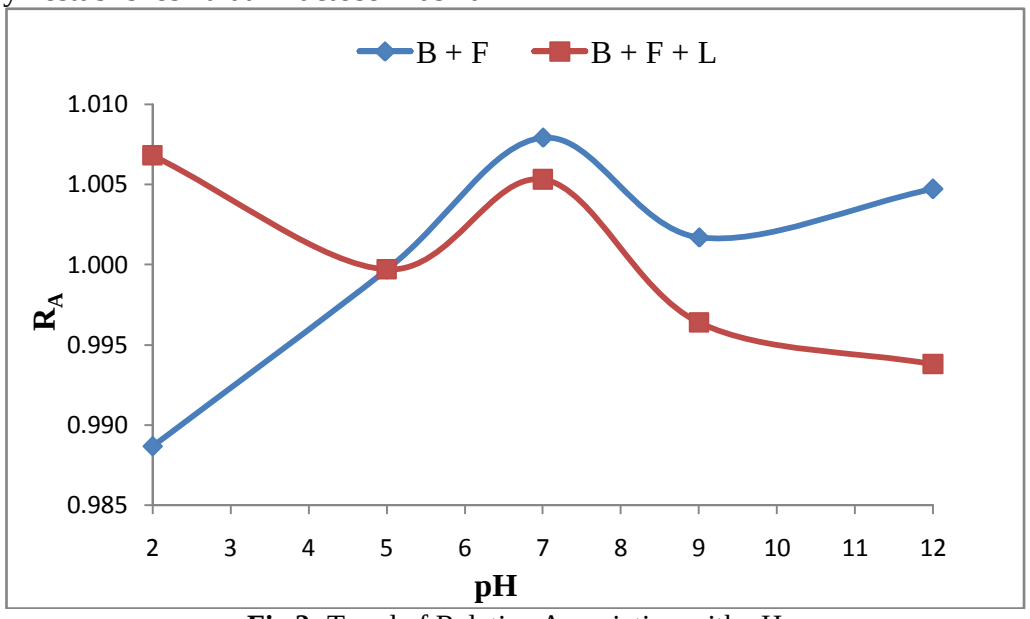

Fig 2: Trend of Relative Association with pH

Further it is also evident that fructose aids in keeping the R_A to be more closer to unity at all pH and the absence of fructose keeps the R_A to deviate from unity. Thus, fructose addition aids the renaturation process. The already stated renaturation mechanism is again holds good. The increased intramolecular binding and the expulsion of water-containing voids and in the protein interior on one hand and the strong interaction between water and cosolvent on the other hand are the main attributes for these observed trends. The collective effect of these two opposing process tends to reduce the denaturation of proteins.

An effect or change produced by sound propagation is never permanent but always transient hence they are only apparent, not absolute. It is worth to note that these apparent changes can demonstrate interesting results. Proteins are only a component of the system and thus the volume and the compressibility in terms of their partial apparent specific values are aimed in the present work.

It was mentioned that both the partial apparent specific volume (φ_v) and partial apparent specific compressibility (φ_k) of the globular protein molecule have three major contributions (Breslow and Guo 1990; Gekko and Noguchi 1979) *viz.*, one due to intrinsic part, the second due to the cavity part and the one more due to salvation.

For globular aqueous proteins, as regards volume, the cavity term is always positive and the solvation term is negative, thereby cancelling each other. This implies that the intrinsic volume of a globular protein mostly represent its partial apparent specific volume (Gekko and Hasegawa 1986).

For globular proteins dispersed in water, as regards the compressibility, the intrinsic term will be very small, the cavity term is highly positive and the solvation term is highly negative (Gekko and Noguchi 1979). Hence, the measured partial apparent specific adiabatic compressibility of a globular protein is mainly determined by a balance of the cavity and solvation contributions.

Keeping these facts in mind and on inspecting Figs 3 and 4, it is clear that for system I, φ_v and φ_k show that the undisturbed compact state of lactalbumin is at pH 7. As the magnitude of φ_v and φ_k is closer to unity, pH 7 assures the most compact state. For other pH values, on protein addition, φ_v got increases but φ_k decreases. Presence of fructose in acidic extremity generates negative values that suggest the reduction in intrinsic volume and uncontrolled formation of cavities. This suggests the acidic and alkaline extremity produced more denaturation compared to other pHs and also cosolvent addition supports the protein renaturation.

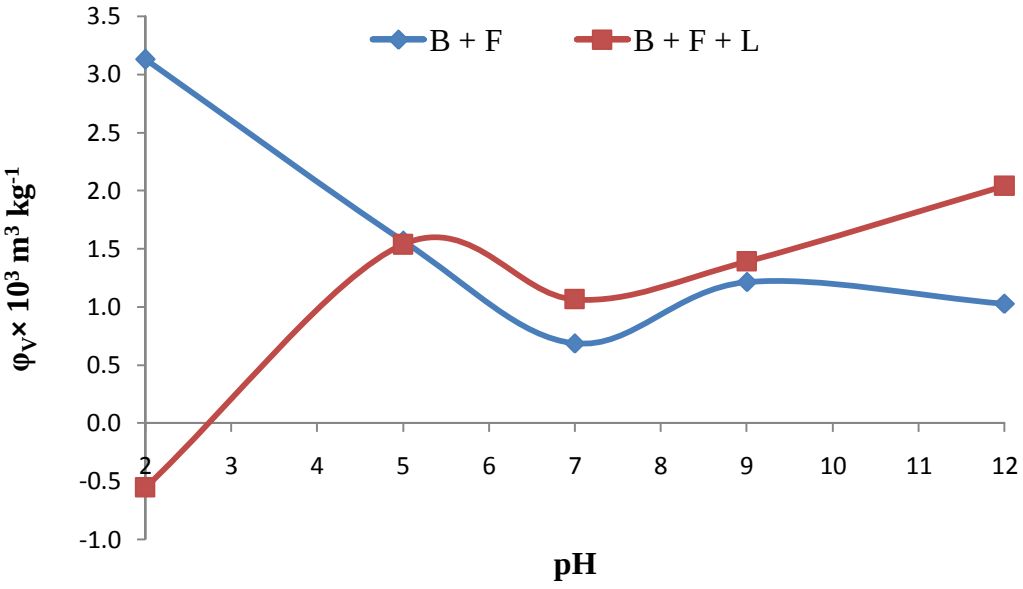

Fig 3: Trend of Partial Apparent Specific Volume with pH

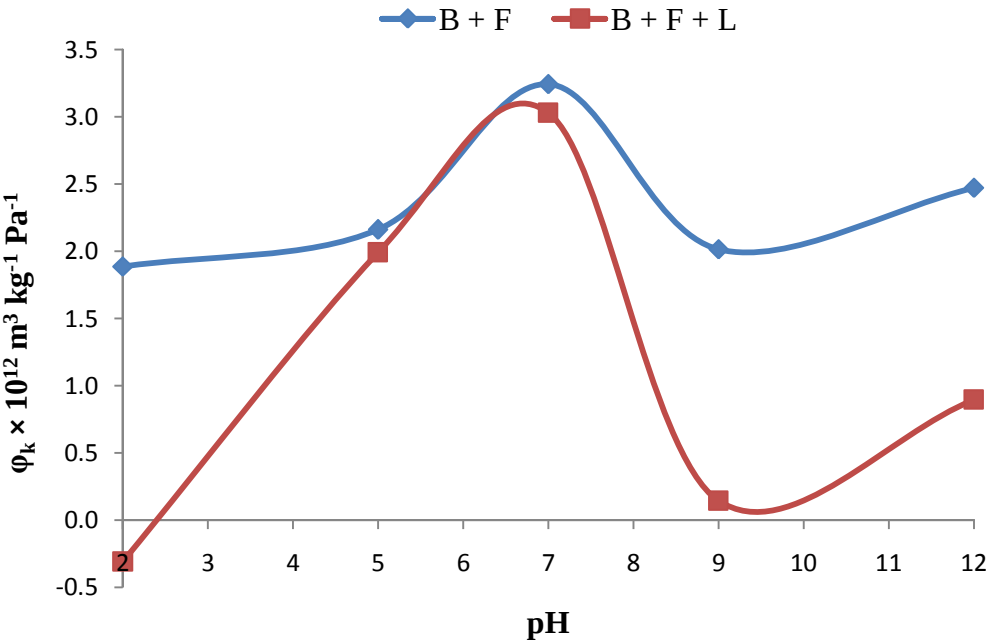

Fig 4: Trend of Partial Apparent Specific Compressibility with pH

All these above discussions generate a clear cut picture about the impact of fructose on the stabilization of lactalbumin. Acidic or alkaline extremity drastically leads to protein denaturation in which the side chains are the most affected part rather than the main chain. Native protein molecules are having non-polar side chain groups whereas the fructose are having polar environment. Thus a tendency would be created so as to enter into the interior of protein. At the same time, this would be also supports protein stability in the chosen solvents, thereby nullifying or reducing the effect of extreme pH factors.

Further, this interpretation also in line with the findings of Timasheff (2002) and Miyawaki (2009) where in the refolding of a protein is said to be by the preferential exclusion of water molecules from the surrounding protein in solution and the preferentially excluded solute stabilizes the protein.

For the confirmation of these ultrasonic observations and findings, FTIR study was made in these systems. Fig 5 shows the primary spectra of both systems at 303 K for the whole FTIR range. Specific variations are found in the spectra for the variation of pH as well as the presence of fructose.

Walter et al (1995) have pointed out that the variations in pH largely affects amide region of proteins and hence for clarity and specificity, among the various characteristic IR bands of α-LA (Bandekar 1992) the amide I region is selected for the present work.

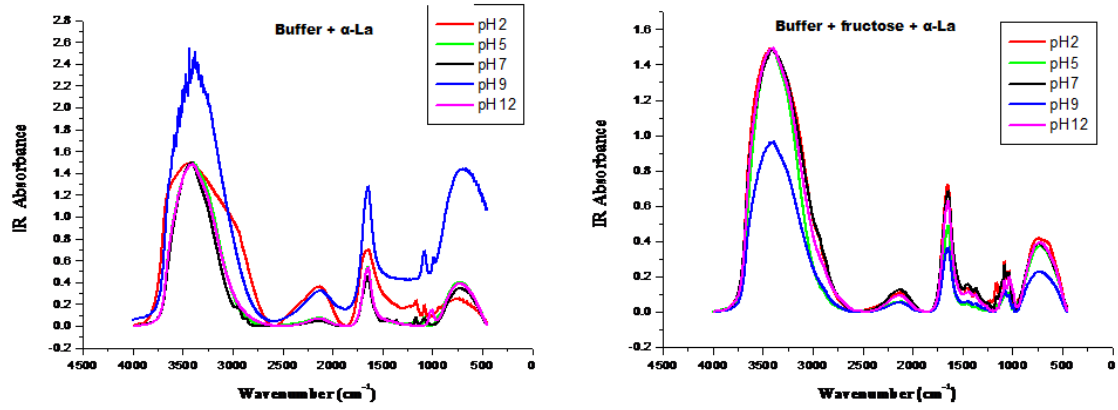

Fig 5. Primary FTIR spectra

Table 3: Amide I frequencies assigned to protein secondary structure in aqueous medium

Secondary structure	Frequency range (cm^{-1})	Average frequency (cm^{-1})
α-helix	1,630	1,630
	1,648-1,660	1,654
3_{10}-helix	1,660-1,666	1,663
β-sheet	1,612-1,641	1,627
	1,626-1,640	1,633
	1,670-1,694	1,682
β-turns	1,662-1,684	1,678
Random coil	1,640-1,650	1,645

Walter et al (1995) have pointed out that the variations in pH largely affects amide region of proteins and hence for clarity and specificity, among the various characteristic IR bands of α-LA (Bandekar 1992) the amide I region is selected for the present work. Amide I bands are supposed to be due to C=O group stretching and are in the range of 1700 to 1600 cm^{-1}. Assigned amide I frequencies to protein are listed in Table 3. (Carbonaro and Nucara 2010; Dong et al., 1990).

Of course the second derivative spectra are not projected here, the second derivative curve fitted spectra were sequentially depicted in Fig6. Here, the counting of structure is made by consolidating all peaks and there is no specific significance in highlighting few peaks, other than clarity.

Origin 7.0 program was used to solve the Lorentzian function. The relative area of each peak was thus obtained. Assignments were made by comparison with literature (Carbonaro and Nucara 2010; Dong 1990; Kuwajima 1996). Table 4 lists these assignments and areas for each pHs and Table 5 shows all the consolidated quantitative measurement in α-LA system.

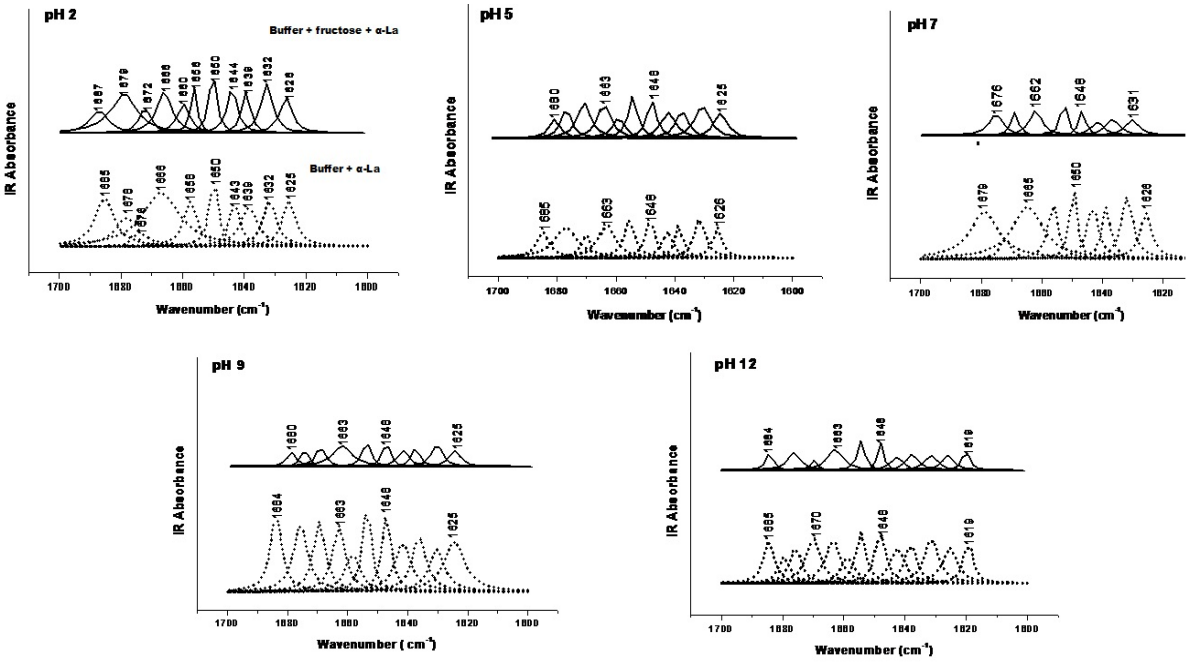

Fig 6. Second derivative curve fitted FTIR spectra at various pH – Amide I region

Table 4. Assignment of peaks and quantitative measurement of structures with and without cosolvent at various pH values

Sl. №	pH 2					
	without cosolvent			with cosolvent		
	υ (cm⁻¹)	Areas (%)	Assignment	υ (cm⁻¹)	Areas (%)	Assignment
1	1625	8	β-sheet	1626	8	β-sheet
2	1632	9	β-sheet	1632	10	β-sheet
3	1639	10	β-sheet	1639	7	β-sheet
4	1643	7	random coil	1644	9	random coil
5	1650	8	α-helix	1650	9	α-helix
6	1658	8	α-helix	1656	6	α-helix
7	1666	28	3_{10}- helix	1660	7	α-helix
8	1676	1	β-tums	1666	12	3_{10}- helix
9	1678	9	β-tums	1672	5	β-turns
10	1685	12	β-sheet	1679	19	β-turns
11	—	—	—	1687	8	β-sheet

Sl. №	pH 5					
	without cosolvent			with cosolvent		
	υ (cm⁻¹)	Areas (%)	Assignment	υ (cm⁻¹)	Areas (%)	Assignment
1	1626	7	β-sheet	1625	9	β-sheet
2	1632	11	β-sheet	1631	12	β-sheet
3	1639	7	β-sheet	1638	9	β-sheet
4	1643	8	random coil	1642	9	random coil
5	1648	9	α-helix	1648	9	α-helix
6	1656	11	α-helix	1654	10	α-helix
7	1663	14	3_{10}- helix	1659	6	α-helix
8	1670	6	β-turns	1663	11	3_{10}- helix
9	1677	18	β-turns	1670	12	β-tums
10	1685	9	β-sheet	1676	8	β-tums
11	—	—	—	1680	5	β-tums

Sl. №	pH 7					
	without cosolvent			with cosolvent		
	υ (cm⁻¹)	Areas (%)	Assignment	υ (cm⁻¹)	Areas (%)	Assignment
1	1626	8	β-sheet	1631	12	β-sheet
2	1632	11	β-sheet	1637	13	β-sheet
3	1639	7	β-sheet	1642	9	random coil
4	1643	9	random coil	1648	10	α-helix
5	1650	8	α-helix	1653	13	α-helix
6	1656	8	α-helix	1659	1	α-helix
7	1665	25	3_{10}- helix	1662	17	3_{10}- helix
8	1672	3	β-tums	1669	9	β-turns
9	1679	21	β-tums	1676	16	β-turns

Sl. №	pH 9					
	without cosolvent			with cosolvent		
	υ (cm⁻¹)	Areas (%)	Assignment	υ (cm⁻¹)	Areas (%)	Assignment
1	1625	10	β-sheet	1625	9	β-sheet
2	1630	8	β-sheet	1631	12	β-sheet
3	1636	9	β-sheet	1639	8	β-sheet
4	1642	9	random coil	1643	9	random coil
5	1648	8	α-helix	1648	9	α-helix
6	1654	9	α-helix	1655	10	α-helix
7	1658	6	α-helix	1663	20	3_{10}- helix
8	1663	10	3_{10}- helix	1670	9	β-tums
9	1669	10	β-turns	1676	7	β-tums
10	1676	11	β-turns	1680	7	β-sheet
11	1684	10	β-sheet	—	—	—

Sl. №	pH 12					
	without cosolvent			with cosolvent		
	υ (cm⁻¹)	Areas (%)	Assignment	υ (cm⁻¹)	Areas (%)	Assignment
1	1619	6	β-sheet	1619	7	β-sheet
2	1625	8	β-sheet	1625	9	β-sheet
3	1631	10	β-sheet	1631	10	β-sheet
4	1638	8	β-sheet	1637	10	β-sheet
5	1642	8	random coil	1642	8	random coil
6	1648	8	α-helix	1648	8	α-helix
7	1654	8	α-helix	1654	10	α-helix
8	1659	5	α-helix	1663	17	3_{10}- helix
9	1664	10	3_{10}- helix	1670	4	β-tums
10	1670	10	β-turns	1676	11	β-tums
11	1676	7	β-turns	1684	6	β-sheet
12	1680	5	β-turns	—	—	—
13	1685	7	β-sheet	—	—	—

Table 5. Consolidated secondary structures in the protein system (**3_{10}**-helices is included in α-helices)

pH	With cosolvent (fructose)				Without cosolvent (fructose)			
	β-sheet	α-helix	random coil	β-turns	β-sheet	α-helix	random coil	β-turns
2	33	34	9	24	39	44	7	10
5	30	36	9	25	34	34	8	24
7	25	41	9	25	26	41	9	24
9	36	39	9	16	37	33	9	21
12	42	35	8	15	39	31	8	22

The strength and the type of the structure can be realized by inspecting the Table 4. This gives a clear idea about the various interactions taking place in the medium and the structural changes that occur due to pH or cosolvent. As an illustration, in the pH 2 second derivative spectrum, 39% contribution is by β-sheets and 16% by α-helix. It is evident that β-sheets are more than the α-helix content indicating the disturbance of stability due to acidic extremity. In other words, this pH doesn't represent native state rather is an indicative of denatured state.

In a similar way, assignments can be made for the second derivative spectra at other pH and the consolidated qualitative measurements obtained are listed in Table 5. An inspection of this Table offers many valid inferences.

As per the explanations earlier cited for Fig 1, the native state is characterized by a large number of α-helix with less number of β-sheet. Further, Permyakov *et al.* (2000) have also reported that the dominant structure in the chosen protein is α-helix in native α-LA.

On this basis, the perusal of Table 5 reveals that pH 7 is found to be native state of the protein which can be well in line with common sense. This is true for both α-helix and β-sheet structures. Further at pH 7, though the cosolvent is found to have no effect in the α-helix value, it slightly reduces the β-sheet value, thereby indicating its renaturation effect. pH other than 7 shows a reduction in α-helix indicating the denatured protein by pH variations and the structure is found to be retained by the addition

of cosolvent. It is to be noted that the apparent variation found in pH 2 is due to the inclusion of 3_{10} helices in to α-helix.

The same is true for the observed trend in β-sheet values and also in other structural components. Cosolvent is unanimously found to support the renaturation process however its role is highly specific in alkaline extremity than in acid extremity.

Kuwajima (1996) has reported that the molten globule state of α-LA has larger volume than that of the native protein. It is a general belief in proteomics that hydrophobic proteins are more compressible due to the presence of voids and absence of hydration. This may be the reason for the observed increase in β-sheets at extreme pHs (Table 5). This point of view leads to guess that the increase in void area of molten globule state tends to increase the hydrophobic interactions existing in the interior. All such predictions declare that the denaturation of protein is due to the existing excess hydrophobic interactions.

A decreasing trend of β-turn is found towards pH extremities as observed by Boye *et al. (1997)* in α-LA analysis. They have attributed this trend to the enhanced surface activity of α-LA.

Random coils are also considered as a well accepted structure of proteins by Pauling and Corey (1950). Though these coils are lack of well defined patterns, cosolvent and pH are found to have least effect on these coils. This may be due to the dominant role of other major secondary structures.

As cited earlier, β-sheets and α-helix are attributed to intermolecular and intramolecular hydrogen bonding interactions respectively. Thus it is here evident that the intra interactions got strengthened as α-helix are increased and inter interactions got improved because of reduction in β-sheet while the overall impact is to improve the protein stability.

Ultrasonic analysis in this study reveals that the addition of cosolvent tends to decrease the overall adiabatic compressibility and volume of the proteins. This is an indication of the expulsion of water-containing voids and increased intramolecular bonding within the protein interior. This entire phenomenon is fully backed by the increased osmotic stress acting on the proteins (Timasheff 1992; Timasheff 2002). Further, the cosolvent tends to stabilize proteins due to steric exclusion effect (Timasheff 2002). All such contrary driving forces support protein stabilization against pH. This trend is supported by the FTIR result that shows the increases of α-helix structure due to the addition cosolvent.

The ends of two adjacent segment of an antiparallel β-sheet are connected by β-turns. Acharya *et al. (1991)* remarked that β-sheet found in α-LA belongs to antiparallel type. In terms of β-turns, an increasing trend is observed in acidic region whereas a decreasing trend is found in alkaline region. Such observations reveal that presence of cosolvent with α-LA supports for higher stability in the acidic region. This is one of the noteworthy findings of the present study.

It is obvious that the secondary structural variations are occurred due to changes in pH and addition of cosolvent in α-LA. Neither the pH nor the cosolvent can offer chances for the covalent interactions. Thus, this trend suggests that the existing interactions are of non-covalent type in the present system.

CONCLUSIONS

Major findings of the present analyses are:

Ultrasonic and FTIR spectroscopic studies both confirm the denaturation of protein due to pH variations. Acidic pH is found to contain high degree of molten-globule state and its impact on denaturating a protein are poor in magnitude due to higher content of α-helix compared to alkaline pH.

Large amount of α-helix is found thereby the major domain in α-LA is ascertained as helical structure.

Protein stability is achieved by the presence of fructose through enhanced non-covalent interactions and preferential exclusion mechanism. This is confirmed by the distortion of structural content in FTIR studies

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