

Response of ovalbumin to fructose addition and pH variations - ultrasonic and FTIR study.

Respuesta de la ovoalbúmina a la adición de fructosa y las variaciones de pH - Estudio ultrasónico y FTIR.

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ABSTRACT

Main aim of this work is to understand how the protein ovalbumin is affected by the presence of cosolvent and variations in pH of the medium. The addition of cosolvent in many cases is found to control the extent of denaturation and pH is one of the main sources of denaturant of proteins. In this work, keeping fructose solution as cosolvent and pH of the solution as main variable, the extent of denaturation is analysed by ultrasonic methods and are further confirmed by FTIR amide-I second derivative spectra 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, though poor, 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, ovalbumin, FTIR, viscosity, pH, non-covalent interactions.

RESUMEN

El objetivo principal de este trabajo es comprender cómo la proteína ovoalbúmina se ve afectada por la presencia de codisolvente y variaciones en el pH del medio. Se encuentra que la adición de codisolvente en muchos casos controla el grado de desnaturalización y el pH es una de las principales fuentes de desnaturalización de proteínas. En este trabajo, manteniendo la solución de fructosa como codisolvente y el pH

de la solución como variable principal, el grado de desnaturalización se analiza mediante métodos ultrasónicos y se confirma mediante el espectro de la segunda derivada de la amida I FTIR a 303 K. Los resultados obtenidos muestran que la desnaturalización es sensible al pH, sin embargo, lo ácido y lo alcalino se comportan totalmente de manera diferente. Se encontró que el impacto del pH alcalino produce una desnaturalización menor y es más lento mientras que el impacto del pH ácido es específico e instantáneo. El análisis ultrasónico muestra que la variación del pH puede desnaturalizar la proteína mientras que la adición de codisolvente favorece la renaturalización. Los espectros FTIR se registraron para las muestras experimentales a partir de las cuales se construyeron los espectros ajustados a la curva de la segunda derivada usando el programa Origin. La asignación cuantitativa de picos y las variaciones en áreas acumulativas calculadas para las estructuras como hélice α , láminas β , etc. confirma las observaciones del análisis ultrasónico de que las variaciones de pH ayudan en la desnaturalización mientras que el codisolvente favorece la renaturalización de la proteína.

Palabras clave: velocidad ultrasónica, fructosa, ovoalbúmina, FTIR, viscosidad, pH, interacciones no covalentes.

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, 1972). 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 2003) have used such techniques at different pH environments. Already we have made an

extensive ultrasonic study on ovalbumin but with glucose (Velusamy and Palaniappan 2016) 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 ovalbumin (Ova) at 303 K using ultrasonic techniques. Further, the outcomes of the analyses are confirmed by FTIR studies in the same systems. Literature gives all necessary background information on fructose (Satyanarayana 2001), protein denaturation / aggregation (Tatsumi et al 1998, Waris et al 2001, Green 1993, Belyakova et al 2003), buffer preparation (Elving et al 1956), thermo-acoustic parameters (Robert et al 1997, Nolting and Sligar 1993, Kharakoz and Sarvazya 1993), fructose quantity (Campbell 2006, Malhotra 2017), FTIR spectroscopy (Noguchi 2007, Schultz 2000, Ramajo et al 2005), etc.

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).

MATERIALS AND METHODS

Materials: Chemicals of purity more than 99% are used in this work. Ovalbumin solution (5 mg/ml) was prepared using the powdered ovalbumin 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 (O) dissolved in these buffers (B) forms System I (B+O) whereas the same with the addition of 1 M fructose (F) solution as cosolvent forms the system II (B+F+O).

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 $\pm 0.1 \text{ ms}^{-1}$. Ostwald's 10 ml viscometer for viscosity measurement (accurate to $\pm 0.001 \text{ mNsm}^{-2}$) and drop (by platinum-iridium Du Nouy ring) weight method for surface tension, accurate to $\pm 0.0001 \text{ kg}$ 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).

FTIR Spectra (resolution of 1.0 cm^{-1}) of proteins were recorded in the region $4000\text{-}450 \text{ cm}^{-1}$ by a FTIR (PERKIN ELMER) spectrometer at Sophisticated Analytical Instrument Facility Lab, Indian Institute of Technology, Madras, India. Earlier FTIR results (Dong et al 2000) show that pure ovalbumin exhibits two amide I bands at nearly 1640 cm^{-1} and 1654 cm^{-1} . Similar peaks noticed for ovalbumin in the present study confirms the purity of the protein sample used.

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)

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

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

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

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

$$R_A = [\rho/\rho_0] [u_0/u]^{1/3} \quad (5)$$

$$\phi_v = 1/\rho_0 + [\rho_0 - \rho]/[C_p \rho_0] \quad (6)$$

$$\phi_k = \beta_0 [2\phi_v - 2[u] - 1/\rho_0] \quad (7)$$

where K_T is 199.53×10^{-8} in S.I system at 303 K, ρ_0 , u_0 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_0 and $[u]$ is the relative specific sound velocity increment given as.

$$[u] = [u - u_0]/u_0 C_p \quad (8)$$

Further, the three relative parameters viz, R_A , ϕ_v and ϕ_k are calculated for the system of B+F+O 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 at 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 1 to 3 respectively. Fig 4 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 5. The assigned amide I frequencies for the secondary structure of protein are listed in Table 3 (Carbonaro and Nucara 2010, Dong 1990). The assignment of peaks and their areas for each pH were listed in Table 4. The consolidated quantitative measurement of these secondary structures in Ova system with and without cosolvent is given in Table 5.

Measured parameters

Table 1: Measured Parameters at various pH

pH	Density (ρ)		Sound velocity (u)		Viscosity (η)		Surface tension	
	kgm^{-3}		ms^{-1}		mNsm^{-2}		$(\tau) \text{Nm}^{-1}$	
	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)
2	1018.4	1098.2	1552.2	1582.6	0.9121	1.1811	0.2343	0.2491
5	1020.2	1072.4	1538.7	1575.2	0.7527	1.0723	0.2492	0.2532
7	1019.6	1068.5	1533.0	1563.9	0.8231	1.2627	0.2477	0.2488
9	1021.4	1073.6	1539.8	1593.3	0.8228	1.3024	0.2426	0.2312
12	1033.2	1078.2	1561.2	1594.4	0.8991	1.2856	0.2014	0.2222

The accumulation of mass per unit volume is density and the negative log of hydrogen ion concentration is termed as pH. The observed variation in density with respect to increase in pH indicates the influence of more number of hydrogen ions. Invariably in all experimental solutions, variation exists only in hydrogen ion concentration. 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 Ovalbumin.

The primary structure has planar and rigid peptide bonds. Free amino group in N terminal and acid group in C terminal allow this structural compactness. 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. The coiling or uncoiling mobility factors, the asymmetric α -helix of Ovalbumin, all suggests the mobility difference of C and N-terminal. The N-terminal with slight positive charge and

C-terminal with slight negative charge (Kadi et al. 2006) 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 into their corresponding acids by pH variations (Kadi et al. 2006, Carbonaro and Nucara 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 as 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, a dynamic 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. This suggests less viscosity at the iso-electric point (pI). 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 systems 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 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 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 Ovalbumin at its iso-electric point is also confirmed by Belton and Twidle (1940) in their work. 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 and hence their stability got increased.

Calculated parameters

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

pH	$\beta \times 10^{10} \text{ Pa}^{-1}$		$L_f \times 10^{11} \text{ m}$		$Z \times 10^{-6} \text{ kgm}^{-2}\text{s}^{-1}$		$t \times 10^{10} \text{ s}$	
	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)	System (B+O)	System (B+F+O)
2	4.0755	3.6356	4.0280	3.8044	1.5807	1.7380	4.9564	5.7253
5	4.1400	3.7581	4.0598	3.8680	1.5697	1.6892	4.1549	5.3731
7	4.1733	3.8265	4.0761	3.9030	1.5630	1.6710	4.5801	6.4423
9	4.1292	3.6691	4.0545	3.8217	1.5727	1.7105	4.5301	6.3715
12	3.9709	3.6484	3.9760	3.8110	1.6130	1.7190	4.7604	6.2538

The calculated values of standard thermo acoustical parameters such as the adiabatic compressibility, intermolecular free length, acoustic impedance and the relaxation time are presented in Table 2.

Compressibility is the space that is available freely in the medium. An orderly structure is always tightly packed and has minimum 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 t 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 7) 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.

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 Ovalbumin 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 Ovalbumin. 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.

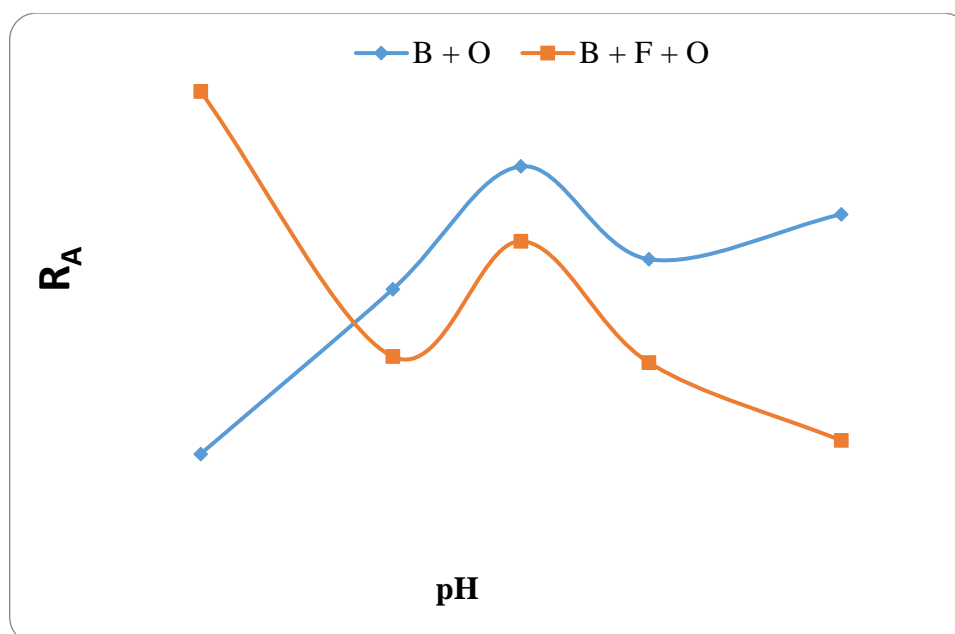


Fig 1: Trend of Relative Association with pH

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 1 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 pH, represent the denaturation.

Further it is also evident that fructose aids in keeping the R_A to be 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.

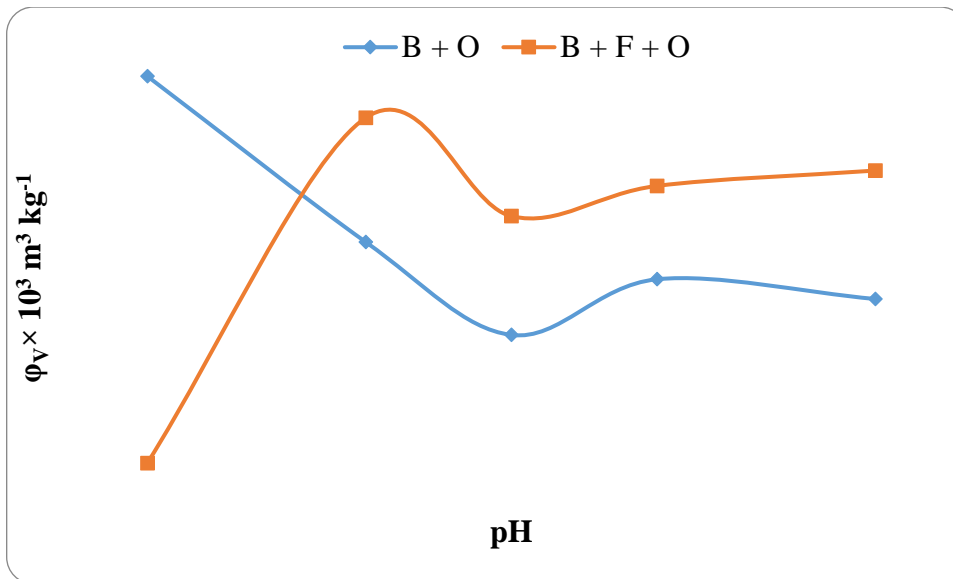


Fig 2: Trend of Partial Apparent Specific Volume with pH

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 solvation.

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).

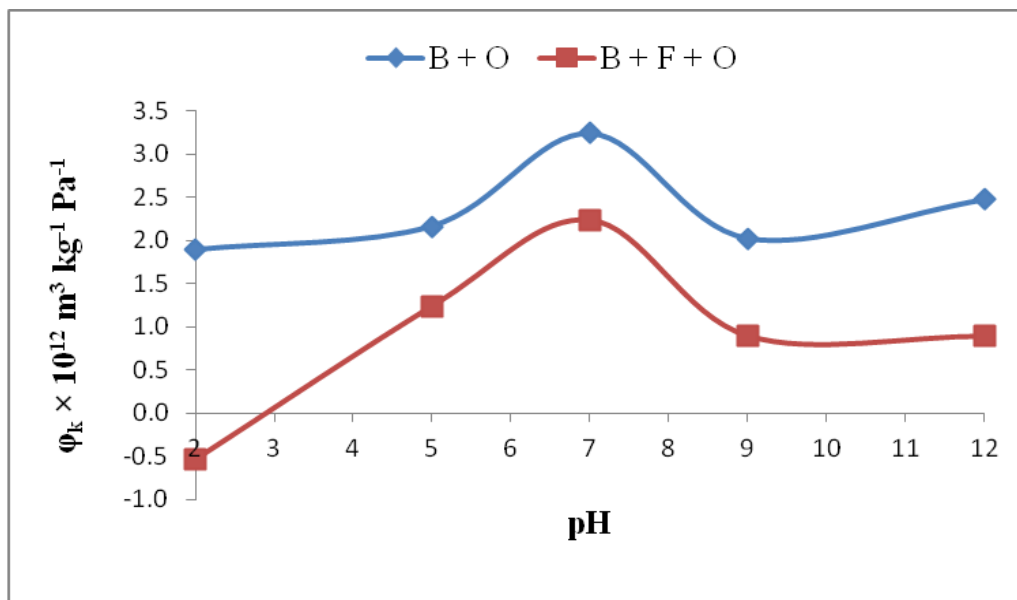


Fig 3: Trend of Partial Apparent Specific Compressibility with pH

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 2 and 3, it is clear that for system I, ϕ_v and ϕ_k show that the undisturbed compact state of Ovalbumin 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.

All these above discussions generate a clear cut picture about the impact of fructose on the stabilization of Ovalbumin. 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 4 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.

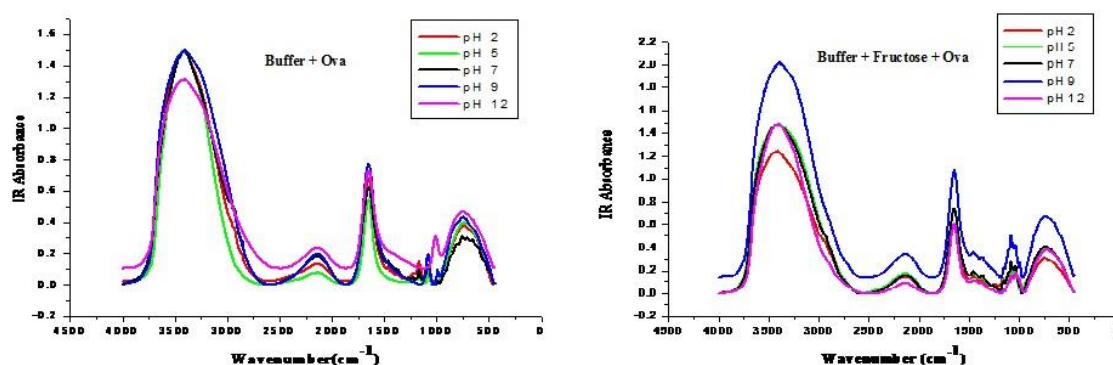


Fig 4: Primary FTIR spectra

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 Ova (Bandeekar 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).

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

Of course the second derivative spectra are not projected here, the second derivative curve fitted spectra were sequentially depicted in Fig 5. Here, the counting of structure is made by consolidating all peaks and there is no specific significance in highlighting few peaks, other than clarity.

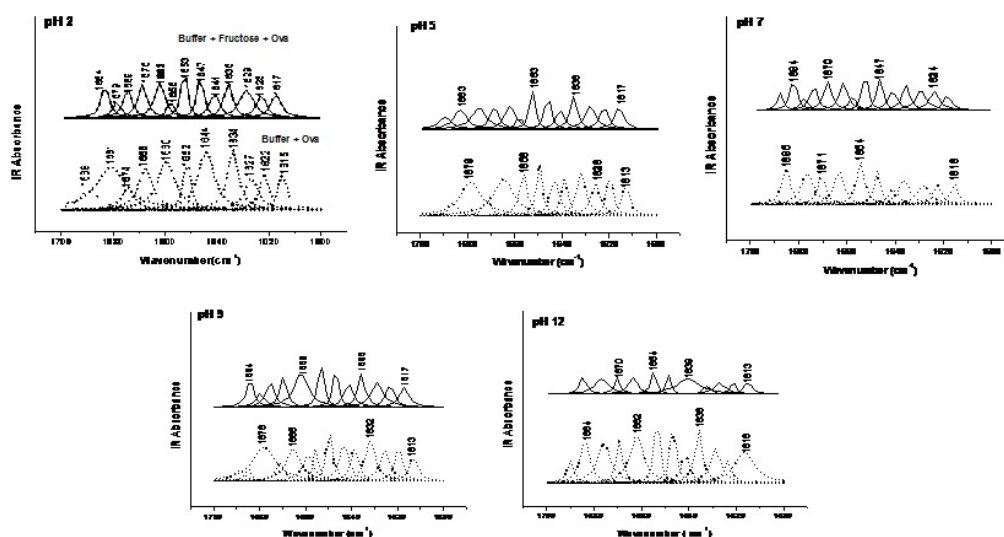


Fig 5. Second derivative curve fitted FTIR spectra at various pH – Amide I region

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 et al. 1990, Kuwajima 1996). Table 4 lists these assignments and areas for each pHs and Table 5 shows all the consolidated quantitative measurement in Ova system.

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

pH 2							pH 5							pH 7						
Sl. №	without cosolvent			with cosolvent			Sl. №	without cosolvent			with cosolvent			Sl. №	without cosolvent			with cosolvent		
	v (cm ⁻¹)	Areas (%)	Assignment	v (cm ⁻¹)	Areas (%)	Assignment		v (cm ⁻¹)	Areas (%)	Assignment	v (cm ⁻¹)	Areas (%)	Assignment		v (cm ⁻¹)	Areas (%)	Assignment	v (cm ⁻¹)	Areas (%)	Assignment
1	1615	5	β-sheet	1617	7	β-sheet	1	1613	6	β-sheet	1617	7	β-sheet	1	1616	5	β-sheet	1618	5	β-sheet
2	1622	5	β-sheet	1623	7	β-sheet	2	1620	6	β-sheet	1623	7	β-sheet	2	1622	6	β-sheet	1624	8	β-sheet
3	1627	7	β-sheet	1629	11	β-sheet	3	1626	7	β-sheet	1630	9	α-helix	3	1628	9	β-sheet	1630	9	α-helix
4	1634	10	β-sheet	1636	9	β-sheet	4	1632	10	β-sheet	1636	8	β-sheet	4	1636	10	β-sheet	1636	8	β-sheet
5	1644	15	random coil	1641	7	β-sheet	5	1639	6	β-sheet	1641	6	β-sheet	5	1642	5	random coil	1641	7	β-sheet
6	1652	5	α-helix	1647	8	random coil	6	1643	8	random coil	1646	7	random coil	6	1648	7	α-helix	1647	8	random coil
7	1660	13	α-helix	1653	9	α-helix	7	1650	7	α-helix	1653	9	α-helix	7	1654	13	α-helix	1653	9	α-helix
8	1668	9	β-turns	1658	4	α-helix	8	1656	8	α-helix	1658	3	α-helix	8	1663	12	3 ₁₀ -helix	1659	4	α-helix
9	1674	5	β-turns	1663	10	3 ₁₀ -helix	9	1665	19	3 ₁₀ -helix	1662	9	3 ₁₀ -helix	9	1671	8	β-turns	1663	9	3 ₁₀ -helix
10	1681	20	β-sheet	1669	9	β-turns	10	1672	3	β-turns	1669	7	β-turns	10	1676	14	β-turns	1670	9	β-turns
11	1689	6	β-sheet	1675	7	β-turns	11	1679	18	β-turns	1676	12	β-turns	11	1685	11	β-sheet	1675	8	β-turns
12	—	—	—	1679	4	β-sheet	12	1686	2	β-sheet	—	—	—	12	—	—	—	1680	4	β-sheet
13	—	—	—	1684	8	β-sheet	—	—	—	—	—	—	13	—	—	—	1684	8	β-sheet	
14	—	—	—	—	—	—	—	—	—	—	—	—	14	—	—	—	1690	4	β-sheet	

pH 9							pH 12												
Sl. №	without cosolvent			with cosolvent			Sl. №	without cosolvent			with cosolvent								
	v (cm ⁻¹)	Areas (%)	Assignment	v (cm ⁻¹)	Areas (%)	Assignment		v (cm ⁻¹)	Areas (%)	Assignment	v (cm ⁻¹)	Areas (%)	Assignment						
1	1613	5	β-sheet	1617	7	β-sheet	1	1616	13	β-sheet	1613	6	β-sheet						
2	1620	6	β-sheet	1623	7	β-sheet	2	1624	4	β-sheet	1619	5	β-sheet						
3	1626	7	β-sheet	1629	11	β-sheet	3	1629	8	β-sheet	1625	8	β-sheet						
4	1632	10	β-sheet	1636	9	β-sheet	4	1636	9	β-sheet	1630	4	α-helix						
5	1639	6	β-sheet	1641	7	random coil	5	1641	5	random coil	1639	23	β-sheet						
6	1643	8	random coil	1648	8	α-helix	6	1647	8	random coil	1647	6	random coil						
7	1650	8	α-helix	1653	9	α-helix	7	1653	8	α-helix	1654	8	α-helix						
8	1656	4	α-helix	1662	17	3 ₁₀ -helix	8	1662	15	3 ₁₀ -helix	1659	2	α-helix						
9	1659	7	α-helix	1669	7	β-turns	9	1669	6	β-turns	1663	11	3 ₁₀ -helix						
10	1665	11	3 ₁₀ -helix	1675	7	β-turns	10	1676	12	β-turns	1670	6	β-turns						
11	1671	4	β-sheet	1679	4	β-sheet	11	1684	9	β-sheet	1676	14	β-turns						
12	1678	19	β-turns	1684	7	β-sheet	12	1690	3	β-sheet	1684	7	β-sheet						
13	1685	2	β-sheet	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	1689	3	β-sheet	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 5. Consolidated secondary structures in the protein system (3₁₀-helices is included in α-helix)

pH	With cosolvent (fructose)				Without cosolvent (fructose)			
	β-sheet	α-helix	random coil	β-turns	β-sheet	α-helix	random coil	β-turns
2	53	23	8	16	53	18	15	14
5	37	30	7	19	37	34	8	21
7	44	31	8	17	41	32	5	22
9	45	34	7	14	43	30	8	19
12	49	25	6	20	46	23	13	18

The strength and the type of the structure can be realized by inspecting the Table 4 as well as Table 5. 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 with cosolvent, the second derivative spectrum reveals that 53% contribution is by β-sheets and 23% by α-helix. It is evident that the presence of more number of α-helix content indicates 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.

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 is α -helix but in native α -lactalbumin.

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 increases 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 Ova 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 lactalbumin analysis. They have attributed this trend to the enhanced surface activity of lactalbumin.

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 increase 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 β -sheet 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 Ova 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 Ova 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 Ova. 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.

As conclusions, the major findings of the present analyses are: 1) Denaturation of protein due to pH variations is confirmed. 2) Acidic pH is found to contain high degree of molten-globule state and its impact on denaturing a protein are poor in magnitude due to higher content of α -helix compared to alkaline pH. 3) Large amount of α -helix is found thereby the major domain in Ova is ascertained as helical structure. 4) Protein stability is achieved by the presence of fructose through enhanced non-covalent interactions and preferential exclusion mechanism.

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