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Flexibility and viscometric studies of globular proteins ovalbumin and ovotransferrin

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The ultrasonic velocity, density and viscosity of two egg proteins, ovalbumin and ovotransferrin in phosphate buffer have been studied at the physiological *p*H values. The thermodynamic functions for unfolding, ellipticity, surface amino acid residues and compressibility have been obtained for thermal and chemical denaturation in these food proteins. The computed values of Huggin's constant and shape factor, at a fixed ionic strength 0.16 *M* are found to be in agreement with the reported values for globular proteins. The slow increase in free-energy of unfolding with temperature at a fixed *p*H 7 suggests uncoiling and in turn, disappearance of biological activity. It has been observed that the effects of temperature and chemical denaturant on the native protein may give rise to different conformational states. In the presence of urea and sodium dodecyl sulphate (SDS), the proteins gave the excessively denatured states at 25°C and *p*H 7, in comparison to the thermal denatured state. The positive values of partial adiabatic compressibility $\bar{\beta}_s$ over the temperature range 45-75°C suggest the possibility of large internal flexibility in ovotransferrin than in ovalbumin.

Several factors contribute to tertiary structure of a protein and changes in any of them could affect its structural integrity and biological activity. When a protein is heated to an extreme temperature, the balance of non-covalent interactions maintaining the native structure is disrupted and the protein unfolds partially. This process disturbs the active site of the enzyme[1]. Similar behaviour is observed with the denaturating agents like urea and sodium dodecyl sulphate (SDS). The characterization of stable intermediate states would be of great help for the proper understanding of the over all process of protein folding[2]. X-ray crystallography data have revealed that some packing defects or cavities in a protein molecule[3] cause fluctuations, which are related to the structural characterization and functional properties of proteins. However, a complete understanding to ascertain the role of fluctuations in protein function requires further investigation on the magnitude of the flexibility of protein molecules in various solvents and at different *p*H values. Since the fluctuation in volume is directly related to the compressibility[4], the flexibility of protein should be reflected in the compressibility and the latter is primarily related to its thermal stability.

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This paper essentially deals with (i) the comparison of the globular nature in terms of intrinsic viscosities, Huggin's constant, shape factor and surface amino acid residues of two proteins, ovalbumin and ovotransferrin; ovalbumin, a member of Serpin family is found in uncleaved form in egg white[5], ovotransferrin is also extracted from egg white[6]; (ii) the methods of determination of protein stability and comparison of extent of denaturation; from the values of thermodynamic functions for thermal as well as for chemical denaturation, and (iii) the role of compressibility studies in order to discuss the flexibility of food proteins. The digestion velocity and ellipticity in relation to flexibility, of both proteins have been reported earlier[7]. The shape factor and stability of proteins are computed in the present work by viscometric studies, and the digestibility is judged by the ultrasonic studies or flexibility.

Experimental procedures

Materials

The proteins, viz., ovalbumin and ovotransferrin from Sigma Co., U.S.A. were used. Five solutions of different concentrations (2-10 mg/ml) were prepared by dissolving the proteins in phosphate buffer (*p*H 7.0), containing 1% SDS or 9 *M* urea. All the measurements were made between the temperature range: 25 to 75°C ± 0.1.

Ultrasonic velocity measurements

The ultrasonic velocity (u), in protein solution was measured by means of single frequency (2 MHz) ultrasonic interferometer (Mittal's Model F-81) (S.D. ± 0.026). The apparatus and the procedure adopted were essentially the same as used earlier[8], the ultrasonic velocities (u) were calculated by using the relation:

$$u = \lambda \times \nu \quad \ldots (1)$$

where, λ is the wavelength and ν is the frequency. The partial adiabatic compressibility, $\bar{\beta}s$ of the solution was calculated by using the relation[9]:

$$\bar{\beta}_s = -(1/\bar{\nu}^\circ)(\partial \bar{\nu}^\circ/\partial P) = (\beta_o/\bar{\nu}^\circ) \lim [(\beta/\beta_o - V_o)/c] \quad \ldots (2)$$

$$\text{where } V_o = (\rho - c)/\rho_o \quad \ldots (3)$$

$$\text{and } \bar{\nu}^\circ = \lim [(1-\nu_o)/c] \quad \ldots (4)$$

in which P is the pressure, β and β_o, are adiabatic compressibilities of solution and solvent; ρ and ρ_o densities of solution and solvent, respectively; c, the concentration in g/ml solution; V, is the apparent volume fraction of solvent in solution and $\bar{\nu}^0$ is the partial specific volume of the solute. The values of β and β_o can be calculated from the ultrasonic velocity, u, and the density ρ, of the solution or solvent using the Laplace equation:

$$\beta = 1/\rho u^2 \quad \ldots (5)$$

Density measurements

The densities of solvents and solutions were measured over the temperature range of 25-75°C ±0.02°C , using a pyknometer (~ 3 ml capacity). These values were least squares fitted to polynomial Eq. $\rho = \rho_o + \rho_1 t + \rho_2 t^2$. The accuracy of the measurement was about ± 0.2 mg/ml.

Viscosity measurements

The viscosity of samples were measured over the temperature range of 25-75°C with the help of Cannon-Ubbelohde type[10], viscometer (2.0 ml capacity). The time of fall of water at 25°C was about 398.2 sec[8,11] and was recorded with a stop watch(accuracy ±0.1 sec).The error in the measurement of viscosity was approx. 0.2%. The intrinsic viscosities were determined by least-squares fitting of the data to the relevant expression. The temperature and concentration effects on the kinematic viscosity were investigated. The intrinsic viscosities [η] were computed by the least-square method, using the following equation:

$$\eta_{red.} = [\eta] + K[\eta]^2 C \quad \ldots (6)$$

where K is Huggin's constant.

The shape factor[12] or ellipticity ν, was evaluated from the computed data of intrinsic viscosity and partial specific volume of solute, $\bar{\nu}^0$, using the equation

$$[\eta] = \nu[\bar{\nu}^o + \Sigma m_i \bar{\nu}_1^0] \quad \ldots (7a)$$

where ν is the shape factor and is 2.5 for spheres and larger for ellipsoids and m_i is the g of a solvent component with the partial specific volume $\bar{\nu}_1^0$, bound to 1 g dry wt of protein. If the native ovalbumin in the phosphate buffer binds only with water for which $\bar{\nu}_1^\circ$, may be taken to be the maximum amount of water bound to 1 g of protein (spherical shaped) may be calculated by setting ν=2.5 in Eq. (7a). Thus, the value of m_i turns out to be 0.67. In this work, ellipsoid shape factor has been presumed in using Eq. (7a) and the last term is taken as zero, i.e. $\Sigma m_i \bar{\nu}_1^\circ = 0$, the calculated value for the ovalbumin at 35°C is in good agreement with that of reported one[13].

The number of amino acid residues, n per polypeptide chain was calculated by empirical relation[1]:

$$[\eta] M_0 = 76.1\, n^{0.66} \quad \ldots (7b)$$

where M^0 is the mean residue weight M/n and M is the molecular weight of the polypeptide.

The free energy of unfolding, ΔG_{un}, was obtained from the viscosity versus temperature plots based on the expression

$$\eta = hN/V_m \exp^{\Delta Gun/RT} \quad \ldots (8)$$

where h is plank's constant, N Avagadros no., and V_m is the molar volume. The computed values of entropy and enthalpy of denaturation were determined by the least-squares fitting of the data to the Eq. (8).

The partial specific volume of a protein in buffer consists of three contributions (Kauzman 1959)[14]:

$$\bar{\nu}^0 = \nu_c + \nu_{cav} + \Delta\nu_{sol} \quad \ldots (9)$$

where v_c is the constitutive atom or group volume, v_{cav}, the volume of cavities due to imperfect packing of the atoms or groups and Δv_{sol}, is the volume change due to solvation or hydration. Since v_c is highly incompressible. v_{cav} consists of incompressible (due to close packing of atoms or groups) and compressible voids (due to random close packing) and contributes positively to $\bar{v}^0$. Δv_{sol} involves: (a) electrostatic solvation of ionic groups; (b) hydrogen-bonded hydration of polar groups, and (c) hydrophobic hydration or non-polar groups. Each of them contributes negatively to Δv_{sol}[15]. Therefore, Δv_{sol} contributes negatively to $\bar{v}°$. The terms v_{cav} and Δv_{sol} tend to cancel each other. This makes it possible to calculate the partial specific volume as the sum of constitutive atomic or group volumes. Then the differentiation of Eq. (9) with respect to pressure under adiabatic conditions gives the following Eqs:

$$\bar{\beta}_s = - (1/ \bar{v}^o) (\delta \bar{v}^o / \delta p) \quad \ldots (10)$$

$$= - (1/ \bar{v}^o) (\delta v_{cav} / \delta p + \delta \Delta v_{sol} / \delta p) \quad \ldots (11)$$

It has been previously reported[16] that the experimentally determined partial adiabatic compressibility of a protein is the contribution of the cavity and hydration. Similarly, in this case the first term on the right hand side of Eq. (11) contributes positively and the second term negatively to $\bar{\beta}s$, then the values indicate that cavity effects overcome the solvation effects to make β_s positive, generally in the case of ovotransferrin (specially in buffer solution with urea) and negative in that of ovalbumin (Tables 1 and 2).

Results and Discussion

The intrinsic viscosity computed from the reduced viscosity, η_{red} of ovalbumin at 35°C was found to be 3.85 ml/g and resembled with the reported values[17,18]. The overall native conformation of ovotransferrin, as indicated by its intrinsic viscosity (7.7 ml/g), differed significantly from that of a typical globular protein. The calculated value of the free energy for ovalbumin at 25°C was found to be 28.98 KJ/mole and was comparable with the reported values[19], suggesting unfolding of the protein. The temperature dependence of intrinsic viscosity Fig. 1A and B of the denatured proteins can be used as a method for the recognition of the randomly coiled states. Both the proteins in native state, appear stable up to 35°C (Fig. 1A and B), but show instability after 40°C[4], that is why viscosity composition profiles show higher $\Delta\eta$ values for low concentrations, than that of higher concentrations.

No regular pattern is observed with computed values of Huggin's constant (K), which vary slightly with temperature due to change of [η]. Such variations may be due to the strength and the nature of the solvent-solute interactions especially at above 55°C. The shape factor values against temperature show a pattern similar to that of intrinsic viscosity [η]. Such results support that the observed event in Fig. 1A for both the proteins is due to collapse of natural organized globular state into a partially organized molten globule state. This state is indicated by decrease in free energy (ΔG) and [η], in ovalbumin, and increase in ΔG and [η] in case of ovotransferrin. This state is also recognized by the formation of a few H-bonds with water molecules, because when a folded polypeptide chain begins to unfold, hydrophobic groups tend to get partially buried. The buried side chains are scattered along the entire polypeptide chain. This causes these residues to be relatively buried during

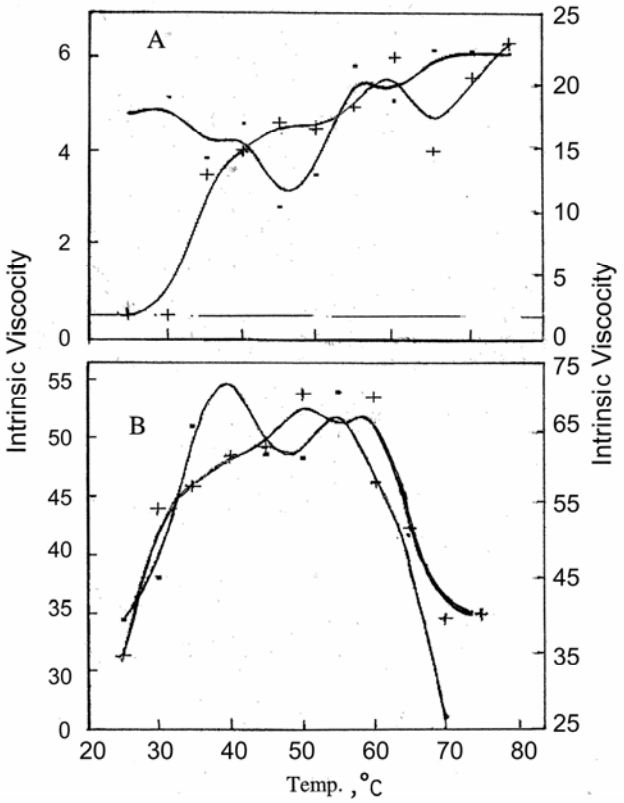

Fig. 1—Intrinsic viscosity [η] *versus* temperature for ovalbumin (-•-), for ovotransferrin (-+-) [(A) in phosphate buffer, (*p*H 7); (B) in 1% SDS (*p*H 7)]

early stage of molten globule, which has closely packed hydrophobic core and some secondary structure. Thus, the molten globule is an ensemble of structurally related molecules that are rapidly interconverging, but slowly change to a single unique conformation. The conversion of molten globule to unfolded state is energetically easy and rapid (Fig. 1B), while transition in reverse direction is energetically unfavorable and slow[20]. Last stage of conformational changes is the completely unfolded state that is characterized by the breaking of a number of bonds — hydrogen, disulphide and electrostatic etc.

Our data in Fig. 1A is in accordance with the above discussion that both the proteins show four states (folded, molten globule, 1st transition, and unfolded state)[20]. The zigzag shape of each curve indicates the extent of internal hydrogen bonds formed during unfolding and extent of secondary structure in each case. In other words, the positions of flexible loops are different in natured, molten globule and denatured states[20]. The compact structure of protein has greater amount of hydrogen bonding and less exposed hydrophobic groups. The increased order of the water molecules in the solvation layer correlates with an unfavourable decrease in the entropy of water. The cluster of non-polar groups decreases the extent of solvation because each group no longer presents its entire surface to the solution. The result is a favourable increase in entropy of protein solution. The entropy is the major deriving force for the association of hydrophobic group in aqueous solution. Hydrophobic amino acids side chains, therefore, tend to be clustered in the protein's interior, away from water[21]. Thus, the hydrophobic groups are not exposed towards solvent ions in natured state, partially exposed in molten globule, but more exposed in the unfolded state, which are reflected by a decrease in compressibility values. The curves in Fig. 2A and C show the shape factor or flexibility (or the ratio of exposed hydrophobic groups to internal hydrogen bonds) of two proteins at different temperatures[20]. At 298.75K, the values {($[\eta]_{int}$=4.68 for ovalbumin) and ($[\eta]_{int}$ = 7.57 for ovotransferrin)} indicate more internal hydrogen bonding in case of the former and more exposure of hydrophobic groups in case of latter, at neutral *p*H. Thus, the zigzag shape of the curves reflects disappearance and exposure of hydrophobic groups. In other words, these curves indicate the slight structural changes (or flexibility) during transition from natured → molten globule → unfolded state. These structural changes are, in turn, related to the functional properties of the proteins[22].

Fig. 2B and D indicate the difference in the nature of two proteins, when treated with denaturant, SDS. The denaturation is more pronounced in ovotransferrin due to its bilobal, flexible structure, but is not so pronounced in ovalbumin due to its rigid, single unit structure. The curves also confirm the presence of the three states during thermal and chemical denaturation. Both the proteins on comparing (Fig. 2A and B) and (Fig. 2C and D), indicate that intermediate denaturation states are more pronounced after the addition of SDS, as compared to phosphate buffer. The addition of 9 *M* urea in ovotransferrin causes almost complete denaturation above 50°C (Fig. 2E). The effects of two denaturants can be compared as follows: (i) When exposed to SDS, deformation of the protein molecule occurs resulting in subunits which are covalently bound, due to spreading of negative charge on polypeptide chains. So we observed high values of shape factor with increasing temperature as shown in Fig. 2B and the shape factor also increases with concentration of SDS; (ii) on the other hand, the three states of protein are clearly observed due to the effect of second denaturant, urea for the given range of temperature (Fig. 2E). Urea has structure breaking effect in denaturation of proteins[23], which results in stabilization of unfolded protein molecule, both by diminishing the hydrophobic interactions between non-polar groups and water, and by increasing the affinity of the solvent for amide and peptide groups[1].

The ovalbumin is globular in nature and less compressible as compared to ovotransferrin, this is confirmed by the plots of intrinsic viscosity-temperature and shape factor-temperature (Fig. 1A and 2A for ovalbumin and Fig. 1A and 2C for ovotransferrin), which show similar pattern. The values of partial specific volume v_o, of ovalbumin at infinite dilution are found to be identical with literature values[4].

The existence of three conformational states on denaturation can also be studied by ultrasonic values[24]. These values as a function of temperature are found to increase up to 40°C, but this pattern changes after 45°C (Tables 1 and 2). Such a change indicates the extent of denaturation beyond 40°C, which increases after the addition of urea and SDS (Tables 1 and 2). The ultrasonic velocity as a function of concentration is found to be typical in nature and indicated by the pattern of thermal and chemical unfolding (Tables 1 and 2). But the special feature of ovalbumin is the

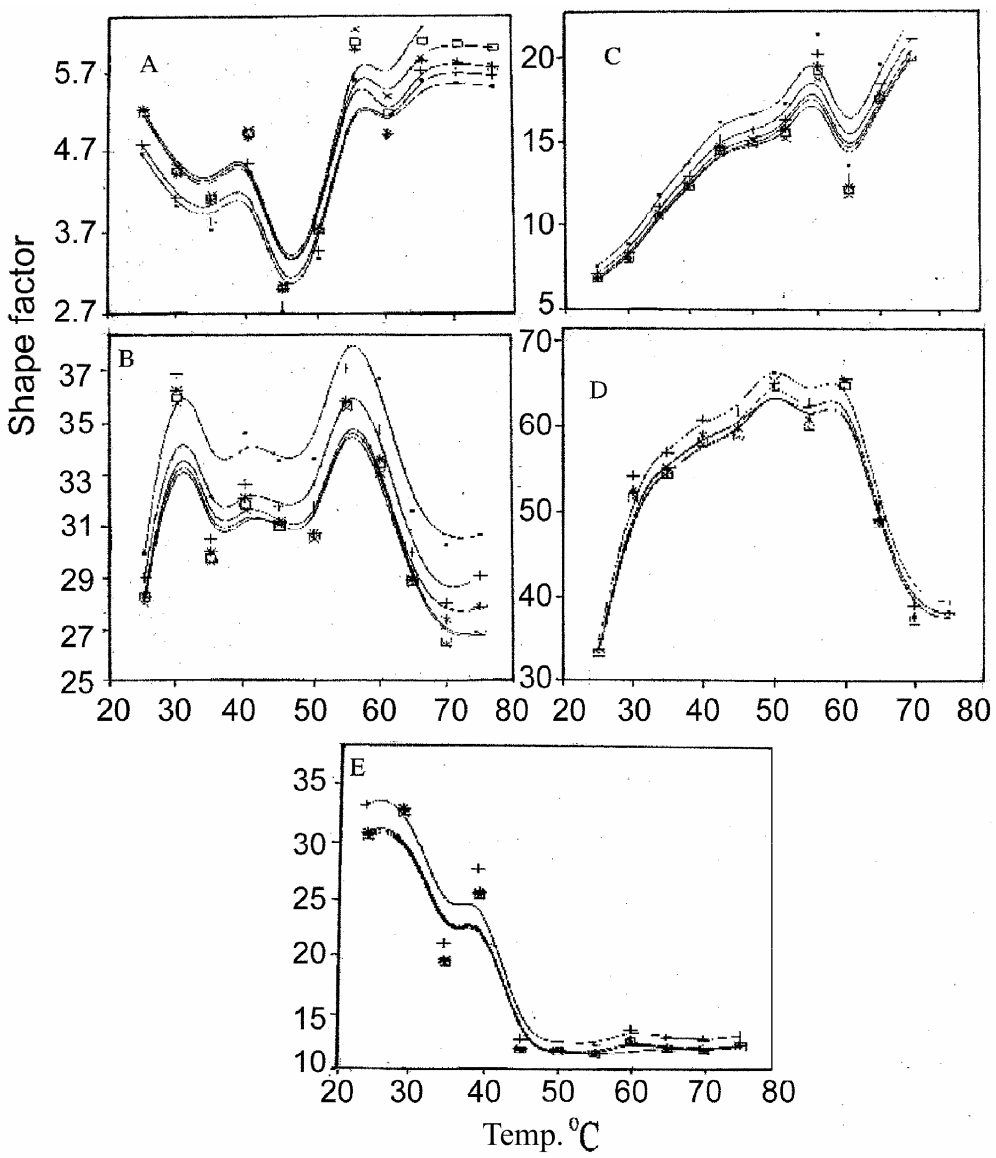

Fig. 2—Shape Factor [v] *versus* temperature at different protein concentrations in different denaturants. (A): Ovalbumin in phosphate buffer, (*p*H 7) [(-•-), 0.2; (-+-), 0.4; (-*-), 0.6; (-□-), 0.8; (–×–), 1.0] g/ml values; (B): ovalbumin in 1% SDS [(-•-), 0.2; (-+-), 0.4; (-*-), 0.6; (-□-), 0.8;(–×–), 1.0] g/ml values; (C): ovotransferrin in phosphate buffer, (*p*H 7) [(-•-), 0.17; (-+-), 0.33; (-*-), 0.54; (-□-), 0.75; (–×–), 0.94;] g/ml values; (D): ovotransferrin in 1% SDS[(-•-), 0.17; (-+-), 0.33; (-*-), 0.54; (-□-), 0.75; (–×–), 0.94;] g/ml values; (E): ovotransferrin in 9 *M* urea., [(-•-), 0.161; (-+-), 0.262; (-*-), 0.462 ; (-□-), 0.665; (–×–), 0.864] g/ml values.

insertion of extra β-strand in the middle of β-sheet, while the adjacent strands in the β-sheet are separated. This involves breaking of many hydrogen bonds, as well as change in a number of hydrophobic packing contacts at the same time. The general folds in the different states are the same during the increase in β-structure, but the position of flexible loop region varies in a novel way[20,25], which may be responsible for more pronounced concentration effect in the case of ovalbumin, compared to ovotransferrin, which lacks such flexible loop regions, and shows less changes with concentration (Fig. 2A and C, 2B and D). This is the reason, why ovalbumin easily aggregates. In addition, we may also say that the concentration effect on the two proteins is different due to the presence of different types of amino acids. The difference between the partial specific volume of native and thermally denatured ovotransferrin in buffer is only 0.003 ml/g and that from the chemically denatured by the addition of urea is 0.035 ml/g. The increase in the

Table 1—Thermodynamic characteristics for ovalbumin (concentration used 0.2×10^{-2} g/ml) in phosphate buffer (*p*H-7.0) (I); 1% SDS (II) and 9 *M* urea (III)

Temp. (°C)	v^o ml/g cm^2/dyne	U ms^{-1}	$\bar{\beta}_S \times 10^{12}$	K	n
25	0.736[a]	1521.8	9.35[(b)]	0.68	19.10
	0.719	1525.8	-3.29	2.17	378.0[(b)]
	0.728	1525.9	5.42	3.24	102.2
30	0.737	1530.6	8.70	0.76	15.30
	0.720	1533.0	-2.86	1.60.	437.0
	0.729	1533.0	1.96	5.70	82.50
35	0.739	1541.0	07.53	0.41	13.70
	0.722	1560.7	-2.56	1.26	638.0
	0.730	1541.0	-18.90	3.65	62.50
40	0.740	1547.8	5.91	0.20	17.9
	0.718	1548.0	-2.29	1.18	806.3
	0.731	1548.0	-15.10	3.32	114.4
45	0.742	1553.6	- 3.29	0.27	8.40
	0.725	1583.8	-2.19	1.35	625.0
	0.735	1553.8	-22.90	2.93	94.20
50	0.744	1558.3	-4.46	0.56	11.40
	0.722	1559.0	- 2.50	1.32	627.2
	0.736	1559.0	-17.6	2.57	90.00
55	0.746	1562.6	-6.20	0.17	25.70
	0.725	1562.5	-2.46	1.26	742.4
	0.737	1562.5	- 11.40	0.31	286.3
60	0.747	1565.4	-6.28	0.55	020.9
	0.726	1565.8	- 2.44	1.37	588.7
	0.738	1565.8	-04.67	0.33	454.3
65	0.799	1569.5	-9.85	0.49	27.8
	0.733	1568.0	-2.43	1.42	502.6
	0.739	1568.0	-6.20	1.68	404.8
70	0.803	1571.4	14.54	0.50	27.7
	0.735	1569.2	-1.98	1.70	246.7
	0.742		-19.00	1.37	571.8
75	0.802	1571.3	-11.90	6.67	27.3
	0.743	1569.1	-1.98	1.81	69.1
	0.796	1569.1	-04.57	1.15	841.1

(a) Ref. 7; (b) Ref. 15 (in 6 *M* Gnd HCl)

latter case indicates that there may be more space available for compression in the protein molecule due to the breaking of hydrogen bonds and other polypeptide bonds of proteins. Hence, the result of protein denaturation depends significantly on the values of temperature and *p*H[26].

The partial adiabatic compressibility, β_s of proteins was obtained from the ultrasonic velocity. The difference between the $\bar{\beta}$s of the native and denatured states was found to be within $\pm 3.44 \times 10^{-12}$. The effect of denaturation by SDS is more pronounced than by urea on $\bar{\beta}$s values (Tables 1 and 2). There is decrease in compressibility, which can also be explained by the increase in partial specific volume. This behaviour of partial specific volume is in accordance with Kauzeman's considerations[14]. These values ind-

Table 2—Thermodynamic characteristics for ovotransferrin in (concentration used 0.2×10^{-2} g/ml) in phosphate buffer *p*H-7.0 (I); 1% SDS (II) and 9 *M* urea (III)

Temp. °C	v^o ml/g cm^2/dyne	U ms^{-1}	$\bar{\beta}_S \times 10^{12}$	K	n
25	0.727[a]	1535.7	004.74[(b)]	1.64	039.1
	0.756	1523.5	-2.34	0.21	389.7
	0.759	1690.0	131.20	3.70	340.1
30	0.728	1539.5	4.68	0.07	049.5
	0.727	1524.5	-1.79	0.46	765.8
	0.760	1688.2	140.60	3.56	376.2
35	0.729	1545.2	3.93	0.14	076.6
	0.758	1536.0	-1.58	0.31	827.5
	0.761	1687.5	125.70	6.35	171.0
40	0.730	1550.0	3.822	1.24	097.8
	0.759	1548.0	-1.62	0.26	918.6
	0.762	1686.0	117.00	4.81	257.1
45	0.732	1556.7	3.743	1.86	124.1
	0.760	1554.0	-1.36	0.30	941.0
	0.774	1684.5	116.50	0.02	081.3
50	0.735	1562.5	2.903	1.07	118.4
	0.761	1559.0	-1.04	0.36	1109.6
	0.776	1683.0	116.50	0.04	080.4
55	0.737	1566.6	2.30	0.74	139.2
	0.762	1563.4	-1.03	0.49	972.3
	0.777	1681.5	114.20	0.03	077.0
60	0.738	1569.2	1.78	0.93	193.2
	0.764	1568.5	-1.36	0.72	1097.4
	0.779	1678.0	148.20	0.02	090.5
65	0.739	1571.5	000.84	0.76	096.7
	0.766	1570.0	-0.972	0.94	708.4
	0.781	1672.5	164.90	0.03	083.8
70	0.742	1571.8	000.98	0.74	171.1
	0.768	1570.1	-0.437	1.23	474.3
	0.784	1668.0	135.50	0.03	082.6
75	0.749	1571.9	1.456	0.69	209.3
	0.770	1570.2	-0.3987	1.25	485.1
	0.792	1663.2	130.00	0.02	087.8

(a) Ref. 36; (b) Ref. 15 (in 6 *M* Gnd. HCl)

cate that: (a), the solvation effect overcomes the cavity effect to make the partial specific compressibility, $\bar{\beta}s$ (Eq. 2) negative, in case of ovalbumin (with some exceptions); (b), the cavity effect overcomes the solvation effects to make the $\bar{\beta}s$ (Eq. 2) positive (with some exceptions), in case of ovotransferrin. The positive values of $\bar{\beta}_s$ may indicate the ratio of hydrophobic amino acids residues on the surface with the polar amino acids residues buried in the interior of the molecule, according to Bigelow[27].

As reported earlier, the hydrophobic interactions are generally considered to be the significant factor in stabilizing the three dimensional structure of protein or the degree of three dimensional water molecular

organization[28], and the solvation is less in the bulk of protein solution due to the presence of non-polar amino acid groups in native protein molecules, while at the surface, these polar amino acid residues are exposed to the solvent (water)[15]. So, the increase and decrease in the compressibility with the amount of surface amino acid residues (n) indicate the pattern of arrangement of non-polar and polar amino acids at the periphery and inside the bulk of the protein structure in different cases studied (Table 1). It is known that the ratio of disulphide bonds, in case of ovalbumin and ovotransferrin is 6-fold in natured state[29,30], while their internal compressibility or their internal flexibility is indicated as 2-fold, up to 35°C (Table 3), but after 35°C, due to thermal denaturation and other factors, the said ratio of disulphide bond first decreases (Fig. 3) and then finally increases. Thus, the above data indicate that the disulphide bonds, which are responsible for linkage of two polypeptide chains or over all shape of the protein molecule may reflect the internal flexibility of the proteins with slightly high magnitude.

Table 3—Compressibility ratio of ovalbumin and ovotransferrin [(a) In phosphate buffer; (b) in the presence of urea]

Temp. (°C)	$\beta \times 10^{12}$ ovalbumin	$\beta \times 10^{12}$ ovotransferrin	$\beta_{oval.}/\beta_{ovotr.}$
25	**a** 9.35	**a** 4.74	**a** 1.9726
	b 5.42	**b** 131.2	**b** 0.0413
30	8.7	4.68	1.8590
	1.96	140.6	0.014
35	7.53	3.9	1.9308
	18.9	125.7	-0.1291
40	5.91	3.822	1.5463
	-15.1	117.0	-0.8882
45	3.29	3.743	0.0775
	-22.9	116.5	-0.8790
50	4.46	2.903	-1.5363
	-17.6	116.5	-0.1511
55	-6.20	2.30	-2.6957
	-11.4	114.2	-0.0998
60	-6.28	1.78	-3.5288
	-4.64	148.2	-0.0315
65	-9.85	00.84	-11.7262
	-6.2	164.9	-0.0376
70	14.54	00.98	-14.8367
	-19.0	135.5	-01402
75	-11.9	1.456	-8.1731
	-04.57	130.00	-0.0352

Fig. 3 clearly indicates three stages of protein due to denaturation in terms of compressibility are similar to that reported in terms of spectra[2]. Our data in Table 3 also show that the compressibility ratio of proteins in presence of urea at *p*H 7, is quite low. This is due to the absence of hydrogen bonds, that decrease the compressibility, while the presence of these bonds increase $\bar{\beta}_s$ values. The values of $\bar{\beta}°_s$ with $\bar{v}°$, first decrease and then increase to show the existence of an intermediate state (Tables 1 and 2). The change is more visible after the addition of urea or SDS, indicating three major conformational states, i.e. native, intermediate and completely denatured states. Finally, increase in $\bar{\beta}_s$ values with the partial specific volume also indicate that the hydrophobicity of protein depending upon the structure, suggesting a decrease in the free energy (ΔG) during unfolding (Fig. 4A and B), which in turn depends on the number of ion pair (buried or unburied), charge/helix dipole interactions and disulphide bonds.

The change in free energy is highly specific for each protein, because it separates the folded and unfolded states in typical proteins under physiological conditions[22,26]. So, ΔG values are related up to some extent with the protein size number and type of residue. The free energy can decompose linearly into its components: $G_{hydrophobic}$, $G_{elect.}$, $G_{s\text{-}s}$ and G_{vw}[22,31]. The change in ΔG values computed in the present work is higher than those reported (10-20 kcal/mol) by means of radial functions[5,22], but is in accordance with the

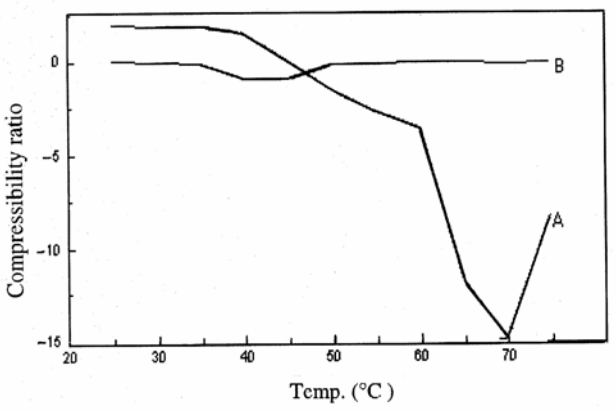

Fig. 3—Ratios of compressibilities of ovalbumin and ovotransferrin against temperature [(-A-) in presence of phosphate buffer (*p*H 7); (-B-) in presence of 9 *M* urea and phosphate buffer (*p*H 7)

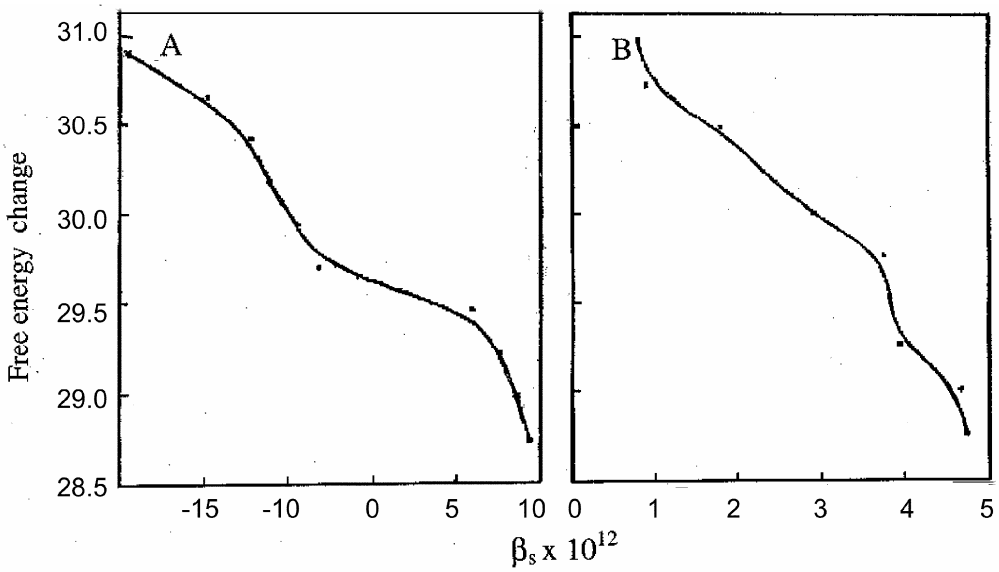

Fig. 4—Free energy change of unfolding *versus* adiabatic compressibility (A): of ovalbumin in phosphate buffer (*p*H 7): (B): ovotransferrin in phosphate buffer (*p*H 7)

values reported by Privalov[19]. The pattern of the plots of ΔG_{unf} *versus* $\bar{\beta}_s^o$ is found in accordance with that reported earlier[7]. The plots (Fig. 4A and B), after the addition of urea and SDS, clearly indicate the partial folding at low temperature and complete unfolding at higher temperatures. Similarly, reducing agents like β-mercapto ethanol (BME) can be used for indicating the flexibility of denatured-globular-protein-molecules by ultrasonic technique in absence of di-sulphide bonds[32].

Globular proteins usually have a rapid turnover rate and their native states have, therefore, evolved to be only marginally stable. Earlier studies have shown that flexibility is related with the biological control through a spring loaded safety catch mechanism for Serpins[33]. Therefore, the flexible proteins take less time to digest in comparison to rigid ones[34]. The present viscometric studies on globular proteins, ovalbumin and ovotransferrin[6,35] show their stability, which in turn, is indicated by the free energy and the flexible nature is indicated by compressibility. This flexibility is related with the extent of denaturation. A rigid protein takes more time to digest (to denature) than a flexible one, because the denaturation of protein at low *p*H in stomach is an essential and important step, during which different bonds buried initially in the native protein are exposed.

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