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Stabilisation of ovalbumin by maltose

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Abstract

Sugars stabilise proteins against extremes of pH and heat denaturation. This was studied by means of density, ultrasonic velocity and viscosity measurements of the following systems (i) ovalbumin–phosphate buffer (pH 2.4, 5.2, 7.0 and 8.9) and (ii) ovalbumin–maltose–phosphate buffer (pH 2.4, 5.2, 7.0 and 8.9) systems as functions of concentration and temperature. The partial specific volumes ($\bar{v}^0$), partial specific adiabatic compressibility ($\bar{\beta}_s$), intrinsic viscosity, [η] and shape factor, v, were calculated for the said systems. The results obtained from such studies suggest that the stabilisation of ovalbumin occurs in the presence of maltose through strengthening of hydrophobic interactions. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The volumetric and compressibility behaviour of solutes in solution provide very useful information related to solute–solvent and solute–solute interactions. The infinite dilution partial specific volumes and compressibilities give the structural information and interaction phenomena associated with solvation processes. Since these properties are independent of solute–solute interactions, they are determined only by the respective intrinsic value and solute–solvent interaction. Various investigations have been done using electrolytes [1,2], carbohydrates [3,4], amino acids [5–7], peptides [8–10] and proteins [11–16] in aqueous as well as in mixed aqueous solvents. During the past 10 years, considerable amount of work has been done on the adiabatic compressibility of proteins. An important result obtained from such studies was that the globular proteins have positive compressibility as compared to their constituent amino acids which have negative compressibility indicating the great contribution of the internal cavity in the structure of proteins.

The study of viscous behaviour of proteins in solutions is important in understanding the mechanism of transport processes. Viscosity and its derived parameters provide valuable information regarding the shape and size of these macromolecules. The sensitivity of viscosity to molecular structure makes it useful for monitoring the processes that result in changes in the shapes and sizes of the molecules such as the denaturation of proteins, intermolecular cross-linking, etc.

Sugar solutions have large effects on the structure and properties of proteins including their solubility, denaturation, etc. In literature, there are reports about the effect of sugars on the stability of proteins and enzymes [17–36] which has also been explained by their effect on the structure of water [21,34]. Despite a

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lot of information obtained on the stability of proteins by different sugars, the compressibility and viscometric behaviour of ovalbumin in the presence of maltose has not been studied so far. Therefore, in order to study the behaviour of ovalbumin in sugar solutions, we have studied the partial specific and viscometric properties of the following systems:

1. Ovalbumin in phosphate buffer (pH 2.4–8.9).
2. Ovalbumin in phosphate buffer (pH 2.4−8.9)+ maltose mixtures.

2. Experimental

2.1. Material and sample preparation

Crystallised and lyophilised ovalbumin from Sigma Chemical Co. (LOT 106 H 7070, Grade V) was used for sample preparation. 0.2 M aqueous solutions of both monobasic and dibasic sodium phosphate (from E. Merck) were mixed in different proportions to prepare phosphate buffers of pH 2.4, 5.2, 7.0 and 8.9. The pH of these solutions was measured by digital pH meter (Elico Pvt. Ltd., Hyderabad, model T-10). Two sets of ovalbumin solutions were prepared. For the first set, the phosphate buffers of pH 2.4, 5.2, 7.0 and 8.9 were used as solvents for preparing the solutions of ovalbumin of concentration ranging from 4–10 mg/ml. For the second set, l.5 M solutions of maltose (S.D. fine chem.) prepared in phosphate buffers of pH 2.4, 7.0 and 8.9, were used as solvents for ovalbumin. The concentrations of ovalbumin remained unchanged.

2.2. Density measurement

The density of solvents and solutions were measured over the temperature range of 25–45°C (±0.1°C) using a 5 ml pycnometer. The accuracy in the measurement was about ±0.4 mg/ml. The pycnometer was immersed in a thermostated paraffin bath to maintain the temperature of the system.

2.3. Ultrasonic velocity measurement

The ultrasonic velocity in protein solutions was measured by a single frequency (4 MHz) ultrasonic interferometer (Mittal's model F-81) which consists of two main parts: (i) the measuring cell; (ii) the high frequency generator.

The measuring cell is a double-walled cell. Water is circulated through the two tubes in the double walled cell to maintain constant temperature of the liquid during the experiment. There is a quartz crystal plate fixed at the bottom of the cell and on the top of it is a movable metallic plate. This metallic plate is attached to the micrometer screw held on the top of the cell. The cell is connected to the high frequency generator by a co-axial cable.

The high frequency generator excites the quartz plate of the cell to generate ultrasonic waves in the liquid filled in the cell. These waves are reflected back by the movable metallic plate kept parallel to the quartz plate. As a result standing waves are produced. This acoustic resonance gives rise to a maximum of the anode current. If the distance between the two plates is exactly a whole multiple of sound wavelength or one-half of the wavelength the current becomes maximum. The changes in current are recorded by a microammeter provided with the high frequency generator. The micrometer screw is slowly raised to record the maximum current. The wavelength is determined by recording the total distance moved by the micrometer for 20 maxima of the anode current. The distance (d) thus moved by the micrometer gives the value of wavelength (λ) from the relation

$$d = \frac{n\lambda}{2}$$

where n is the number of maxima in the current. Knowing the values of frequency (v) and wavelength (λ) the ultrasonic velocity (u) in the solution is obtained from the relation

$$u = v\lambda \tag{1}$$

The accuracy in the measurement was found to be ±0.3 m/s.

The partial specific adiabatic compressibility of the protein was calculated by using the relation given by Shiio [37]:

$$\overline{\beta}_s = -\frac{1}{\overline{v}^0}\left(\frac{\partial \overline{v}^0}{\partial P}\right) = \frac{\beta_0}{\overline{v}^0}\lim_{c\to 0}\left[\frac{\beta/\beta_0 - V_0}{c}\right] \tag{2}$$

$$V_0 = \frac{\rho - c}{\rho_0} \tag{3}$$

$$\overline{v}^0 = \lim_{c\to 0}\left[\frac{1 - V_0}{c}\right] \tag{4}$$

where P is the pressure, c the protein concentration in gram per millilitre of the solution, V_0 the apparent volume fraction of the solvent in solution, ρ and ρ_0 the densities of the solution and solvent, respectively, β and β_0 the adiabatic compressibilities of the solution and solvent, respectively and $\overline{v}^0$ is the partial specific volume of the protein. The adiabatic compressibility was calculated using the relation

$$\beta = \rho^{-1}u^{-2} \tag{5}$$

According to Kauzmann [38]

$$\overline{v}^0 = V_c + V_{cav} + \Delta V_{soln} \tag{6}$$

where V_c is the constitutive atomic or group volume, V_{cav} the volume of the cavities due to imperfect packing of the atoms or groups and ΔV_{soln} the volume change due to solvation. Here 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 $\overline{v}^0\Delta V_{soln}$ involves (i) electrostatic solvation of ionic groups; (ii) hydrogen-bonded hydration of polar groups and (iii) hydrophobic hydration of non-polar groups. Each of them contribute negatively to ΔV_{soln} [12]. Therefore, ΔV_{soln} contributes negatively to $\overline{v}^0$. The terms V_{cav} and ΔV_{soln} have been known to tend to cancel each other. This makes it possible to calculate the partial specific volume as the sum of constitutive atomic or group volumes [39–42].

Since V_c is highly incompressible, the differentiation of Eq. (6) with pressure under adiabatic conditions gives

$$\overline{\beta}_s = -\left(\frac{1}{\overline{v}^0}\right)\left(\frac{\partial \overline{v}^0}{\partial P}\right)$$

or

$$\overline{\beta}_s = -\left(\frac{1}{\overline{v}^0}\right)\left[\frac{\partial V_{cav}}{\partial P} + \frac{\partial \Delta V_{soln}}{\partial P}\right] \tag{7}$$

Thus, the partial specific adiabatic compressibility obtained experimentally for different systems is mainly contributed from the cavities and solvation. The first term in Eq. (7) contributes positively while the second term contributes negatively to $\overline{\beta}_s$.

2.4. Viscosity measurement

Viscosity was measured with a cannon-ubbelohde viscometer whose time of fall varied from 250 to 400 s depending on the temperature. The time of fall was recorded with a stopwatch of accuracy ±0.1 s.

Poiseuille's equation

$$\eta = \pi\rho g h\frac{r^4 t}{8LV} = \rho B t \tag{8}$$

was employed to calculate the viscosity of the test solutions. The error in the measurement of viscosity was approximately 0.2%. Temperature was controlled in constant temperature bath to ±0.1°C. The intrinsic viscosity $[\eta]$ was calculated by using the relation [43,44]:

$$[\eta] = \lim_{c\to 0}\frac{\eta_{sp}}{c} = \lim_{c\to 0}\frac{\eta' - \eta}{\eta c} \tag{9}$$

where η' and η are the viscosities of solution and pure solvent, respectively. Intrinsic viscosity is obtained by plotting $(\eta' - \eta)/\eta c$ versus c and extrapolating to $c = 0$. An alternate expression for calculating $[\eta]$ is

$$[\eta] = \lim_{c\to 0}\left[\frac{1}{c}\ln\left(\frac{\eta'}{\eta}\right)\right] \tag{10}$$

Shape factor (v) was calculated by using the relation [43,44]

$$v = \frac{[\eta]}{\overline{v}^0 + 0.29} \tag{11}$$

3. Results and discussion

The partial specific volume, $\overline{v}^0$, was determined as the extrapolated value of the apparent specific volume to zero protein concentration from Eq. (4) by the least-squares method and the results are presented in Table 1. The value of $\overline{v}^0$ at 25°C and pH 7.0 for compact native form of ovalbumin is 0.743 which is very near to the reported value [15] of 0.744 ml/g.

The partial specific adiabatic compressibility, $\overline{\beta}_s$, was determined by the linear extrapolation of $(\beta/\beta_0 - V)/c$ to zero protein concentration. The $\overline{\beta}_s$ values, thus, obtained, are listed in Table 2.

As apparent from Table 2, the $\overline{\beta}_s$ values for all the systems studied is positive suggesting the presence of highly compressible cavities in the protein molecules

Table 1
Effect of maltose on the partial specific volume, $\bar{v}^0$ (ml/g), of ovalbumin as functions of pH and temperature[a]

pH	Temperature (K)				
	298.15	303.15	308.15	313.15	318.15
2.4	0.7703	0.7802	0.8448	0.8371	0.8080
	0.7413	*0.7665*	*0.7745*	*0.7498*	*0.7598*
5.2	0.8786	0.8462	0.8383	0.9236	0.9918
7.0	0.7430	0.7350	0.7575	0.7967	0.8060
	0.7523	*0.7610*	*0.7617*	*0.7942*	*0.7942*
8.9	1.0373	0.8650	0.8635	0.8715	0.9038
	0.7590	*0.7588*	*0.7743*	*0.7663*	*0.7237*

[a] The values given in italics represent the ovalbumin–maltose–phosphate buffer system. The non italicised values represent the ovalbumin–buffer system.

and that the effect of cavity has overcome the solvation effect.

An examination of Table 1 shows that the partial specific volume, $\bar{v}^0$, of ovalbumin at pH 7.0 is less than that at pH 2.4, 5.2 and 8.9. This shows the native or compact form of protein at pH 7.0 upto 40.0°C. At pH 2.4, 5.2 and 8.9 the protein is denatured and the random coils are formed. This is evident from the higher values of $\bar{v}^0$. The effect of maltose sugar has been studied at the extremes of pH and also at pH 7.0. It has been observed that after the addition of maltose to the protein solutions there is a decrease in the values of partial specific volume as well as compressibility of the solutions. This may be attributed to the fact that the addition of maltose to the protein increases the hydrophobic, electrostatic and hydrogen-bonding interactions giving rise to the compact form of protein. In the absence of sugar, the extremes of pH cause denaturation of protein and the random coils are formed. Due to the random coiling of protein, the values of partial specific volume as well as compressibility are increased. Therefore, by observing a decrease in the compressibility of the solution and the partial specific volume of the protein after the addition of maltose we can say that the extent of denaturation of protein is reduced and its stabilisation has taken place.

Table 2
Effect of maltose on the partial specific adiabatic compressibility ($\bar{\beta}_s \times 10^{12}$ cm^2/dyne) of ovalbumin as functions of temperature and pH[a]

pH	Temperature (K)				
	298.15	303.15	308.15	313.15	318.15
2.4	11.8065	15.7412	20.8916	26.2210	14.0037
	12.1352	*14.3606*	*15.8636*	*15.0490*	*16.5338*
7.0	10.2139	10.2174	11.4957	13.0105	18.6215
	11.8954	*12.2924*	*12.1029*	*14.8612*	*16.8930*
8.9	34.2240	22.1682	14.7291	16.9894	27.5892
	13.0261	*11.6493*	*13.8546*	*14.8888*	*14.6808*

[a] The values given in italics represent the ovalbumin–maltose–phosphate buffer system. The non italicised values represent the ovalbumin–buffer system.

Proteins are stabilised by a combination of hydrogen-bonding interactions, electrostatic interactions and hydrophobic interactions. In some proteins there is an additional contribution from cross-linking, metal complexing and specific binding of ions and cofactors. In discussing the effect of maltose on the stability of ovalbumin, we have to consider the effects of maltose sugar on these various forces and interactions.

In aqueous solutions of proteins there is a co-operative hydrogen-bonded structure [21] in which water competes as both donor and acceptor with the backbone and side chain groups of the protein. When sugar is added to the protein solutions, the OH groups of sugar may also compete for hydrogen-bonding [21]. Now we have to consider the respective interactions between protein, water and additive (maltose) molecules. The additives interacting more strongly with protein than with water will tend to stabilise the denatured states by the formation of protein-additive complexes. They will, therefore, have a denaturing effect. However, additives interacting more strongly with water molecules than with protein will favour the stabilisation of protein molecules [29]. In the present case, the sugar maltose interacts more strongly with water molecules than with protein by forming hydrogen bonds with water molecules. This will favour an increase in the degree of organisation of water molecules by the formation of clusters (as in ice) and will thus limit the unfolding of protein [29]. This was supported by the view that the addition of sugars results in a higher resistance of proteins to denaturation by an increase contribution of water extrusion entropy change [49].

The aqueous solutions of sugars have lower dielectric constant than pure water indicating that the electrostatic interactions are stronger in these

solutions than in pure water as reported in the literature [45]. The above mentioned interaction between sugar and water molecules create a polar environment near the protein due to which the hydrophobic interactions increase. These hydrophoic interactions are generally considered to be the significant factor in stabilising the three-dimensional structure of proteins [21,46]. In aqueous-organic mixed solvents, hydrophobic interactions depend on the solvent structure with maximum hydrophobic interactions occurring in those solvents mixtures in which the three-dimensional structure of water is most developed or the degree of water molecules organisation is increased [29,47]. The evidence for the same fact was derived by Tait et al. from both spectroscopy and thermodynamics [48]. The protective action of sugars on proteins can be attributed to the fact that sugars may replace a certain number of water molecules that are hydrogen-bonded to the structure in a way similar to water itself. This would result in a solvent system where the already exposed side chains attached with non-polar groups in the native protein molecules would have a tendency to enter into the interior of the protein due to the polar environment produced by sugar molecules. This phenomenon would be responsible for higher stability of the protein molecule in these solvents and would reduce the extent of denaturation of protein molecules induced thermally or by extremes of pH. The decrease in the values of $\overline{v}^0$ and $\overline{\beta}_s$ for systems (ii) when compared with those of systems (i) at pH 2.4 and 8.9 shows that the denaturation of protein is reduced by adding maltose to the solutions. When the proem undergoes denaturation, random coiling occurs, therefore, there is an increase in the values of $\overline{v}^0$ and $\overline{\beta}_s$ at all other pH values except pH 7.0 in the system (i). It was also found that $\overline{\beta}_s$ is highly correlative with the partial specific volume of the protein.

The intrinsic viscosity, $[\eta]$, is obtained by plotting $1/c \ln(\eta'/\eta)$ as a function of concentration and extrapolating it to $c = 0$. An examination of Table 3 shows that the value of $[\eta]$ lies between 3–4 ml/g in the temperature range of 25.0–40.0°C at pH 7.0. This represents the native state of ovalbumin. The value of $[\eta]$ increases with the increase in temperature of the system and its value goes beyond 4.0 ml/g after 40.0°C. This shows that denaturation has just started. It is also clear from Table 3 that ovalbumin undergoes denaturation at the extremes of pH, hence the value of $[\eta]$ is very large. The addition of maltose to the protein stabilises it through increased hydrophobic interactions, therefore, in this case the trend of variation of $[\eta]$ is the same as that at pH 7.0. This indicates that the extent of stabilisation is nearly the same from pH 2.4–8.9 and the stabilisation is independent of pH.

Table 3
Effect of maltose on the intrinsic viscosity ($[\eta]$, ml/g), of ovalbumin at different pH and temperatures[a]

pH	Temperature (K)				
	298.15	303.15	308.15	313.15	318.15
2.4	9.274	10.559	9.655	9.791	10.153
	3.063	*3.258*	*3.616*	*3.899*	*4.235*
5.2	11.781	9.254	9.924	8.585	8.955
6.0	10.258	10.607	7.734	7.070	8.130
7.0	3.079	3.388	3.712	3.971	4.063
	3.189	*3.308*	*3.316*	*3.731*	*4.123*
8.9	7.220	5.784	5.095	9.751	6.005
	3.285	*3.413*	*3.816*	*4.180*	*4.263*

[a] The values given in italics represent the ovalbumin–maltose–phosphate buffer system. The non italicised values represent the ovalbumin–buffer system.

The values of shape factor v (Table 4) obtained from Eq. (9), follow exactly the same pattern as that of $[\eta]$. Fig. 1 shows that the thermal unfolding of the protein occurs in steps while the stepwise stabilisation of modified protein is not so pronounced (Fig. 2).

Table 4
Effect of maltose on the shape factor, v, of ovalbumin at different temperatures and pH[a]

pH	Temperature (K)				
	298.15	303.15	308.15	313.15	318.15
2.4	8.747	9.867	8.508	8.687	9.247
	3.026	*3.044*	*3.387*	*3.923*	*4.108*
5.2	13.563	8.343	8.796	7.074	6.986
6.0	10.449	11.058	7.709	6.909	7.680
7.0	3.003	3.359	3.714	3.936	3.954
	3.001	*3.049*	*3.055*	*3.433*	*3.690*
8.9	5.366	5.053	5.014	9.917	5.092
	3.442	*3.496*	*3.793*	*4.178*	*4.380*

[a] The values given in italics represent the ovalbumin–maltose–phosphate buffer system. The non italicised values represent the ovalbumin–buffer system.

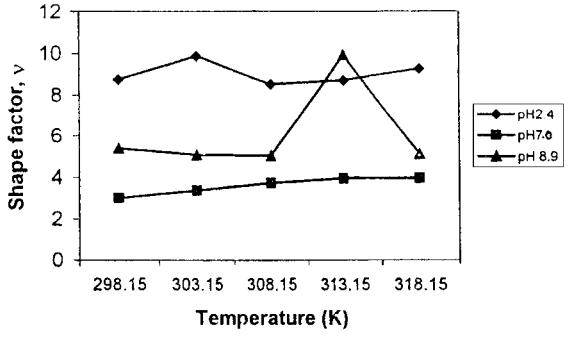

Fig. 1. Plots of shape factor vs. temperature for ovalbumin–buffer system at different pH values.

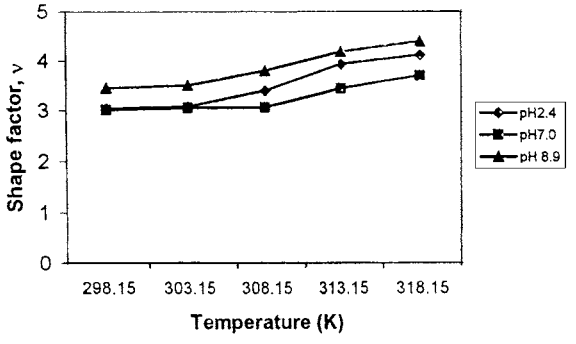

Fig. 2. Plots of shape factor vs. temperature for ovalbumin–maltose buffer system at different pH values.

All the parameters evaluated here justify the stabilising action of maltose on ovalbumin and it is suggested that the structural stabilisation is mainly due to

1. Primary interaction between sugar and water molecules leading to the formation of clusters (as occurs in ice) in the vicinity of protein. This will favour an increase in the degree of water molecule organisation and will thus limit the denaturation of protein.
2. Strengthening of hydrophobic interactions caused by the unfavourable (or polar) environment produced by sugar molecules.

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