

Structural stabilization of bovine β -Lactoglobulin in presence of polyhydric alcohols

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β -Lactoglobulin (β -Lg) is the major protein present in bovine milk whey. Addition of hydrogen-bonded cosolvents to proteins is known to modify the thermodynamic properties of proteins. Preferential interaction parameters of β -Lg were determined in various concentrations of cosolvents like sorbitol, glycerol and sucrose using the precision densimetry. The apparent partial specific volumes determined at 20°C under both isomolal and isopotential conditions in 0.02 M phosphate buffer (pH 7.9) were 0.743 ± 0.001 and 0.744 ± 0.001 , respectively. From the partial specific volume data with cosolvents the preferential interaction parameter and other thermodynamic parameters were calculated at different cosolvent concentrations. The values increased with solvent concentration up to 40% and reached a maximum of -0.190 ± 0.02 and -0.140 ± 0.02 in sucrose and sorbitol, respectively. In glycerol, the value increased up to -0.213 ± 0.03 in 20% glycerol and then decreased with the increase in the cosolvent concentration. There were no changes in secondary structure of the protein as reflected by far UV-CD spectra. The fluorescence spectra showed changes in the fluorescence intensity due to perturbations in the tryptophan moiety without changes in the emission wavelength. The above data was further supported by the degree of hydrolysis in the presence of cosolvents with the enzyme α -chymotrypsin. The degree of hydrolysis reduced to 12% from the control value of 18% in the presence of sorbitol and glycerol and increased by 3% in the presence of sucrose. All the cosolvents also enhanced thermal stability to various extents.

Keywords: β -Lactoglobulin (β -Lg), cosolvents, sorbitol, glycerol, sucrose, preferential hydration, apparent thermal transition temperature (Apparent T_m).

Introduction

About 80% of bovine milk proteins consist of caseins (2.7g/100g milk), a group of phosphate containing proteins named α , β and κ -caseins. Whey proteins amount to about 20% of bovine milk proteins (0.6 g/100 g milk). The whey protein fraction consists of α -Lactalbumin, β -Lactoglobulin, bovine serum albumin, immunoglobulins, lactoferrin and the protease-peptone fraction. β -Lactoglobulin is the major protein present in bovine whey. At physiological pH, it exists, as a dimer with a molecular weight of 36,400 Da. Two disulfide bonds and a free thiol group exists within β -Lactoglobulin¹. Bioactive peptides liberated from this protein could be used as dietary supplements and for pharmaceutical preparations².

Protein molecules in aqueous solution are surrounded by a hydration shell, which is composed

of water molecules attached to the protein surface. If an organic molecule is present, the solvent molecule tends to displace the water molecules both in the hydration shell and in the interior of the protein thereby distorting the interactions responsible for maintaining the native conformation of the proteins^{3,4}.

Glycerol and other polyhydric alcohols have been shown to affect both dynamic and thermodynamic properties of proteins⁵. These cosolvents enhance protein stability. Stabilizing effect of sucrose stems from preferential hydration of proteins in this medium and that this, in turn, may be related to the increase in free energy of cavity formation by the addition of sucrose to water⁶. It has been proposed that the mechanism of this phenomenon is preferential hydration of the protein, which is exclusion of cosolvent molecules from the protein surface. Various studies on different proteins suggest that the glycerol modifies protein interior in a way, which leads to decrease in protein volume and compressibility⁷. Stabilization effects are due to solvent medium effects

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or to the alteration of the water structure in the solvent. This view seems to explain why protein stabilization is generally enhanced with increasing concentration of polyhydric alcohols. The preferential hydration of protein in aqueous glycerol must be due to repulsive forces between glycerol and non-polar regions located on the protein surface⁸. Since glycerol is essentially hydrophilic and that glycerol may penetrate on to the solvation sheath of protein through delicate balance between repulsion from non-polar regions and attraction from polar regions of the protein surface. The stability of proteins is also temperature dependent. A variety of attempts have been made to elucidate the nature of thermostabilization of proteins. Effect of cosolvents on the thermostability of the proteins is also studied by others⁹⁻¹². The basic and fundamental observation in the stabilization of macromolecules in a three-component system has a great potential for stabilization of enzyme and proteins¹³⁻¹⁵. The behaviour of certain proteins in polyols underlines a very fundamental concept of preferential exclusion and preferential interaction of such cosolvents with these macromolecules. The thermal stability, conformation and aggregation of globular proteins depend on the concentration and type of cosolvents present in the surrounding aqueous phase¹⁶.

In this paper, the interaction of solvent components with β -Lg in aqueous solutions of some polyhydric alcohols, glycerol, sorbitol, and sucrose has been measured by densitometric method, with the application of multicomponent theory, supported with the data of degree of hydrolysis, Circular Dichroism, Fluorescence measurements and UV-Difference spectra.

Materials and Methods

The chemicals used were: sucrose, D-sorbitol, glycerol, TNBS (Trinitro benzene sulphonic acid), and α -chymotrypsin from Sigma Chemicals Co, MO St Louis, USA and used without further purification. All other chemicals were of analytical grade. Triple glass distilled water was used throughout the experiments.

Isolation of β -Lg

β -Lg was isolated from defatted bovine milk in accordance with the procedure of Fox *et al*¹⁷. A local breed of cow was selected and milk was always freshly collected. Whey was prepared from bovine

milk collected from the local breed of cow. One litre of milk was used. The pH of the milk was adjusted to 4.6 using 1N HCl. The precipitate was removed by centrifugation at 10000xg for 30 min at 4°C. The supernatant obtained was the whey. To 500 mL of whey 15 g of TCA was added and kept for 30 min. This was centrifuged at 8000xg for 30 min at 4°C. To the filtrate 0-60% ammonium sulfate was added with stirring in the cold room and kept for 1 h. The precipitate was separated by centrifugation at 8000xg for 30 min at 4°C. The precipitate was dialyzed against triple distilled water for 24 h at 4°C. The dialyzed sample was lyophilized and stored at 2-8°C. The yield of β -Lg from whey was approximately 1.2g/L of whey. The homogeneity was established by SDS-PAGE¹⁸ and amino acid composition, which had 99% correlation with the literature values. $E^{1\%}$ at 280 nm of β -Lg was 9.5.

Fluorescence Emission Spectra

Protein solution of concentration 2.7×10^{-6} M containing different cosolvents was used for fluorescence measurements using Shimadzu Spectrofluorimeter (model RF-5000) at 25°C. The protein in phosphate buffer (0.02 M; pH 7.9) was used as the control. The cosolvents of various concentrations required in the experiments were dissolved in the phosphate buffer. The protein was excited at 280 nm and after 10 sec the emission was scanned from 300-400 nm. Slit width was maintained at 5 nm for excitation and 10 nm for emission. Corrections were applied both for inner filter effect for protein concentration and solvent effect¹⁹.

UV-Difference Spectra

UV-Difference spectra with respect to protein solution without cosolvents were recorded using double beam Shimadzu UV visible recording spectrophotometer in the wavelength region of 370-250 nm at 25°C. The base line was obtained using a reference solution in matched quartz cuvettes. Solutions having a final concentration of 2.7×10^{-5} M and containing desired concentrations of cosolvents were prepared and incubated at 25°C for 4 h before recording UV difference spectra.

Partial Specific Volume of β -Lg

For the partial specific volume studies, β -Lg was dissolved in triple glass distilled and deionized water and dialyzed against the same for 36 h at 8°C. The

proteins were centrifuged at 6000 \times g for 30 min and the clear supernatant was lyophilized. Apparent partial specific volume Φ of β -Lg was determined by measuring densities of protein solutions in the presence of cosolvents at 20 \pm 0.05 $^{\circ}$ C²⁰.

The densities of the solvents and of the protein solutions were determined on a precision density meter DMA-55 (Anton paar, Gratz, Austria)²¹. The apparent partial specific volume ' Φ ', was calculated from the obtained density data using the following equation,

$$\Phi = 1/\rho_0 (1 - (\rho - \rho_0)/C) \quad \dots (1)$$

where, C is the concentration, ρ is the density of the protein solution and ρ_0 is that of the solvent (g/mL). The densities of the protein solutions were measured at 20.00 \pm 0.05 $^{\circ}$ C at conditions such that the molality of the solvent composition and the chemical potential are kept in turn, identical in the solvent and in the protein solution. The apparent partial specific volumes of the protein, at the isomolal conditions and at the isopotential conditions, were measured as a function of protein concentration, and extrapolated to zero protein concentration to obtain partial specific volumes at isomolal condition (Φ_2°), and at isopotential condition ($\Phi_2^{\prime\circ}$), respectively.

Preferential Interactions

The preferential interaction parameter (ξ_3)(g/g), for this three-component system was calculated from the following equation,

$$\xi_3 = (\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3} = 1/\rho_i (\Phi_2^{\circ} - \Phi_2^{\prime\circ}/1 - V_3 \rho_3) \quad \dots (2)$$

where ρ_3 is density of the third component (cosolvent), ρ_i are the densities of the solvent/protein solutions and T is the thermodynamic temperature, g_i is the concentration of component, i, in grams per gram of water, and μ is its chemical potential, and V_3 is the partial specific volume of the component 3.

The preferential interaction parameter in mole/mole basis was calculated from the following equation,

$$(\delta m_3/\delta m_2)_{T, \mu_1, \mu_3} = M_2/M_3 (\delta g_3/\delta g_2)_{T, \mu_1, \mu_3} \quad \dots (3)$$

where m_i is the molal concentration of component i, Where M_2 is the molecular weight of the protein and M_3 is the molecular weight of the cosolvent.

The values of ϕ were plotted as a function of protein concentration and the value extrapolated to infinite dilution and was the partial specific volume of the protein, V_{app} . In preferential interaction measurements, two types of apparent specific volume were measured. The first (Φ_2°) was measured under the conditions at which the molal concentration of diffusible component is kept identical in the solvent and the solutions. The second ($\Phi_2^{\prime\circ}$) was measured under the conditions at which the chemical potential of the component 3, is kept constant between the solution and the reference solvent, which can be attained to a close approximation by dialyzing the protein solution against the solvent. Here the components 1, 2 and 3 are water, protein and the cosolvent, respectively, following the standard notations^{22, 23}.

Hydrolysis of β -Lg with α -Chymotrypsin

β -Lg was hydrolyzed using α -chymotrypsin in the ratio 1:100 at 37 $^{\circ}$ C, pH 7.9 in 0.02 M phosphate buffer. The samples were withdrawn at particular intervals of time and checked for degree of hydrolysis. The degree of hydrolysis was calculated for the protein in 20% cosolvent concentration. The protein was dissolved in various cosolvents and hydrolyzed and the samples withdrawn at particular intervals of time.

Degree of Hydrolysis

The degree of hydrolysis was measured by TNBS method²⁴. The TNBS reaction was carried out as follows: 0.250 mL of the sample containing in between 0.25 \times 10⁻³ and 2.5 \times 10⁻³ aminoacqui/L, was mixed in a test tube with 2.00 mL of phosphate buffer at pH 8.2. Two milliliters of TNBS solution was added and the test tube shaken and placed in water bath at 50 $^{\circ}$ C for 60 min. During incubation the test tubes and the water bath must be covered with aluminium foil because the blank reaction is accelerated with the exposure to light. After 60 min, 4.0 mL of 0.1 N HCl was added at room temperature to terminate the reaction. Absorbance was taken after 30 min and read against water at 340 nm. The reaction in the blank and the standard solution was carried out by replacing the sample with 1% SDS and 1.5 \times 10⁻³ M L-Leucine in 1% SDS. The absorbances of the blank and the standard were determined as the averages of three individual determinations.

Far UV-Circular Dichroic Spectra

Far UV Circular dichroism measurements were performed at 25°C in a Jasco J-810 Spectropolarimeter equipped with a Xenon arc lamp. The far UV-CD measurements were made from 200-260 nm with 1 mm path length in a quartz cell, at a protein concentration of 0.2 mg±0.05 mg/mL (5.2×10^{-6} M) in phosphate buffer 0.02 M, pH 7.9²⁵. The slit width was 1 nm. The protein solution was dialyzed against various concentrations of glycerol and sorbitol (10 to 30%) for 12 h in cold and centrifuged at 6000×g for 30 min to obtain a clear solution. The rotations were converted to molar ellipticity values, with a mean residue weight of 113 calculated from the amino acid composition of β-Lg²⁶. The analysis of the data for the secondary structural elements were done^{27, 28}.

Measurement of T_m of β-Lg

Thermal denaturation profile of β-Lg in different buffer conditions and different concentrations of third component (polyhydric alcohols) were measured using Cary-100 Bio-UV-Visible spectrophotometer (Varian INC, Australia). β-Lg in buffer solution of 1 mg/mL concentration was equilibrated with respective concentration of cosolvents. The UV absorbance at 287 nm was followed in the temperature range of 25-90°C by 1°C increment in temperature. Apparent thermal transition temperature and other thermodynamic parameters were calculated²⁹. The fraction unfolded was calculated using the formula:

$$F_D = (A_T - A_N) / (A_D - A_N) \quad \dots (4)$$

where,

F_D is the fraction of the protein unfolded.

A_N is the absorbance of the protein sample at 25°C.

A_D is the absorbance of the protein sample at 90°C.

A_T is the absorbance at different temperature from 25°C - 90°C.

The temperature at which F_D is 0.5 is taken as the apparent melting temperature of the protein.

From the van't Hoff plot the standard enthalpy (ΔH°) and entropy change (ΔS°) for thermal unfolding of the protein in buffer and in the cosolvent were calculated²⁹. The slope of van't Hoff plot gives the ΔH° . From this ΔS° was calculated using the formula.

At transition temperature, $\Delta G = 0$,

$$\Delta G = \Delta H - T\Delta S \quad \dots (5)$$

using this equation, ΔS was calculated.

Results and Discussion

The concentration dependence of apparent partial specific volume of β-Lg in 0.02 M phosphate buffer (pH 7.9) is shown in Fig. 1a. The extrapolated isomolal and isopotential values at zero protein concentration were 0.743 ± 0.001 mL/g and 0.744 ± 0.001 mL/g, respectively. Similar plots of apparent partial specific volumes versus β-Lg concentrations were obtained in three different cosolvents namely glycerol, sorbitol and sucrose. In all the cases, the apparent partial specific volume was found to be independent of protein concentration under both isomolal and isopotential conditions. A representative plot is shown in Fig. 1b. The extrapolated values were calculated in all the solvent concentrations.

The preferential interaction parameters were calculated for these cosolvents from the partial specific volumes (Table 1). In all the cases, (ξ_3) values were negative suggesting preferential exclusion of the solvent components from the domain of the protein molecule, the protein is said to be preferentially hydrated. In the two states, the equilibrium between the native and denatured states of the protein was shifted towards the native and

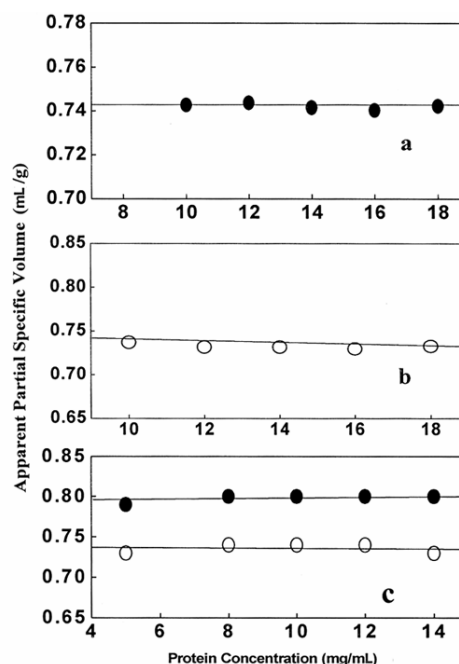


Fig. 1—Apparent partial specific volume of β-Lg in: (a) Buffer (Isomolal), (b) Buffer (Isopotential), & (c) 20% sucrose. (○) Isomolal, (●) Isopotential (Representative plot of β-Lg in sucrose). Phosphate buffer of 0.02 M, pH 7.9. Cosolvent dissolved in the same phosphate buffer.

stable state of the protein. The value of preferential interaction was highest in case of 20% glycerol being -0.213 ± 0.03 g/g and the lowest being -0.010 ± 0.005 g/g in 10% glycerol. In sorbitol, the value of preferential interaction was lowest at 10% sorbitol being -0.043 ± 0.01 g/g, and highest being -0.140 ± 0.02 g/g at 40% sorbitol. In sucrose, the value was the least at 10% being -0.020 ± 0.05 g/g and highest at 30% being -0.190 ± 0.03 g/g.

In the case of both sucrose and sorbitol, the (ξ_3) value reached to maximum at 40% solvent concentration. On the other hand, in the case of

glycerol, the (ξ_3) value increased to maximum at 20% of solvent concentration and gradually decreased with the increase in solvent concentration. The results from the densitometer measurements showed that the surface properties of proteins contribute to the extent of hydration by these cosolvents.

The effect of cosolvents on the extent of change in the microenvironment of tryptophan residues has been monitored by intrinsic fluorescence measurements. It will be evident from this, whether the tryptophan groups of β -Lg are experiencing either a more polar or non-polar environment in the presence of

Table 1—Preferential interaction parameters of β -Lg as a function of cosolvents**

Conc. (%) (W/V)	Φ_2°	$\Phi_2'^\circ$	ξ_3 (g/g)	$\partial \xi_3 / \partial g_2$ (g/g)	$\delta m_3 / \delta m_2$ (mol/mol)
Buffer*	0.743 ± 0.001	0.744 ± 0.001	—	—	—
Glycerol					
10	0.723 ± 0.002	0.725 ± 0.001	0.108	$-0.010 \pm .005$	-2 ± 1
20	0.719 ± 0.003	0.762 ± 0.001	0.240	-0.213 ± 0.03	-43 ± 7
25	0.741 ± 0.001	0.774 ± 0.002	0.330	-0.167 ± 0.05	-33 ± 7
30	0.722 ± 0.001	0.748 ± 0.001	0.390	-0.140 ± 0.04	-28 ± 3
40	0.770 ± 0.002	0.783 ± 0.001	0.580	-0.069 ± 0.02	-14 ± 2
Sorbitol					
10	0.751 ± 0.002	0.765 ± 0.001	0.107	-0.043 ± 0.01	-5 ± 1
20	0.713 ± 0.001	0.733 ± 0.002	0.232	-0.066 ± 0.05	-13 ± 3
30	0.758 ± 0.002	0.793 ± 0.001	0.375	-0.117 ± 0.03	-12 ± 4
35	0.795 ± 0.001	0.830 ± 0.001	0.460	-0.126 ± 0.02	-13 ± 5
40	0.715 ± 0.002	0.754 ± 0.002	0.546	-0.140 ± 0.02	-14 ± 3
Sucrose					
10	0.740 ± 0.002	0.748 ± 0.002	0.106	-0.020 ± 0.05	-1 ± 0.05
20	0.736 ± 0.002	0.790 ± 0.001	0.160	-0.150 ± 0.02	-9 ± 1
30	0.738 ± 0.001	0.798 ± 0.002	0.230	-0.164 ± 0.05	-10 ± 1
35	0.760 ± 0.002	0.828 ± 0.001	0.370	-0.190 ± 0.04	-10 ± 1
40	0.780 ± 0.002	0.842 ± 0.002	0.530	-0.190 ± 0.03	-10 ± 1

*Phosphate buffer 0.02 M, pH 7.9, 20°C; All data are the mean \pm SD of triplicates.

**Cosolvents in buffer

cosolvents. In Fig. 2a, the fluorescence emission spectra of β -Lg in the presence of varying concentrations of sucrose are shown. As seen in Fig. 2b, the fluorescence intensity shows slight increase in the intensity with the concentration of sucrose from the control. As also observed there is no shift in the emission maxima. The same spectra were reproducible with glycerol and sorbitol with decrease in the fluorescence intensity without change in emission wavelength.

The results indicated a change in the emission intensity with various concentrations of cosolvents. This indicated a modification of microenvironment of tryptophan residues in the presence of cosolvents. But there was no shift in the emission maxima indicating no structural changes of the protein in presence of cosolvents. Similar results were obtained in all the cosolvents used. The

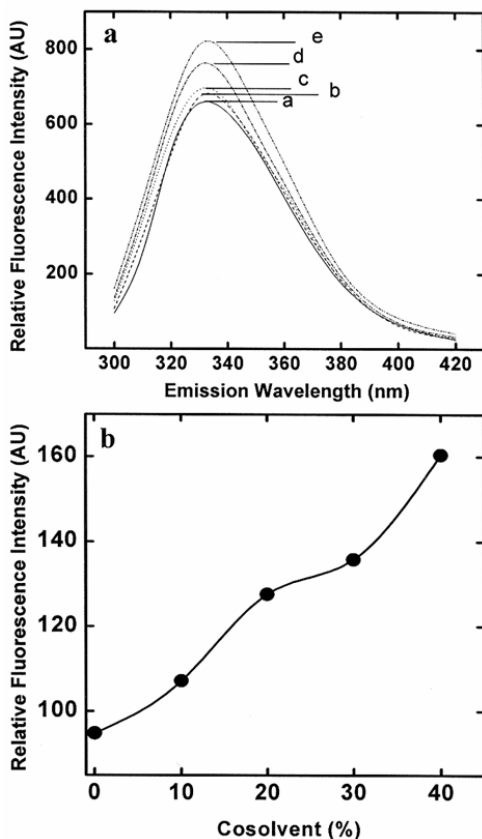


Fig. 2—a, Fluorescence spectra of β -Lg in presence of various concentrations of sucrose: (a) control, (b) 10% sucrose, (c) 20% sucrose, (d) 30% sucrose, & (e) 40% sucrose (Representative plot). Sucrose dissolved in phosphate buffer of 0.02 M, pH 7.9; b, Fluorescence spectra of β -Lg as a function of cosolvent concentration. Protein was dissolved in various concentrations of sucrose in phosphate buffer of 0.02 M, pH 7.9.

perturbations of aromatic chromophores in solvents such as sucrose alter the absorbance of the aromatic chromophores, and have calculated the extent of such perturbations on UV-Difference spectra^{30,31}. Model compounds, L-Tryptophanyl ethyl ester show changes in the fluorescence spectra at 334 nm in the presence of cosolvents. This may be one reason why fluorescence intensity of β -Lg changes in the presence of cosolvents without a shift in the emission maxima. The solvent viscosity has a greater effect on protein dynamics³². The high viscosity is the dominant factor, which reduces the conformational changes in myoglobin³³. The difference in viscosity of sorbitol and glycerol causes the variation in the emission intensity of the protein.

The UV-Difference spectra of β -Lg in varying concentrations of cosolvents also showed increase in the intensity without changes in the absorption maxima, in the UV absorption, with the increasing concentrations of cosolvents. There were no changes in the absorption maxima either (Graph not shown).

The secondary structure values were calculated from CD spectra of β -Lg in phosphate buffer (Table 2). The control protein had $24 \pm 1\%$ α -helix, $50 \pm 1\%$ β sheets and $26 \pm 2\%$ aperiodic structures. The values did not show significant changes in the secondary structure data from the control values in the presence

Table 2—Secondary structural content of β -Lg as a function of cosolvents

Cosolvents	α - Helix (%)	Beta Sheets (%)	Aperiodic (%)
Buffer*	24 ± 1	50 ± 1	26 ± 2
Sorbitol (%)			
10	25 ± 1	49 ± 1	26 ± 2
20	26 ± 1	46 ± 1	28 ± 1
30	25 ± 1	42 ± 2	33 ± 1
Glycerol (%)			
10	23 ± 1	48 ± 1	29 ± 1
20	24 ± 1	48 ± 1	28 ± 1
30	26 ± 1	48 ± 2	26 ± 1
Sucrose (%)			
10	25 ± 1	50 ± 1	25 ± 2
20	20 ± 1	49 ± 1	31 ± 1
30	23 ± 1	49 ± 1	28 ± 1

*Phosphate Buffer 0.02 M, pH 7.9

All data are the mean \pm SD of triplicates

of sucrose, sorbitol and glycerol as seen in Table 2. There was no considerable change in the α -helix content in the presence of these cosolvents. Helix element appears to be a dynamic domain to increase adiabatic compressibility³⁴. These results conclude that the protein did not show any structural modifications in the presence of various cosolvents used in this study. This clarifies the fact that the changes in the fluorescence spectra were due to the conformational fluctuations and not due to the structural changes in the molecule. The far UV spectra did not show any changes (Fig. 3). Glycerol protects the tertiary structure of protein against aggregation as reported in the case of rabbit creatine kinase³⁵. Polyols restrict the conformational changes by forming hydrogen bonds with the surface groups of the protein. Such interactions would preserve the native conformation to protect the buried groups as described for lysozyme³⁶. Polyols strengthen the bridge interaction between polypeptide to stabilize the triple helix replacing water³⁷.

The degree of hydrolysis of β -Lg with α -chymotrypsin in phosphate buffer has been shown in Fig. 4 as 18%. The degree of hydrolysis of β -Lg in glycerol is shown in Fig. 4a. The value decreased to 12% in this cosolvent with the enzyme. The value for degree of hydrolysis of the protein in sorbitol also

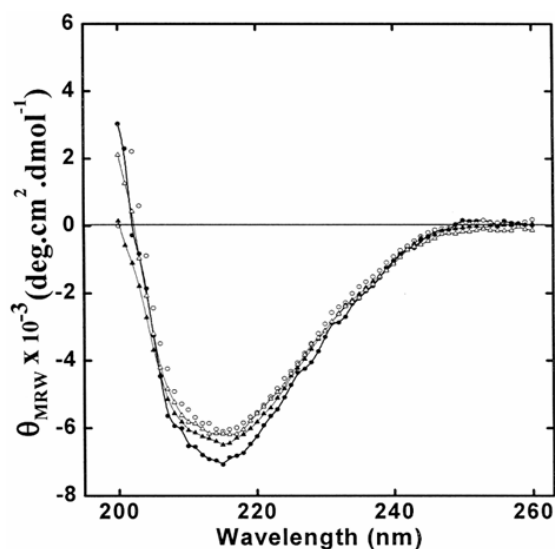


Fig. 3—Far UV-CD spectra of β -Lg in the presence of sucrose (Representative plot of β -Lg in presence of sucrose). The protein dissolved in various concentration of sucrose in phosphate buffer. Temperature maintained at 25°C. A 1 cm pathlength cell used. A protein concentration of 0.3 mg/mL used in all the experiments. (●) Control in buffer (○) 10% sucrose, (▲) 20% sucrose, (△) 30% sucrose.

decreased to 12%, (Fig. 4b). But in case of sucrose, the degree of hydrolysis increased by 3% from the control value (Fig. 4c).

An important aspect of milk protein is protease digestibility. Such a property has been used as a measure of the flexibility of proteins by assuming that the flexible proteins would be more easily digested by proteases. The high hydrophobic proteins such as BSA and β -Lg are susceptible to proteases. The hydrophobic proteins seem to cause an unstable conformation, which increases flexibility of proteins³⁸. Since flexible proteins are more

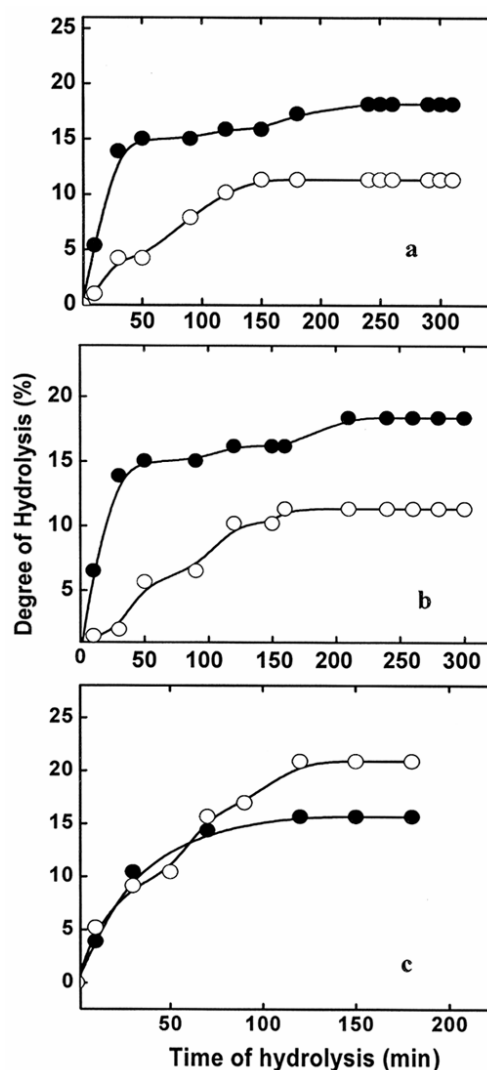


Fig. 4—Degree of hydrolysis of β -Lg with α -chymotrypsin in: (a) 20% glycerol, (b) 20% sorbitol, (c) 20% sucrose. Sucrose dissolved in phosphate buffer of 0.02 M, pH 7.9, and at temperature of 37°C. Enzyme to protein ratio used 1:100. Samples withdrawn at various time intervals and degree of hydrolysis estimated by TNBS method.

susceptible to proteases than rigid proteins, the protease susceptibility method seems to be promising in detection of protein flexibility. Protein susceptibility was positively correlated to adiabatic compressibility, which shows large internal flexibility³⁹. In these studies, flexible proteins are assumed to be more easily digested by proteases than rigid ones. Internal cavities have been associated with conformational flexibility⁴⁰. Importance of flexibility has been pointed out earlier^{41,42}. Moreover solvent addition modulates biochemical reactions, if during the course of the reaction; there is a change in the preferential interactions of solvent components with the reacting system⁴³.

Globular proteins undergo considerable volume fluctuations⁴⁴. Conformational fluctuations play an important role in the structural stability of the globular proteins. Glycerol modifies the protein interior in a way⁷, which leads to decrease in protein volume and compressibility. Glycerol affects protein interior, which is measured by apparent partial specific volume. Glycerol dehydrates the protein by means of displacing some hydration water and with preferential hydration. Thus, it is plausible to conclude that the glycerol induced reduction in the protein volume and compressibility is due to the release of water. This leads to the enhanced intermolecular bonding, which locally rigidifies the protein. The tendency of water to leave the protein interior in the presence of glycerol could be the result of it being squeezed out by the compression of the protein⁴⁵, following the process of reducing the surface area of contact of the protein with the solvent.

The results obtained with α -chymotrypsin digestion of β -Lg in the presence of glycerol and sorbitol further supports the fact that the cosolvents rigidify the protein, which offers the protein, protection against enzymatic attack. Similar results were obtained when β -Lg was hydrolyzed by the protease in the presence of glycerol and sorbitol. But in the presence of sucrose the result was opposite. The results from fluorescence spectra in the presence of sucrose show some perturbations in the protein molecule. So it can be concluded that sucrose makes the protein more flexible for enzymatic attack.

The stabilization of β -Lg by different cosolvents to different extents is evidenced by apparent thermal denaturation temperature measurements of the protein at 287 nm over the range of 25-90°C. The thermal denaturation curve of β -Lg in the presence of sucrose

is shown in Fig. 5. The van't Hoff plot of β -Lg in the presence of sucrose is shown in Fig. 6. The standard enthalpy (ΔH°) could be obtained from the slope of van't Hoff plot. The standard enthalpy (ΔH°) and entropy change (ΔS°) at the transition temperature were calculated. The ΔH° for β -Lg in buffer was calculated and found to be 26 Kcal/mol and ΔS° was 400 cal/mol degree. Both the parameters tend to increase with increasing concentration of cosolvents. In the case of sucrose (representative plot), ΔH° was 58 Kcal/mol and that of ΔS° was 846 cal/mol degree. Maximum stabilization was observed with glycerol when compared to sorbitol or sucrose (Table 3).

These results suggest that the cosolvents exert a stabilizing effect on β -Lg at various concentrations. Preferential hydration restricts the thermal motions in the molecule. This is reflected by the increased thermal stability of β -Lg in cosolvents. The thermal transition temperature shifted from 65°C to various higher levels being highest of 88°C in 10% glycerol. From the partial specific volume, it is clear that the polyols induce structuring of water around the protein molecules. This additional hydration shell protects the protein from thermal denaturation. The extent of stabilization by the cosolvents depends on the nature of the cosolvent used. Sugars are found to act as stabilizers at elevated temperature. This stabilization may be attributed to the strengthening of the hydrophobic interactions, which makes the protein more rigid, and resist thermal unfolding^{46,47}.

Presence of glycerol could help the protein prevent against heat induced unfolding. Glycerol could efficiently prevent the change of the native secondary structure induced by heat. Thus, glycerol maintains the structural stability of the molecule. The polyols induced transition in the melting temperature is more effective for glycerol than sorbitol. Unfavourable interaction of polyols with non-polar amino acid residues has resulted in exclusion of polyols from protein surface. Thus, glycerol stabilizes by strengthening hydrophobic interactions by overcoming the electrostatic interactions between charged residues⁴⁸. So stabilization by glycerol is by a delicate balance between repulsion of non-polar regions, attraction of the polar region on the protein surface and interaction of glycerol and water could lead to an enhancement of internal hydrophobic interaction⁴⁹.

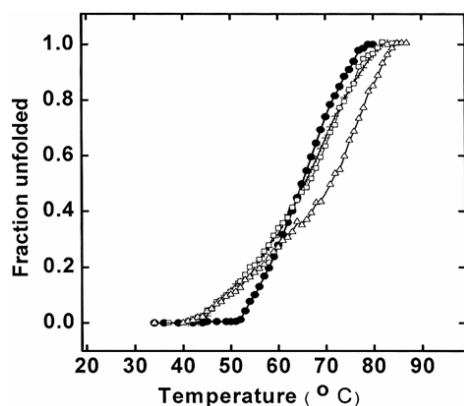


Fig. 5—Thermal denaturation profile of β -Lg in presence of cosolvent as monitored at 287 nm as a function of temperature. (Representative plot of β -Lg in presence of sucrose). The change in absorbance monitored from 25°C-90°C. The increment in temperature 1°C per min. (●) Control in buffer (□) 10% sucrose (×) 20% sucrose (Δ) 40% sucrose. The protein was dissolved in various concentrations of sucrose in phosphate buffer of 0.02 M, pH 7.9. Protein concentration of 1mg/mL used.

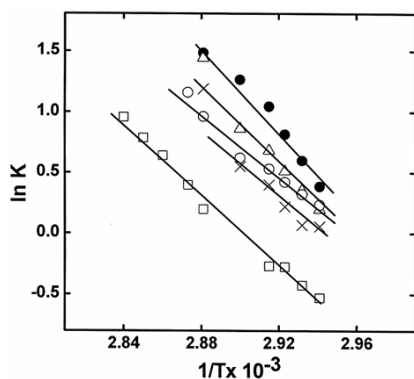


Fig. 6—The van't Hoff plot for the thermal denaturation curve of β -Lg in presence of cosolvents. (Representative plot of β -Lg in presence of sucrose). (●) Control in buffer (□) 10% sucrose (×) 20% sucrose (○) 30% sucrose (Δ) 40% sucrose. The protein dissolved in various concentrations of sucrose in phosphate buffer of 0.02 M, pH 7.9. Protein concentration of 1 mg/mL used.

Table 3—Apparent T_m of β -Lg as a function of cosolvents

Cosolvents Conc. (%) w/v	Apparent T_m (°C)		
	Glycerol	Sucrose	Sorbitol
Buffer*	65 ± 2	65 ± 2	65 ± 2
10	88 ± 3	66 ± 1	66 ± 1
20	87 ± 2	67 ± 1	67 ± 1
30	85 ± 1	67 ± 1	68 ± 1
40	84 ± 1	71 ± 1	68 ± 1

* Phosphate Buffer 0.02M, pH 7.9

All data are the mean ± SD of triplicates

The nature of both the proteins and polyols are important in governing the thermal stability of the protein. An increase in surface tension of the medium in the presence of polyols appears to be the dominant factor. A delicate balance of interactions between the surface hydrophobic, hydrophilic and charged residues with water and the ability of polyols to alter the physico-chemical properties of water plays a significant role⁵⁰. Sugars affect the hydrogen bonded network around the protein molecule. So surface tension is not the only criteria for protein stabilization⁵¹.

Conclusions

Partial specific volume measurements suggest that the flexibility of the protein depends on the type and the concentration of the polyols used. Glycerol and sorbitol makes the protein structure more rigid and gives protection against protease digestion. Sucrose, on the other hand, makes the protein more flexible and prone to protease digestion. All the polyols, in fact, make the protein more stable in solution. Looking at the behaviour of the protein in different concentrations of cosolvents, there can be a unique concentration of the cosolvent, where the protein can be more stable or is being stabilized as compared to the higher concentrations of the cosolvents. Therefore, it is vital to observe that at what concentration of the cosolvent the protein is more stabilized and need not be necessary that only at higher concentrations of cosolvents only the protein is stabilized. Stabilization can also be at lower concentration of the cosolvent depending on the protein and the cosolvents used.

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