

The Viscosity and Opacity of Heated β -Lactoglobulin Solutions: The Effect of Salts, and Oxidizing and Reducing Reagents

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The viscosity of heated β -lactoglobulin solutions is pH-dependent. When solutions are salt-free the increases in viscosity on heating in the pH range 6.2 to 7.5 are relatively slight with a maximum at pH 6.8. In general, the viscosities are increased by salts with the greatest increases at the low pH values. There are, however, specific salt effects; sodium phosphate and citrate prevent the aggregation and concomitant opacity in the low pH region and bring about regular increases in viscosity. Sodium chloride, on the other hand, increases the viscosity but does not prevent opacity at low pH values, and there is a viscosity maximum at pH 6.7 with 0.025 *M* sodium chloride. In general, however, increases in viscosity and opacity are parallel. The increases in viscosity in the presence of salts are greatest with high concentrations of protein indicating a high degree of cross-reaction. The more viscous solutions also show structural viscosity. Treatment of β -lactoglobulin with iodine leads to some increase in the viscosity and clearing of the solutions which is consistent with sulfhydryl, disulfide participation in formation of opaque gels. Very large increases in viscosity are obtained in the presence of excess sulfhydryl reagents. The probable explanation of this increase is the opening of loops formed by disulfide bridges with consequent elongation of the molecule.

Protein solutions denatured by heat or urea become highly viscous or gel, a property which is strongly pH-dependent^{2,3} with maximal gelling near the isoelectric point. The diminished gelling tendency at more remote pH values is viewed as a consequence of the increased electrostatic repulsion, and thereby decreased interaction, between the like-charged molecules.^{2,3} In view of this interpretation of the pH dependence, the presence of salts would be expected to increase the viscosity by reduction of the electrostatic repulsion. The present studies with β -lactoglobulin and chiefly with the salts sodium phosphate and chloride, revealed in general the expected increase in viscosity

and aggregation with increase in salt concentration. There were, however, exceptions to this generalization, apparent at low salt concentrations and low pH values, which indicated that there were also specific salt effects probably due to binding of the salt ions to the protein. The effects of treating β -lactoglobulin with iodine, and mercaptoethanol and other reducing agents before heating, were also studied because of the participation of sulfhydryl and disulfide groups in gel formation.^{2,3}

Materials

β -Lactoglobulin.—This protein was prepared from raw milk by the method of Palmer⁴ and recrystallized once. It was electrophoretically homogeneous at pH 8.6. This material was dried from the frozen state and stored as described previously.⁵

β -Lactoglobulin, Iodine-treated.—Ten milliliters 3.6% β -lactoglobulin in water at pH 7.5 was mixed with 1.0 ml.

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(2) (a) E. V. Jensen, V. D. Hospelhorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950); (b) V. D. Hospelhorn and E. V. Jensen, *THIS JOURNAL*, **76**, 2830 (1954).

(3) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *ibid.*, **75**, 5157 (1953).

of 0.1 *M* sodium phosphate buffer, pH 7.5, and treated with 0.6 ml. of 0.1 *N* iodine in 0.15 *N* KI at 30°. The pH dropped 0.5 unit and was adjusted to 7.5 with NaOH. The molar ratio of iodine to protein is 7, or approximately 2 per sulfhydryl group. In the studies of Jensen, *et al.*,³ with serum albumin the molar ratio was 2.6.

Methods

Viscosity.—The viscosity and other properties of the heated β -lactoglobulin solutions were measured without further adjustment of protein concentration, pH or ionic composition. The viscosity was measured in a Bingham type⁶ viscometer with a variable pressure head. The characteristics of this viscometer have been described previously.⁵ The results are recorded as relative viscosities, compared with water at 30°. The viscosities were determined with pressures of 15 to 50 cm. of water. The precision is $\pm 0.5\%$, with the principal error in the pressure reading. With relative viscosities up to about 3 the product of pressure (centimeters of water) and time (seconds) for a particular sample was constant. For higher viscosities the pressure-time product was not constant, indicating a yield point or structural viscosity. Solutions showing structural viscosity are indicated and viscosities are given for a pressure of 30 cm. of water.

Heating.—The β -lactoglobulin solutions were heated in a constant-level water-bath at 90°, in 18 \times 150 mm. Pyrex rimless test-tubes. Small-flanged test-tubes containing ice-water were placed in the top of the large tubes to prevent the loss of water by evaporation. The volume of solution heated was 6.0 ml. and the time of heating 30 minutes.

pH Values.—The pH values of the β -lactoglobulin solutions with all reagents present were measured at 25°, before and after heating.

Light Absorbance.—Spectrophotometer readings were made in the same tubes used for heating, with a Beckman model B photometer, at 600 m μ . The apparent absorbance readings serve to measure the opacity or gross visible aggregation of the heated solutions.

Results

Effect of pH and Salts on Viscosity of Heated β -Lactoglobulin Solutions.—The effect of pH on the viscosity of heated 1.8% β -lactoglobulin solutions was determined in the pH range of 6.2 to 7.7. The results are shown in Fig. 1 for systems containing sodium chloride or phosphate. The phosphate data are presented at constant ionic strength (μ) rather than molarity, for comparison with the sodium chloride results. The viscosity increases sharply at low pH values when phosphate is present. Sodium chloride on the other hand at low concentrations increases the viscosity at all pH values to about the same extent, but at higher concentrations the greatest increase in viscosity is found at the lower pH values. Sodium sulfate behaved similarly; at a concentration of 0.01 *M* (0.03 μ) the viscosity curve closely paralleled the 0.025 μ sodium chloride curve.

The effect of higher concentration of phosphate on viscosity with several concentrations of β -lactoglobulin is shown in Fig. 2. In this case reduced viscosity (η rel. - 1/concn. of protein) is plotted against concentration of β -lactoglobulin for pH 7.5 and 6.2. It is apparent that the viscosity is not only dependent on phosphate concentration and pH but is also sharply dependent on the protein concentration, particularly at high salt concentration and low pH values. The viscosity at pH 7.5 is depressed slightly by low concentrations of phosphate, an effect that is also shown by citrate.

Effect of pH and Salts on Absorbance of Heated β -Lactoglobulin Solutions.—The decline in vis-

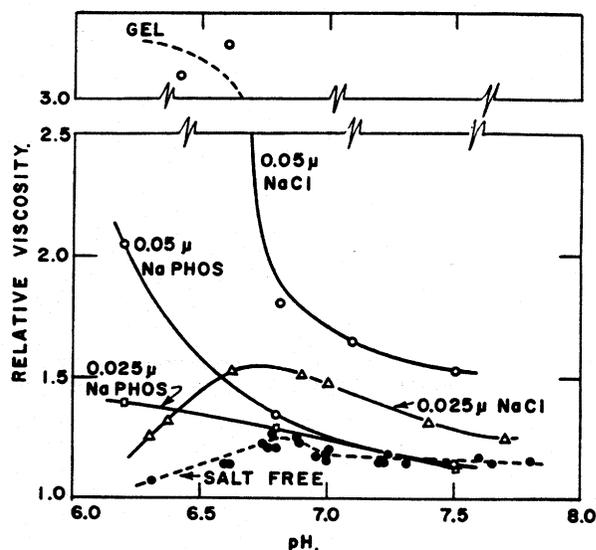


Fig. 1.—Influence of pH on the viscosity of heated β -lactoglobulin solutions, salt-free and with sodium phosphate and chloride present. The results given are for 1.8% β -lactoglobulin. The solutions containing 0.05 μ NaCl show slight structural viscosity at low pH values. For example, at pH 6.4 the viscosity at 30 cm. pressure is 3.26, at 50 cm. pressure it is 5% less.

cosity in salt-free solutions below pH 6.8 shown in Fig. 1 is accompanied by a sharp increase in apparent absorbance, that is, aggregation, as shown in Fig. 3. The presence of phosphate reduces the aggregation in this pH range. Sodium citrate tested at pH 6.2 kept the heated solutions as clear as did phosphate. With sodium chloride, on the other hand, the aggregation at low pH values is almost as great as in salt-free solutions, as is also true of sodium sulfate.

The aggregation, measured by the absorbance, in heated 1.8% β -lactoglobulin solutions with a range of phosphate concentrations is shown in Fig. 4, as well as the results with sodium citrate at pH 7.5. At low pH values the aggregation, compared to salt-free solutions, is decreased by small concentrations of phosphate, although the viscosity increases (Fig. 1). Sodium citrate also has a like effect.

Effect of Oxidizing and Reducing Reagents on Heated β -Lactoglobulin Solutions.—Treating the β -lactoglobulin with iodine did not change its behavior greatly. Solutions heated at pH 7.5 in 0.2 μ phosphate, however, had a relative viscosity about 25% greater than the untreated, and were considerably clearer (Fig. 4). The presence of low concentrations (0.01 *M*) of the sulfhydryl compound mercaptoethanol when β -lactoglobulin solutions were heated had no influence on the viscosity or clarity of the solutions. With high concentrations of this and several related compounds, however, there were striking increases in the viscosity as shown in Fig. 5. Most of these solutions show structural viscosity, that is, the apparent viscosity changes with the pressure, and in these instances apparent viscosities are given for pressures of 30 cm. The magnitude of this effect was evident from a plot of volume/time against pressure for the solu-

(6) E. C. Bingham and R. F. Jackson, *Bur. Stds. Sc. Paper No. 298*, 1917.

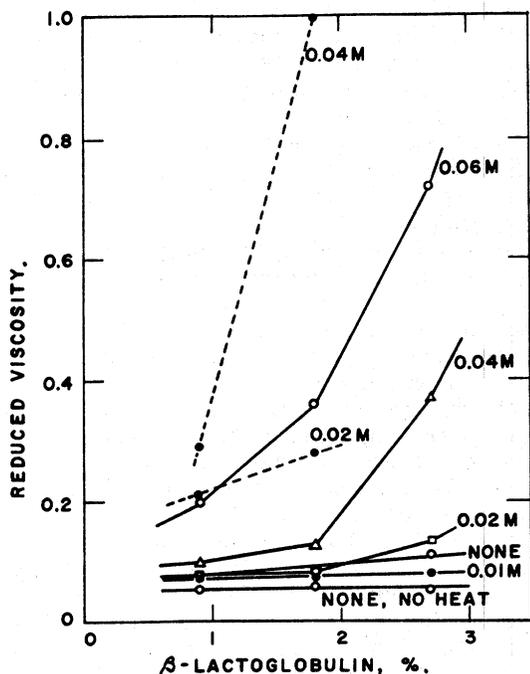


Fig. 2.—Reduced viscosity of heated β -lactoglobulin solutions as a function of protein concentration, sodium phosphate and pH. The sodium phosphate concentration is indicated on the graph (at pH 7.5, $\mu = 2.8 \times M$; at pH 6.2, $\mu = 1.7 \times M$). Data given by the solid lines are for pH 7.5, the dashed lines are for pH 6.2.

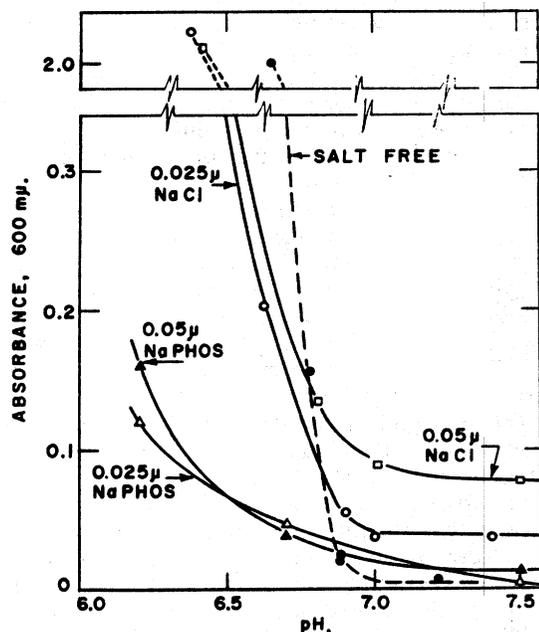


Fig. 3.—Influence of pH on absorbance of heated β -lactoglobulin solutions, salt-free and with sodium phosphate and chloride present.

tion containing 0.24 M mercaptoethanol which gave a yield point (intercept of the pressure ordinate) of 15 cm. At 60 cm. the value for volume/time was 0.04. Comparison of the viscosity of 0.9 and 1.8% β -lactoglobulin solution shows an extreme dependence on the concentration of the protein.

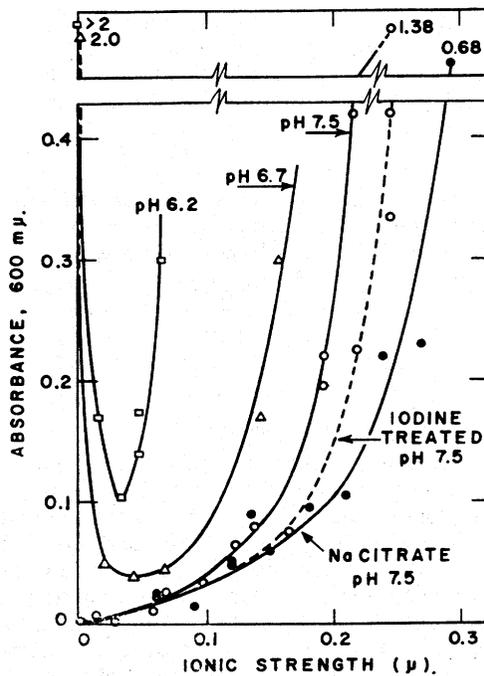


Fig. 4.—Influence of ionic strength on absorbance of heated β -lactoglobulin solutions at several pH values. Concentration of β -lactoglobulin is 1.8%. Where not indicated the solutions contain sodium phosphate.

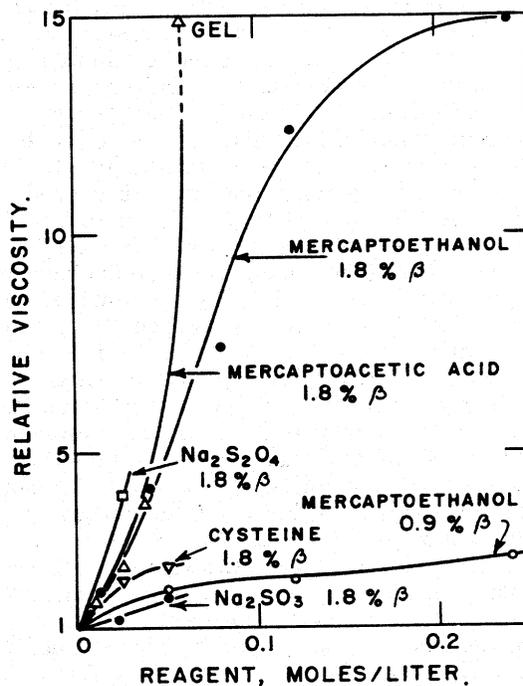


Fig. 5.—Viscosity at 30 cm. pressure of heated β -lactoglobulin solutions containing various reducing agents at pH 7.5. All the solutions, except those containing cysteine and Na_2SO_3 , show structural viscosity. This is marked for the more viscous solutions. For example, the apparent viscosity of 1.8% β -lactoglobulin containing 0.24 M mercaptoethanol is 33% less at 50 cm. pressure.

These solutions are clear, with absorbance readings of 0.1 or less. They also become more viscous when sodium phosphate is present; 2% β -lacto-

globulin with 0.12 *M* mercaptoethanol and 0.025 *M* sodium phosphate forms a clear gel at *pH* 7.5. The effect of sodium phosphate has been studied in some detail with 0.9% β -lactoglobulin as shown in Table I.

TABLE I
INFLUENCE OF MERCAPTOETHANOL AND SODIUM PHOSPHATE ON VISCOSITY OF 0.9% β -LACTOGLOBULIN SOLUTIONS AT *pH* 7.5 HEATED WITH THE REAGENTS PRESENT

Mercaptoethanol, <i>M</i>	None	Sodium phosphate	
		0.025 <i>M</i>	0.050 <i>M</i>
Relative viscosity (30 cm. pressure)			
None	1.07	1.07	1.13
0.12	2.1	3.1	3.1
0.24	2.6	3.1	4.7

Discussion

The viscosities of heated protein solutions, in general, are very much greater than the viscosities of native protein solutions, particularly when salts are present (compare Fig. 2). The heat not only alters the shape of the protein molecule by denaturation with concomitant viscosity increases, but cross reactions between the protein molecules affected by the heat are increased by the presence of salts and are greatest near the isoelectric point. These cross reactions increase the viscosity. The present studies of the effect of *pH* with heated β -lactoglobulin solutions containing phosphate are in general agreement with the behavior of human and bovine plasma albumin reported by Jensen, *et al.*² The extent of the cross reactions which lead to viscosity increases is viewed² as limited by the like electric charges on the molecule since these lead to electrostatic repulsion between molecules. The present results show that, as would be expected, salts increase the viscosity, presumably by reduction of electrostatic repulsion with concomitant increase in cross reaction. This is observed with sodium phosphate, but only above concentrations of 0.02 *M* (Fig. 2), and for sodium chloride at all concentrations (Fig. 1). The maximal effect of salts is exerted where the charge is smallest, that is, toward the isoelectric region. There are, however, specific salt effects particularly apparent at low concentrations (<0.05 μ) of salts, which indicate that the electric charge of the ionic environment cannot explain the total change in viscosity and aggregation. Sodium chloride, for example, does not prevent the aggregation shown by salt-free β -lactoglobulin below *pH* 6.8 (Fig. 3). Sodium phosphate and citrate do prevent the aggregation. In this instance presumably the polyvalent anions are bound leading to increased negative charge and preventing the isoelectric aggregation; simultaneously the viscosity increases. A related example⁵ of a marked effect due to ion binding is shown by heated β -lactoglobulin solutions containing calcium chloride. In this instance binding of the positive calcium ion reduces the net charge on the protein molecule and precipitation at *pH* 7.4 occurs with concentrations of 0.005 *M* or less of calcium chloride.

Other specific salt effects are observed when β -lactoglobulin solutions containing dilute salts are heated. The nature of the observed drop in viscosity at *pH* 7.5 with low concentrations of sodium

phosphate (Figs. 1 and 2) and citrate is not clear. It too may be due to the binding of the polyvalent anions for all concentrations of sodium chloride at this *pH* increase the viscosity. Heated salt-free solutions of β -lactoglobulin aggregate at the lower *pH* values, as shown by the great increase in absorbance (Fig. 3). Simultaneously a reduction in viscosity occurs which may indicate that the aggregates are compact and symmetrical. Aggregation of β -lactoglobulin heated in the presence of low concentrations of calcium chloride also is accompanied by a reduction in viscosity⁵ and calcium chloride both aggregates and reduces the viscosity of unheated casein solutions (unpublished studies).

The use of reagents that oxidize or combine with the sulfhydryl groups of proteins has shown that these groups participate in protein gel formation.^{2,3} In the case of heated proteins² the gels are clearer and firmer after treatment with sulfhydryl reagents, leading to the conclusion that the sulfhydryl groups participate in lateral association with opaque gel formation. Other groups, perhaps principally hydrogen-bond forming, form the three-dimensional clear gels. The influence of iodine treatment on β -lactoglobulin gels fits this picture, although the effects appear to be less striking than with heated plasma albumin.²

Treatment of β -lactoglobulin with a sulfhydryl compound like mercaptoethanol, in slight excess of equivalence, had no detectable effect on the viscosity or appearance of heated β -lactoglobulin solutions. When excess amounts of these and similar reagents were added, as shown in Fig. 5, large increases in viscosity resulted and the solutions remained clear. Presumably with excess of the reagent, loops of the β -lactoglobulin molecule formed by disulfide (four disulfide and four sulfhydryl groups are present in β -lactoglobulin⁷) were opened to give a greatly elongated molecule, as Kauzmann, *et al.*,⁸ have postulated for the effect of sulfhydryl compounds on serum albumin in urea. The effect with heated β -lactoglobulin is much more striking than for serum albumin in urea, and the viscosity is sharply dependent on the concentration of β -lactoglobulin. Greater increases in viscosity might be expected with heat as the denaturant than with urea. Elongation of the protein molecule by excess reducing agent would lead to greater opportunity for hydrogen bonding but this bonding would not occur in urea. Cross bonding is also evident from the structural viscosity of these solutions. The effect of sodium phosphate on this reaction (Table I) suggests that the phosphate not only functions to reduce electrostatic repulsion, but that it also enhances the reduction by mercaptoethanol. The viscosity of 3.1 attained in 0.12 *M* mercaptoethanol and 0.025 *M* phosphate is not increased by doubling either reagent but, if both reagents are doubled, the viscosity increases to 4.7 with a considerable increase in structural viscosity (these viscosity values of 3.1 and 4.7 at 30 cm. pressure are 3.4 and 6.6, respectively, at 20 cm. pressure).

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