

VISCOSITY AND FLOCCULATION OF HEATED β -LACTOGLOBULIN SOLUTIONS: EFFECT OF CALCIUM CONCENTRATION AND pH

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A full knowledge of the effect of heat on milk is important for the understanding and control of the physical properties of heat-processed milk. The factors involved in the instability of milk to heat, including the time of heating and the influence of various salts, have been studied intensively (17). Since milk is a complex substance, it appears desirable to amplify and interpret the data accumulated on it by a supplemental study of its individual components. Milk proteins, because of their lability, concentration, and diverse and numerous reactive groupings, deserve first consideration. It is expected that the proteins in heated milk will interact with other components of milk. It is important, therefore, to study the interaction of the various components.

This paper describes the interaction of two important components of milk, β -lactoglobulin and calcium, when heated within a range of calcium concentrations and pH values. The β -lactoglobulin has also been modified with a number of reagents in order to obtain information about the nature of the calcium effect. Light transmission, viscosity, gelling, and precipitation have been determined on the heated solutions. The results obtained are discussed in relation to the physical changes observed when milk itself is heated.

PROCEDURE

MATERIALS

β -lactoglobulin. This protein was prepared from raw milk by the method of Palmer (12) and recrystallized once. It was electrophoretically homogeneous at pH 8.6. One preparation was dried in the frozen state after dialysis against dilute ethanolamine (about 0.2 ml. per 10 l.) at pH 7.0. A second preparation was dried in the frozen state in the crystalline, isoelectric condition. This material was compact and easier to weigh than the fluffy material obtained when the solution at pH 7.0 was dried; when used, it was adjusted to pH 7.0 with ethanolamine to be comparable with the first preparation. Other pH adjustments of both preparations were made with HCl and NaOH. The dry preparations were stored in a desiccator at a relative humidity such that the moisture content was 10.0%. The concentrations given in the results are for the moisture-free product.

For some experiments the β -lactoglobulin was modified, particularly with reagents affecting the amino groups. The following reagents were used in the manner indicated.

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a) *Formaldehyde*. To 10.0 ml. of 4.5% β -lactoglobulin at pH 7.7 were added 3.8 ml. of 36.6% formaldehyde, reducing the pH to 5.9. The mixture was held 2 or 18 hours at 25° C. and finally dialyzed for 18 hours against water to remove the unreacted formaldehyde. The pH was raised to 7.5 and suitably diluted for the experiments with calcium. An estimation of the isoelectric point of heated 1.8% β -lactoglobulin solutions by adjustment of the pH to maximum precipitation showed that untreated β -lactoglobulin precipitated at pH 5.5, close to the expected pH of 5.2; the formaldehyde-treated solution precipitated at pH 5.0.

b) *Nitrous acid*. 0.9 g. of β -lactoglobulin was dissolved in 12.5 ml. 0.5 *M* acetate buffer, pH 4.3. To this were added 12.5 ml. of 2 *M* sodium nitrite. After the reaction had proceeded for 45 minutes at 25° C., the mixture was dialyzed against water for 18 hours. The β -lactoglobulin was precipitated from this solution with 3.2 *M* ammonium sulfate and dialyzed again. The solution was finally adjusted to pH 7.5 for the calcium experiments. A dry-weight determination established that no protein was lost in this procedure. An isoelectric point estimated from the maximum precipitation of a heated solution was at pH 4.7.

METHODS

pH values. The pH values of the β -lactoglobulin solutions with all reagents present were measured at 25° C., before and after heating. The pH values at and above 7.0 dropped slightly on heating, sometimes several tenths of a unit. Below pH 7.0 the pH values rose several tenths on heating. The pH values after heating are given in the data.

Light transmission. Light transmission was measured in a Beckmann model B spectrophotometer at 600 $m\mu$ in the same tubes in which the solutions were heated.

Heating. The β -lactoglobulin solutions were heated in a constant-level water bath at 90° C., in 18 × 150-mm. Pyrex rimless test tubes. Small flanged test tubes containing ice water were placed in the top of the large tubes to reduce the loss of water vapor by evaporation. The volume of solution heated was 6.0 ml. and the time of heating, 30 minutes. In occasional experiments the time of heating was extended to 60 minutes, but no further changes were observed. This is to be expected from the rapid rate at which β -lactoglobulin is denatured at this temperature (9, 10).

Viscosity. The viscosity was measured in a Bingham-type viscometer (3) at pressures of 15 to 50 cm. of water and at a temperature of 30° C. With water in the viscometer the product of the pressure (centimeters of water) and the time (seconds) was 517. The volume between marks was 2.97 ml., the length of the capillary 10.5 cm., and the radius of the capillary, calculated (3) from the flow time of water at 30° C., was 0.0335 cm. The air pressure was regulated with a Nullmatic² regulator and measured with a water manometer. The pressure was applied somewhat as described by Bingham (2). The measurements were

² Mention of products does not imply endorsement of the U. S. Department of Agriculture or recommendation over any other products of a similar nature not mentioned.

made with the solution flowing upward or downward and a correction was made for the lack of symmetry (pressure head) (β) of the two arms of the viscometer. The readings recorded are averages of up and down readings at three different pressures. Absolute viscosities can be obtained with this viscometer, but the data in this report are expressed as viscosities relative to water at the same temperature. With the solutions studied in the present report the pressure-time product was always a constant, with a precision of $\pm 0.5\%$, and there was no evidence for structural viscosity.

RESULTS

A series of experiments was performed in which calcium chloride concentration and the pH were varied in 0.9 and 1.8% concentrations of β -lactoglobulin. The relative viscosity of unheated β -lactoglobulin solutions containing no calcium is 1.05 and 1.09 for the 0.9 and 1.8% concentrations, respectively, at pH 7.4. These values are increased slightly to 1.07 and 1.18, respectively, on heating. The concentrations of calcium used in this study have a negligible effect on the viscosity of unheated β -lactoglobulin. When calcium is present in heated solutions of β -lactoglobulin, however, marked changes in the viscosity are obtained. The determining effect of pH on the viscosity is shown in Figure 1, A (1.8% β -lactoglobulin) and B (0.9% β -lactoglobulin), with calcium concentrations up to those required for precipitation. The final viscosity increase with the high calcium concentrations is followed abruptly by gelling and the formation of a flocculent precipitate when the gel is disturbed.

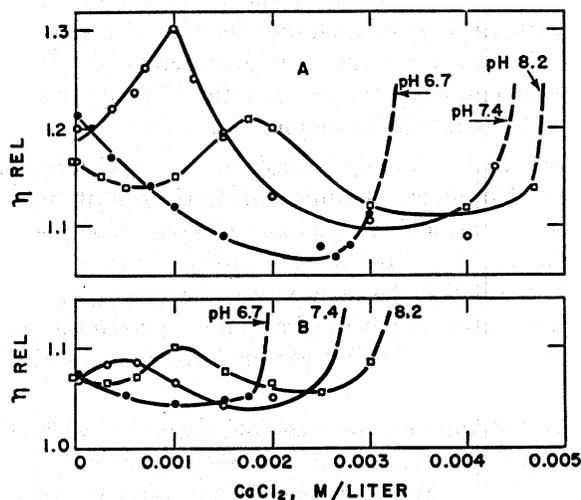


FIG. 1. The influence of calcium concentration and pH on the viscosity of β -lactoglobulin solutions heated for 30 minutes at 90°C . The calcium, added as calcium chloride, is present when the β -lactoglobulin is heated. The termination of the dashed lines is placed at calcium concentrations at which precipitation occurs and does not represent a viscosity value.

- A. Viscosities of solutions containing 1.8% β -lactoglobulin at the pH values indicated.
- B. Viscosities of solutions containing 0.9% β -lactoglobulin at the pH values indicated.

The heated solutions of β -lactoglobulin without calcium are clear, above pH 7, whereas below this pH they are increasingly opalescent as the isoelectric point (pH 5.2) is approached. When β -lactoglobulin solutions containing calcium are heated, those that show initial viscosity increases are slightly opalescent (absorbance readings less than 0.1 at 600 $m\mu$). Solutions in which the viscosity decreases, i.e., "thinning" occurs, are white, and layers less than 1 mm. in thickness are very opalescent (absorbance readings at 600 $m\mu$ are greater than 2 and have no significance). Study of these solutions in the ultracentrifuge has shown that the protein in the white, "thin" solution was in a more aggregated condition, that is, it sedimented more readily, than in the clear, more viscous solutions.

Studies of the pH region between 7.3 and 6.7 have shown a gradual drop in the viscosity peak with a shift in the peak to lower concentrations of calcium. Experiments performed at pH 6.2 with a full range of calcium concentrations gave a more abrupt drop in the viscosity than at pH 6.7, and precipitation at a lower concentration of calcium. If calcium is added to solutions of β -lactoglobulin after heating, the solutions do not become white nor does the viscosity decrease.

The sharp dependence of the viscosity peak on pH suggested that a group dissociating in this pH region might be involved in a reaction with calcium. The α -amino group, with a dissociation pK of 7.8 (16), appeared to be a possibility. The β -lactoglobulin contains four of these groups (5). To obtain information on this point, β -lactoglobulin was treated with α -amino reagents (described under *Materials*), and the effect of calcium on the treated protein was determined.

Formaldehyde treatment (2 or 18 hours) had a striking influence on the reactivity of calcium in heated solutions of the treated protein. Experiments performed at pH 7.5 with 1.8% protein showed no viscosity peak; there was a slight drop in relative viscosity from 1.25 with no calcium to 1.12 with 0.006 *M* calcium. With larger concentrations of calcium the viscosity increased, and a precipitate was obtained with 0.0083 *M* calcium.

Nelson (11) has observed the stabilizing influence of small concentrations of formaldehyde on heat-treated milks. To approximate the conditions he used, 0.26 ml. of 36.6% formaldehyde was added to 25 ml. of 3.6% β -lactoglobulin. After 2 hours the pH was adjusted to 6.7 and the effect of calcium on a 1.8% solution was studied. With 0.005 *M* calcium the solution was opalescent, but no precipitate was obtained. At a concentration of 0.0067 *M* calcium a precipitate was obtained, a concentration of calcium over twice that required for precipitation of the untreated protein.

Parallel results were obtained on β -lactoglobulin treated with nitrous acid. A 1.8% solution of treated protein at pH 7.5 showed no viscosity peak with calcium. The viscosity of the solution with no calcium was 1.16, and about a 3% decrease in viscosity occurred in going to a concentration of 0.005 *M* calcium. A viscosity increase occurred above 0.007 *M* calcium and a precipitate was obtained at 0.01 *M* calcium.

In view of the possible role of amino groups glycine was added to give a concentration of 0.1 *M* to 1.8% β -lactoglobulin at pH 7.5 to see whether it would

influence the calcium reaction. There was no influence on the precipitation with 0.005 *M* calcium, and the usual viscosity peak was obtained with 0.001 *M* calcium.

The effect of lactose was determined at concentration of 5% in the 1.8% β -lactoglobulin solution at pH 7.5 with 0.003 *M* calcium. There was no apparent influence on the rate at which the solution turned white when heated, and the final viscosity could be estimated from addition of the specific viscosity of the components. Although it is well known that carbohydrates (1, 7) and other compounds with a large fraction of hydroxyl groups (15) inhibit denaturation rates, apparently the concentration of lactose present in milk is not high enough to have an appreciable effect.

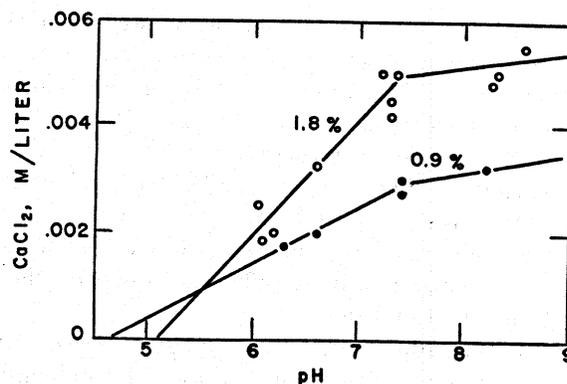


FIG. 2. The concentrations of calcium and pH values required for maximum precipitation of heated β -lactoglobulin solutions. Data are shown for 0.9 and 1.8% β -lactoglobulin solutions.

The relationship between the pH and the calcium concentration required to give a precipitate of heated β -lactoglobulin is shown in Figure 2. These results were obtained by heating a series of mixtures in the neighborhood of the precipitation point. White, gel-like precipitates were obtained, which on agitation gave "curds and whey." Maximum precipitation was evident from a water-clear whey. Actually, maximum precipitation occurs within a narrow range of calcium concentrations. A solution at pH 7.5 with 0.005 *M* calcium, which visually was maximally precipitated, was centrifuged at $2,500 \times G$ for 10 minutes. The protein in the supernatant fluid was estimated from the nitrogen content, and 97.7% of the protein was in the precipitate. With a concentration of 0.0045 *M* calcium the supernatant fluid was slightly opalescent, but 90.0% of the protein was in the precipitate. With a concentration of 0.0040 *M* calcium a precipitate was evident when the heated solution was centrifuged, but a satisfactory separation could not be made. Probably incipient precipitates of this type account for the viscosity increases with concentrations of calcium slightly less than those required for precipitation (See Figure 1). There is considerable scatter of the points in Figure 2 because of the sharp dependence on pH and calcium concentration, but a consistent picture is obtained relating these variables. The 1.8% β -lactoglobulin solution consistently requires somewhat less than twice as much

calcium for precipitation as the 0.9% solution. The amount of calcium required for precipitation of β -lactoglobulin is proportionately greater as the pH of the solution increases above the isoelectric pH of 5.2 for β -lactoglobulin. Beyond pH 7, however, the requirement diminishes considerably.

DISCUSSION

The sharp dependence of calcium precipitation of heated β -lactoglobulin on the pH is of considerable interest and may be of importance for understanding the effect of heat on milk. A recent paper by Pyne and McHenry (13) on the heat coagulation of different milk samples established that the lower the effective calcium concentration, the longer the heating period required for coagulation. It was further established that all heated samples showed a drop in pH, and this was greatest (as much as one unit) for the samples that required the longest heating for coagulation. It can be assumed that if the pH had not dropped, some of these milks would have remained stable indefinitely. The authors discussed the key role of the pH on heat coagulation but the mechanism of the effect could only be surmised. The present studies with β -lactoglobulin show that the pH directly determines the protein precipitation at a given concentration of calcium, and presumably this will be true of other proteins also. A small drop in pH, probably less than 0.1 unit, is sufficient to change the system from a heat-stable to a heat-unstable condition.

In general, values for ionizable calcium and magnesium in milk (4, 6, 13) are at least 0.003 *M*, more than enough to precipitate β -lactoglobulin when milk is heated. Ramsdell and Whittier (14), however, have observed that in milk heated to 100° C. for 10 minutes, although the whey proteins were denatured, they were not aggregated to the extent that they were sedimented with the casein in an ultracentrifuge. The β -lactoglobulin may be held in solution by other components of milk.

Probably the major sites of calcium binding on the β -lactoglobulin molecule are the negatively charged carboxyl groups; the extent of binding at these sites is determined, however, by the net negative charge, that is, binding increases as the pH is raised. The need for heat to bring about the precipitation of β -lactoglobulin by calcium shows, however, that the calcium reaction is not strictly electrostatic. The binding of the positive calcium ion at the negative carboxyl sites would, with proteins at a pH above the isoelectric point, lead to a reduction in the negative charge on the molecule. The proper concentration of calcium would bring the protein to the isoelectric condition where the net electrical charge is zero and isoelectric precipitation occurs. The data presented in Figure 2 support this interpretation of calcium precipitation. As the isoelectric point is approached, that is, as the net negative charge approaches zero, the amount of calcium required for precipitation decreases proportionately. The greater calcium concentration required for the precipitation of casein (unpublished studies) than for β -lactoglobulin, at a given pH, also supports this interpretation. The isoelectric point of casein is at pH 4.7, considerably lower than the pH 5.2 of β -lactoglobulin.

Although binding of calcium at carboxyl groups of β -lactoglobulin and reduction of the negative charge seem to explain calcium precipitation, other groups also appear to assist in the binding. The viscosity data with low calcium concentrations (0.001 M) show a high viscosity at pH 7.4 and a low viscosity at pH 6.7. This suggested that a group dissociating in this pH region might be participating. The α -amino group with a dissociating pK of 7.8 (16) fills this requirement. At pH 7.8 half of the amino groups would be in the free form, presumably the form assisting in the binding of calcium, whereas at pH 6.7 most of these groups would occur as the positively charged ion, apparently unreactive with calcium. The disappearance of the viscosity peak when β -lactoglobulin is treated with formaldehyde or nitrous acid lends support to this interpretation. Klotz (8) has reported data indicating that α -amino acids bind magnesium, and presumably calcium, more strongly than the corresponding aliphatic acid, which is further evidence for the probable participation of the amino groups of proteins in calcium binding.

The greater calcium requirement for precipitation of β -lactoglobulin after treatment with formaldehyde or nitrous acid can be interpreted in terms of net electrical charge on the protein. By altering the amino groups (ϵ -amino groups of lysine as well as α -amino groups) so that they are no longer positively charged, the net negative charge is increased, and the amount of calcium required to neutralize these groups, and thereby bring about precipitation, is increased correspondingly. The lowering of the isoelectric point (0.5 to 0.8 pH unit) by the chemical treatment and the magnitude of the pH effect shown in Figure 2 (about 0.0015 M calcium per pH unit) indicate, however, that the drop in isoelectric point alone does not quantitatively account for the increased amount of calcium required for precipitation.

Nelson (11) has found that small amounts of formaldehyde increase the heat stability of milk. Heat coagulation could be obtained by adding calcium to the formaldehyde-treated milk. The present studies indicate that the formaldehyde intervenes directly, by altering the charge on the proteins, in the amount of calcium required for precipitation of the milk proteins.

SUMMARY

The effect of calcium concentration, pH, and heat on the physical properties of β -lactoglobulin solutions was investigated. Viscosity, light transmission, and precipitation were measured. The dependence of viscosity on pH and the effect of certain α -amino group reagents suggested that α -amino groups assist in the binding of calcium ions. Precipitation in calcium-containing heated β -lactoglobulin solutions was sharply dependent on pH; the lower the pH, the smaller the concentration of calcium required. Calcium appears to function in large part to bring about an isoelectric precipitation by neutralizing negatively charged groups. The effects of heat on β -lactoglobulin solutions are discussed in relation to the heat stability of milk.

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