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RESEARCH PAPERS

κ-CASEIN—β-LACTOGLOBULIN INTERACTION IN SOLUTION WHEN HEATED

SUMMARY

Heating a mixture (0.5% of each) of β-lactoglobulin and κ-casein at pH 7 at 90 C 15 min led to interaction between the two proteins. Electrophoresis at pH 2.1 showed that, before heating, the components moved independently; whereas, after heating, a single component of intermediate mobility was observed. Ultracentrifuge examination of the heated mixture showed a new component with an S_{20} value of 45, three times greater than the S_{20} value of κ-casein. The ability of the κ-casein to stabilize calcium-sensitive casein was considerably reduced in the heated mixture. The clotting time of κ-casein by rennin was increased by the addition of β-lactoglobulin and was increased still further when the mixture had been heated. The clot formed by the action of rennin on the heated mixture contained the β-lactoglobulin as well as the κ-casein.

The effect of heat on solutions of mixtures of β-lactoglobulin and whole casein has been described in an earlier publication (2). In systems containing calcium chloride in which the β-lactoglobulin precipitated, there was no evidence for complex formation. In systems in which precipitation did not occur, the use of electrophoresis at pH 6.5 suggested that complex formation had occurred. The results of electrophoresis are equivocal, however, for demonstrating complex formation since, under certain conditions of heating, the electrophoretic mobility of β-lactoglobulin increases to about the same value as for α-casein. Thus, it could not be decided whether the single peak obtained after heating represented a complex of α-casein—β-lactoglobulin or a mixture of the two proteins, each with about the same mobility. Electrophoresis in acid solution, which has been useful in resolving the components of the α-casein complex (7, 12), has been used in the present experiments in the hope that an unambiguous answer to the question of casein–β-lactoglobulin interaction might be obtained. κ-Casein was used in the present experiments. The electrophoresis experiments were supplemented with ultracentrifuge studies. In addition, the effect of heating the κ-casein—β-lactoglobulin mixture on clotting time and precipitation by rennin, and the ability of the κ-casein in the mixture to stabilize the calcium-sensitive (α_s) casein, were explored.

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MATERIALS AND METHODS

Where not described, the materials and methods are the same as in the previous paper (2). The protein solutions were heated at 90 C 15 min. The concentrations (0.25 to 1.0% total) are indicated for each experiment.

Several κ-casein preparations were used. All gave a symmetrical peak in free-flow electrophoresis (see later), and all were good stabilizers (11) (i.e., 50% stabilization of α_s-casein at κ/α_s ratio of 0.04 to 0.05). Some were prepared by fractionation in urea solution with trichloroacetic acid (8). Others were prepared by fractionation in ethanol (6). This procedure was simplified by precipitating the calcium-κ-casein by saturating with sodium chloride instead of using the potassium oxalate–sodium sulfate steps. Although seemingly homogeneous in free-flow electrophoresis, the κ-casein preparations contained a small amount of material that sedimented more rapidly than did the κ-casein. This heavy fraction did not stabilize α_s-casein. The preparation used in the ultracentrifuge and some of the electrophoretic experiments had had this heavy fraction removed. The very sensitive starch gel electrophoresis in 7 M urea (9) showed that the κ-casein preparations were contaminated with small amounts of the lambda-casein components.

The β-lactoglobulin (Species A) was crystallized from the albumin fraction of Species A pooled milk (3) and recrystallized twice. A suspension of the crystals was freeze-dried.

Solutions of casein and β-lactoglobulin were prepared at pH 7.0 by the addition of NaOH.

The rennin was a commercial preparation rated 1:30,000.

1. *Electrophoresis.* The free-flow Tiselius electrophoretic technique was used with Perkin-Elmer[2] equipment, Model 38. All experiments were performed in a mixture of 1% formic acid and 6% acetic acid at pH 2.1 (ionic strength 0.01). The protein solutions (0.6% of each) at pH 7.0 were acidified with HCl to about pH 2 and were then dialyzed for 24 to 48 hr against the acid mixture.

2. *Ultracentrifugation.* The protein solutions were centrifuged in a Spinco Model E analytical ultracentrifuge.[2] The centrifuge was operated at 25 C, with a 4-degree sector cell. The sedimentation values obtained at 25 C were recalculated to 20 C (S_{20}). The protein solutions were at a total concentration of 1% and adjusted to pH 7.0 with 0.1 N NaOH. These solutions, heated or not heated, were dialyzed against phosphate buffer, pH 7.0 (two one-liter portions in a period of 24 hr) until equilibrium was attained. The phosphate buffer was that described by Waugh and von Hippel (10), but the concentrations were one-half as great, i.e., the ionic strength was 0.1.

3. *Stabilization test.* This test has been described (11). It measures the amount of κ-casein required to maintain α_s-casein in solution with 0.020 M $CaCl_2$ at pH 6.8. The test was applied to heated and unheated mixtures of κ-casein and β-lactoglobulin.

4. *Clotting time with rennin.* The rennin was permitted to act on the κ-casein, or the mixture with β-lactoglobulin, at pH 6.4 and 30 C in the presence of 0.020 M $CaCl_2$. The rennin was added to the protein solution in a ratio of 1:2,000 of casein; 0.1 M $CaCl_2$ was added to give the designated concentration. The clotting time was determined by a visual method (1). Maintenance of a constant pH is very important. The solutions were heated at pH 7.0; after the $CaCl_2$ was added, the pH was adjusted to 6.4 with 0.01 N HCl or NaOH.

5. *Estimation of amount of protein clotted by rennin.* In these experiments the concentration of κ-casein was 1.0%, of β-lactoglobulin, 0.8%. These solutions, heated and unheated, were mixed with water and 0.1 M $CaCl_2$; 5 ml protein solution, 3 ml water, and 2 ml 0.1 M $CaCl_2$. The pH was 6.6. Two milligrams of rennin in 0.1 ml of water were added at 30 C. The solutions were stirred occasionally to observe clot formation. After clotting was complete (5 to 10 min), the solutions were centrifuged in 15-ml graduated centrifuge tubes 5 min. The volume of the sediment was recorded and the protein remaining in the supernatant solution was determined from the ultraviolet absorption at a wave length of 280 mμ. For this purpose, the solutions were clarified by the addition of a drop of 0.5 N NaOH.

[2] It is not implied the USDA recommends the above company or its product to the possible exclusion of others in the same business.

RESULTS

1. The electrophoretic behavior at pH 2.1 of κ-casein and of β-lactoglobulin, and of their mixtures, both unheated and heated, is shown in Figure 1. The tracings shown were obtained after about 35 min electrophoresis at 130 v and 4.5 ma (field strength 10.0 v cm^{-1}). The descending boundaries in the cell are illustrated. The κ-casein gives a sharp peak, with a slight suggestion of inhomogeneity on the advancing limb of the peak. The mobility is 7.3 units (7.3×10^{-5} cm^2 v^{-1} sec^{-1}). The mobility is positive, i.e., all these proteins are positively charged at pH 2.1. The average mobility of the heated κ-casein is 8.3. For β-lactoglobulin the mobility is 15.5, and when heated 13.5. In the unheated mixture the mobilities are 7.3 and 15.4. When the mixture is heated, the major component has a mobility of 10.1. When κ-casein alone is heated and then mixed with β-lactoglobulin, electrophoresis gives mobilities of 8.4 (average) and 15.5, values expected for an

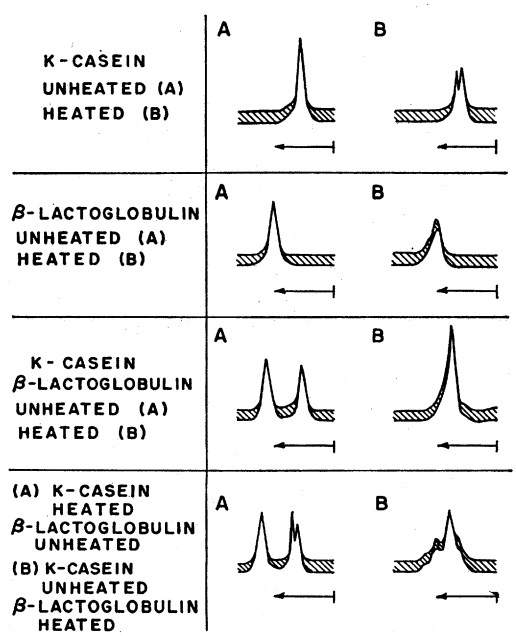

FIG. 1. Electrophoresis at pH 2.1 of κ-casein and of β-lactoglobulin, and of mixtures, unheated and heated.

unreacted mixture from the results given above. When β-lactoglobulin alone is heated and then mixed with κ-casein, the major component has a mobility of 9.8, i.e., similar to that in the heated mixture above. Minor components may be unreacted κ-casein (8.8) and heated (12.8) β-lactoglobulin.

2. Sedimentation values (S_{20}) of 2.7 and 15.1 were obtained for β-lactoglobulin and κ-casein, respectively. When these proteins were heated individually the κ-casein contained a fraction with an S_{20} of 32, but the bulk of the κ-casein sedimented with the characteristic value of 15; β-lactoglobulin A sedimented with an S_{20} of 5.3. In the unheated mixture of these two proteins, the S_{20} values were 2.7 and 17.4, respectively. When the mixtures were heated, however, and this was done for κ-casein:β-lactoglobulin ratios of 1:2 and 1:1, ultracentrifugal examination showed a rapidly sedimenting component with an S_{20} value of 45. Components with sedimentation values corresponding to uncomplexed β-lactoglobulin and κ-casein were still present in both instances.

3. A test of the ability of κ-casein to stabilize α_s-casein was made of an unheated and heated mixture of κ-casein and β-lactoglobulin. The results are shown in Figure 2.

4. The influence of β-lactoglobulin on the clotting time of κ-casein, in unheated and heated mixtures, is shown in Table 1. A marked influence on the clotting time is apparent.

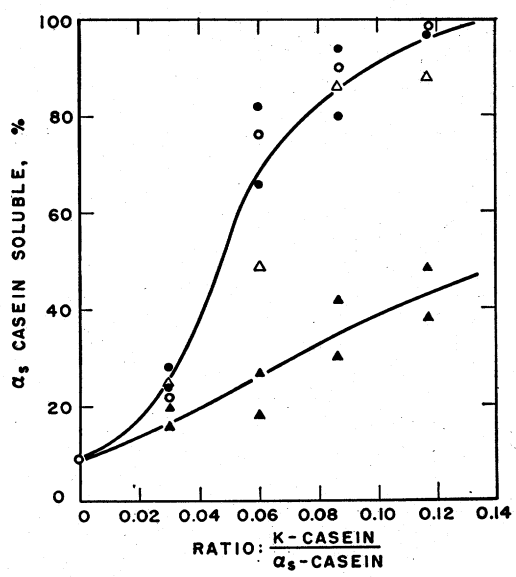

FIG. 2. The stabilization of α_s-casein in the presence of 0.020 M $CaCl_2$ by κ-casein, and κ-casein mixed with β-lactoglobulin, both unheated and heated (90 C for 15 min). κ-Casein (○), κ-casein, heated (●), κ-casein plus β-lactoglobulin (△). These three are represented by a single curve. κ-Casein plus β-lactoglobulin, heated (▲).

TABLE 1

Rennin clotting time of κ-casein (κ), of κ-casein with β-lactoglobulin (β), unheated and heated, pH 6.4, 30 C, with 0.020 M $CaCl_2$

Sample	Treatment	Concentration	Clotting time
		(%)	*(sec)*
κ	Unheated	0.25	645
κ	Heated	0.25	250
κ + β	Unheated	0.25 each	1,040
κ + β	Heated	0.25 each	1,240

5. The precipitation by rennin of various combinations of κ-casein and β-lactoglobulin, unheated and heated, is given in Table 2. The light absorbance of κ-casein is 1.0 for a concentration of 1 mg per milliliter; for β-lactoglobulin, the value is 0.93. The soluble products resulting from rennin acting on κ-casein, however, have a negligible ultraviolet absorption. Thus, the ultraviolet readings do not assess the total soluble proteinaceous material, but they do show that the β-lactoglobulin in the heated mixture was precipitated by rennin action, together with the κ-casein. Some β-lactoglobulin apparently has been coprecipitated in the clot from the unheated mixture. The nature of this association is not apparent from the present experiments. The volumes of the clots were markedly larger for the heated solutions than for the unheated. This was true even for κ-casein alone. Thus, heat had altered the κ-casein, even though with this preparation the ability to stabilize α_s-casein had remained the same.

TABLE 2

Precipitation of κ-casein (κ), of κ-casein with β-lactoglobulin (β), unheated and heated, by the action of rennin, pH 6.6, 30 C, measured by UV_{280} absorption

Sample	Vol of sediment	UV_{280} absorption per milliliter	
		Original	Supernatant
	(ml)		
κ, unheated	0.1	5.0	0.25
β, heated	0	4.35	4.35
κ + β, unheated	0.1	9.35	3.75
κ + β, heated	0.6	9.35	0.35
κ, heated	0.4	5.0	0.35

DISCUSSION

Electrophoresis at pH 2.1 provides unequivocal evidence that β-lactoglobulin and κ-casein interact when heated together at pH 7 at 90 C for 15 min. Electrophoresis shows also that heating β-lactoglobulin before mixing with κ-casein brings about interaction when subsequently mixed with κ-casein at 20 to 25 C. Long (5) also, in his extensive studies of β-lactoglobulin—κ-casein interaction found that heating β-lactoglobulin alone was sufficient for interaction. Presumably, the β-lactoglobulin is activated by heating, perhaps by the exposure of SH groups, so that interaction occurs at the lower temperature. Ultracentrifuge examination of the heated mixtures shows that a new component has been formed which sediments rapidly with an S_{20} of 45, three times larger than the S_{20} value of κ-casein, the largest component of the mixture. Long (5) observed a complex with an S_{20} value of the same magnitude. Heating κ-casein alone does not lead to interaction when subsequently mixed with β-lactoglobulin. κ-Casein is altered by heating, as shown by the split in the electrophoretic pattern, by decrease in the clotting time (Table 1), and increase in the volume of the clot (Table 2). Heated κ-casein solutions, however, frequently retain their ability to stabilize calcium-sensitive casein (11).

It is evident from the results in Figure 2 that the ability of κ-casein to stabilize the calcium-sensitive casein has been considerably reduced by its interaction with β-lactoglobulin. This does not necessarily mean that heating milk would lead to a destabilization by such a mechanism, for there the κ-casein is already associated with the calcium-sensitive casein before heat is applied. The work of others (4) indicates that β-lactoglobulin does complex with the caseins when milk is heated.

The mere presence of β-lactoglobulin lengthens the clotting time of κ-casein by rennin (Table 1). This may be a competitive effect, since rennin is a protease and presumably will have a tendency to associate with all proteins concomitant to peptide bond splitting. Heating the β-lactoglobulin—κ-casein mixture brings about a still further increase in the clotting time, presumably because of the combination of the β-lactoglobulin with the κ-casein, with perhaps spatial interference. Kannan and Jenness (4), in their studies on the clotting time of the calcium caseinate–calcium phosphate complex heated with β-lactoglobulin, observed an increase in the clotting time and considered this evidence for interaction between casein and β-lactoglobulin. The clot obtained by the action of rennin on the heated mixture of κ-casein and β-lactoglobulin contains both proteins (Table 2), as would be expected if heat had caused κ-casein and β-lactoglobulin to combine. Presumably, if conditions are found for satisfactory clotting of heated milks, the β-lactoglobulin, and perhaps other whey proteins also, would be found with the casein clot.

REFERENCES

(1) Berridge, N. J. Some Observations on the Determination of the Activity of Rennet. Analyst, 77: 57. 1952.

(2) DellaMonica, E. S., Custer, J. H., and Zittle, C. A. Effect of Calcium Chloride and Heat on Solutions of Mixtures of β-Lactoglobulin and Casein. J. Dairy Sci., 41: 465. 1958.

(3) Gordon, W. G., and Semmett, W. F. Isolation of Crystalline α-Lactalbumin from Milk. J. Am. Chem. Soc., 75: 328. 1953.

(4) Kannan, A., and Jenness, R. Relation of Milk Serum Proteins and Milk Salts to the Effects of Heat Treatment on Rennet Clotting. J. Dairy Sci., 44: 808. 1961.

(5) Long, J. E. The Physical Properties and Interactions of Some Minor Milk Proteins. Dissertation Abstr., 19: 2242. 1959.

(6) McKenzie, H. A., and Wake, R. G. An Improved Method for the Isolation of κ-Casein. Biochim. et Biophys. Acta, 47: 240. 1961.

(7) McMeekin, T. L., Hipp, N. J., and Groves, M. L. The Separation of the Components of α-Casein. I. The Preparation of α_1-Casein. Arch. Biochem. Biophys., 83: 35. 1959.

(8) Swaisgood, H. E., and Brunner, J. R. A Method for the Isolation of the Calcium-Insensitive Casein Fraction from Whole Casein. J. Dairy Sci., 43: 855. 1960.

(9) Wake, R. G., and Baldwin, R. L. Analysis of Casein Fractions by Zone Electrophoresis in Concentrated Urea. Biochim. et Biophys. Acta, 47: 225. 1961.

(10) Waugh, D. F., and von Hippel, P. H. κ-Casein and the Stabilization of Casein Micelles. J. Am. Chem. Soc., 78: 4576. 1956.

(11) Zittle, C. A. Stabilization of Calcium-Sensitive (α_s) Casein by κ-Casein: Effect of Chymotrypsin and Heat on κ-Casein. J. Dairy Sci., 44: 2101. 1961.

(12) Zittle, C. A., Cerbulis, J., Pepper, L., and DellaMonica, E. S. Preparation of Calcium-Sensitive α-Casein. J. Dairy Sci., 42: 1897. 1959.