

Influence of Heat on κ -Casein

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Abstract

An aqueous solution of κ -casein at pH 7 is not affected by heating (100 C for 5 min). If, however, sodium chloride (0.05 M) or other salts are present the κ -casein is labile to heat, judged by loss of ability to stabilize α_s -casein against precipitation by calcium ions. In experiments at several pH values (6.2 to 7.9) in sodium chloride-imidazole buffer the heat lability was somewhat greater at the lower pH values. At pH 6.2 about 75% of the κ -casein precipitated when the solution was heated. This did not occur in sodium chloride alone or in cacodylate buffer at pH 6.2. The κ -casein in solutions (pH 6.2) containing 0.01 M CaCl_2 did precipitate when they were heated. Reducing compounds like mercaptoethanol alone had little or no effect on the heat lability of κ -casein; when present with salts, however, the combined effect was greater than that of the salts alone. Mercaptoethanol, cysteine, and dithiothreitol enhanced the heat lability. The dithiothreitol was effective at a concentration of 5×10^{-5} M. κ -Casein, reduced and stabilized by alkylation, was somewhat heat labile in sodium chloride-imidazole buffer, but this lability was not enhanced by addition of mercaptoethanol.

Previous studies (11) showed that some preparations of κ -casein were heat labile, judged by loss of ability to stabilize α_s -casein against precipitation by calcium ions. Recent studies, however, showed that none of the preparations made by the urea-sulfuric acid method (12) were heat labile when heated under the same conditions. These preparations of κ -casein were, however, found to be heat labile in the presence of various salts and in the presence of mercaptoethanol and related compounds. The nature of this heat lability is reported in the present paper.

Material and Methods

κ -Casein was prepared from pooled milk by

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the urea-sulfuric acid method (12). Polyacrylamide gel electrophoresis at pH 8.5 of the reduced κ -casein showed that it was a mixture of the A and B types.

α_s -Casein was prepared from pooled milk by a modified urea procedure (10) followed by a final purification with ethanol (12).

Stabilization test. The procedure described previously (13) was followed with minor modifications. The 0.5% α_s -casein solution was prepared in a buffer solution containing 0.050 M NaCl and 0.050 M imidazole adjusted to pH 7.3 with HCl. This facilitated preparation of the α_s -casein solution and also maintained the pH of the test mixture at 7.3 when CaCl_2 was added. Although heating studies suggested that imidazole might associate with κ -casein, there was no evidence that it interfered with the precipitation of α_s -casein by CaCl_2 . The precipitation curves (increasing concentrations of CaCl_2) were identical with and without imidazole (0.015 M) present. Occasional erratic values in the stabilization results (α_s -casein solubility versus κ/α_s ratio curves) were thought perhaps to be due to delay between adding CaCl_2 and manually mixing. However, vigorous mixing with the tubes in contact with an oscillating neoprene cup as the CaCl_2 was added led to increased precipitation (25 to 35%), i.e., destabilization, of the casein. Mixing of this type used after the CaCl_2 was added also led to some destabilization (12 to 15%).

Heating experiments. A stock 0.5% solution of the κ -casein at pH 7.5 was used. This was diluted with an equal volume (usually 2.0 ml plus 2.0 ml) of the solvent system under study in a 15-ml centrifuge tube. The tubes were immersed in a boiling water bath to the 10-ml mark, usually for 5 min. The effect of the heat was fast; this was apparent in the experiments that showed visible changes which were evident in less than one minute.

Results

A 0.25% solution of κ -casein at pH 6.5 to 7.5, heated for 5 min at 100 C, was not changed in its ability to stabilize α_s -casein against precipitation by CaCl_2 . (Some loss of stabilization ability occurred at pH 6.1.) If, however, some NaCl was added to a solution of κ -casein before heating, some of the stabilizing ability was lost.

Results of such experiments with NaCl present are shown in Fig. 1. A study of several concentrations of NaCl showed that a 0.05 M concentration was sufficient to exert the maximum effect. Other salts such as sodium phosphate and sodium sulfate were also effective in imparting heat lability to κ -casein solutions. (Stabilization tests with the phosphate-containing solutions were attempted with extra calcium chloride to combine with the phosphate. The calcium phosphate precipitate, however, adsorbed the α_s - and κ -caseins from solution. Satisfactory results were obtained by dialyzing the κ -casein solutions before doing the stabilization tests.)

The influence of pH on the effect of heat on κ -casein was studied in solutions containing 0.05 M NaCl plus 0.05 M imidazole adjusted to pH 8.2, 7.3, and 6.2 with HCl. The respective pH values measured after heating were 7.9, 7.2, and 6.2. All the solutions were clear before heating, but a flocculent precipitate appeared in the pH 6.2 solution after heating. Since a solution of κ -casein containing only NaCl at the same or lower pH values did not give a precipitate when heated, this was considered to be a specific imidazole cation (concentration 0.025 M) effect at this pH. A κ -casein solution buffered with cacodylate at pH 6.2 did not give a precipitate when heated. On the other hand, κ -casein solutions containing calcium ions did precipitate when heated. The presence of ammonium ions when heated did not cause precipitation of κ -casein. Typical

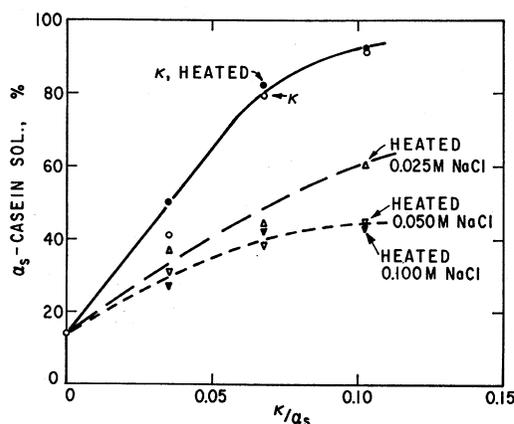


FIG. 1. Influence of heat (5 min at 100 C) on the stabilizing ability of κ -casein. κ -Casein (○); κ -casein heated (●); κ -casein heated with 0.025 M NaCl present (△); with 0.050 M NaCl (▼); with 0.100 M NaCl (▽). The stabilization curve for unheated κ -casein is shown in this and subsequent graphs for comparison. It was repeated with each set of experiments.

results of these experiments at various pH values are shown in Fig. 2. The flocculent precipitate in the imidazole-containing solution pH 6.2 readily sedimented, leaving a clear supernatant solution containing 25% of the κ -casein. When the stabilization curve for this κ -casein experiment was based on the κ -casein remaining in solution it came close to the unheated curve, suggesting that the part remaining in solution was undamaged by heat.

Experiments were also done with solutions of κ -casein in the pH 6.2 cacodylate buffer with CaCl_2 present. The stabilization obtained with these solutions after heating is shown in Fig. 3. The solution containing the least CaCl_2 remained almost clear during heating, the next two were increasingly opalescent, and the two most concentrated solutions gave flocculent precipitates. Seventy-five per cent of the κ -casein was precipitated in both solutions. In an experiment without the cacodylate-NaCl buffer (pH was 6.95) a flocculent precipitate (71%) was obtained with the 0.0048 M concentration of CaCl_2 . In the various experiments in which κ -casein precipitated, the part remaining soluble was about 25% of the total; this suggested that a part of the κ -casein was resistant to heat-aggregation. Analyses of both fractions for sialic acid showed that both contained 1.8% and, hence, there was no fractionation in respect to this component. κ -Casein that had been heated at pH 6.2 (cacodylate) so that considerable of its stabilizing ability was lost did not, however, precipitate on addition of CaCl_2 .

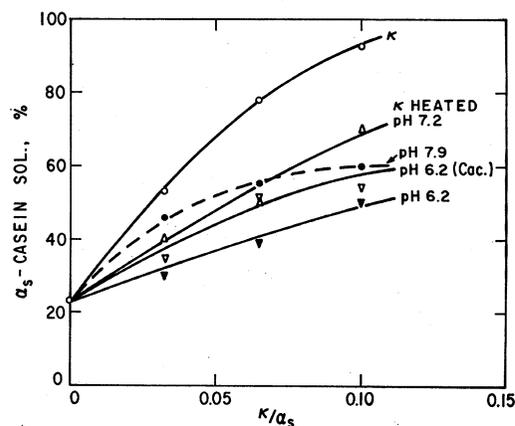


FIG. 2. Influence of heat (5 min at 100 C) on κ -casein at various pH values with imidazole (pH 6.2, 7.2, 7.9) and with cacodylate (pH 6.2) present. κ -Casein (○); κ -casein heated, imidazole, pH 6.2 (▼); κ -casein heated, imidazole, pH 7.2 (△); κ -casein heated, imidazole, pH 7.9 (●); κ -casein heated, cacodylate, pH 6.2 (▽).

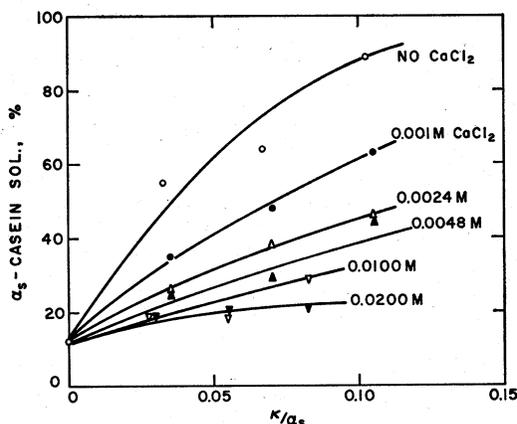


FIG. 3. Influence of heat on solutions of κ -casein containing various concentrations of CaCl_2 at pH 6.2 (0.05 M cacodylate plus 0.05 M NaCl).

Other factors that might influence the effect of heat on κ -casein were explored. Solutions of κ -casein (no extra salts present) adjusted to pH 3.0 or to pH 10.5 and heated showed no loss in stabilizing power. Mercaptoethanol added to κ -casein solutions (no extra salts) had little or no effect when these solutions were heated. Its detrimental effect was greatly increased, however, with some NaCl. Results of experiments of this type are shown in Fig. 4. When similar experiments were performed in the series of imidazole (0.025 M plus 0.025 M NaCl) buffers (pH 6.2, 7.2 and 7.9) with 0.032 M mercaptoethanol, all solutions were affected by heat to about the same degree.

Cysteine and dithiothreitol were as effective as mercaptoethanol in comparative tests at pH

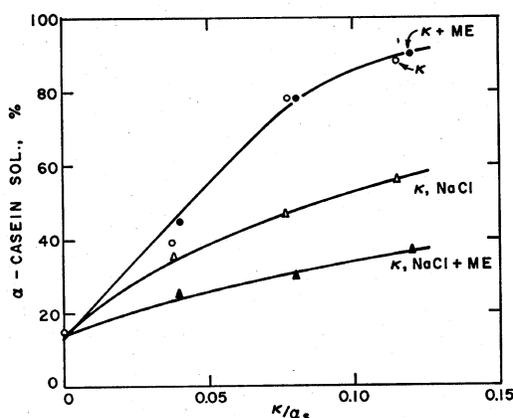


FIG. 4. Influence of heat on solutions of κ -casein containing mercaptoethanol. κ -Casein (○); κ -casein, 0.032 M mercaptoethanol (ME), pH 7.0 (●); κ -casein, 0.050 M NaCl, pH 7.0 (△); κ -casein with both mercaptoethanol and NaCl (▲).

7.9. The latter two were effective, however, at much lower concentrations than cysteine. These results are summarized in Fig. 5, which relates concentration of reducing agent and the amount of heated κ -casein required for 50% stabilization. These values are obtained from stabilization curves such as those shown in Fig. 4. It is evident that dithiothreitol has some effect at a concentration as low as 5×10^{-5} M.

Mercaptoethanol, with which most of the experiments were done, had no effect on stabilization by κ -casein unless the solutions were heated. The effect, thought to involve disulfide cross-bonding formed when the solutions were heated was, however, not reversible. In an experiment to test this, the concentration of mercaptoethanol was 0.00032 M when the κ -casein solution was heated; after cooling, mercaptoethanol was added to a concentration of 0.032 M. Stabilization tests performed 30 min and 24 hr later showed that no significant reversal had occurred. The use of a sulfhydryl reagent (dithionitrobenzoic acid) (2) showed that a positive sulfhydryl reaction was still obtained after heating the κ -casein solution even with dilute mercaptoethanol (0.00032 M).

Experiments were done with κ -casein reduced and stabilized by alkylation (prepared by the action of iodoacetamide on reduced κ -casein (6); although this material is monomeric in respect to disulfide bonds, aggregation of non-covalent type occurs in solution) which contains no sulfhydryl groups. This was heated in the pH 7.9 imidazole buffer; some loss of

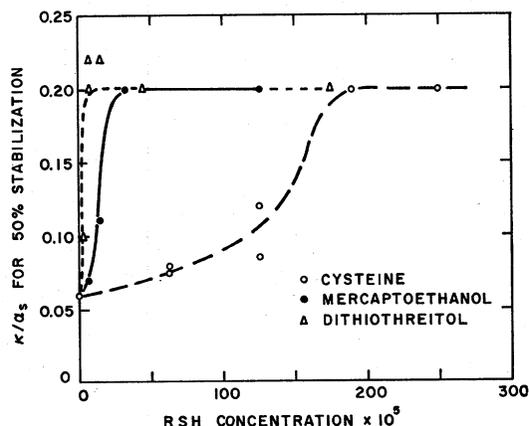


FIG. 5. Relation between the concentration of sulfhydryl present when κ -casein is heated in the pH 7.9 imidazole buffer and the amount of the heated κ -casein (expressed as κ/a_s ratio) required to give 50% stabilization. The zero concentration of sulfhydryl compound value given is for κ -casein heated in the buffer alone. ○: cysteine; ●: mercaptoethanol; △: dithiothreitol.

stabilizing power resulted (Fig. 6). A similar experiment with 0.032 M mercaptoethanol present, however, resulted in no further loss. These results indicate that the sulfhydryl groups in reduced κ -casein have a direct role in heat lability and that in addition other reactions are involved.

A portion of heated κ -casein was dialyzed and freeze-dried. One hundred and fifty milligrams were dissolved in the pH 7.2 imidazole-NaCl buffer, treated with 0.00032 M mercaptoethanol, and heated under the conditions described in the previous experiments. The stabilization of this heated solution was comparable to that shown by the bottom curve, Fig. 4. This solution was dialyzed three days against three changes of water. The solution was adjusted to pH 8.0 and freeze-dried. This material, suspended in imidazole-NaCl buffer, was only 70% soluble. This sample of heated κ -casein was reduced with mercaptoethanol and examined in polyacrylamide gel electrophoresis in 4.5 M urea at pH 8.7; a small portion of the total had the mobility of unheated κ -casein, but the bulk of the material remained in the slot where it had been applied to the gel. In comparative runs in the ultracentrifuge in an imidazole-NaCl buffer, pH 7.0, the heated κ -casein sedimented more rapidly (about two times) than the unheated κ -casein.

Discussion

The inability of heated κ -casein solutions to stabilize α_s -casein against precipitation by CaCl_2 may result from cross-bonding or association of a special type, and not from conformational changes. Optical rotatory dispersion measurements have shown (4) that κ -casein has a low

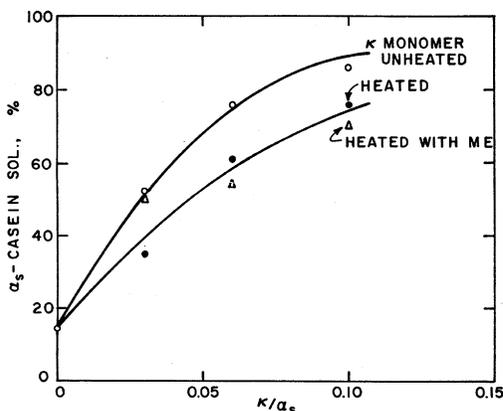


FIG. 6. Influence of heat on alkylated κ -casein monomer in pH 7.9 imidazole buffer. \circ Unheated; \bullet heated; \triangle heated with 0.032 M mercaptoethanol (ME) present.

degree of regular structural organization; hence, conformational changes from heating are unlikely. Organization without regularity must, however, be considered a possibility. The association resulting from heating κ -casein must differ from that involved in the normal polymer-to-monomer relationship brought about by reduction, since both polymer and monomer preparations are equally effective in stabilizing α_s -casein (5). Heating may bring about cross-bonding such that the sites on κ -casein which interact specifically with α_s -casein are inaccessible.

The major effect of salts, particularly neutral salts like NaCl, is probably to reduce the electrostatic repulsion between the charged κ -casein molecules by providing an ionic envelope. The effect of pH leads to the same conclusion. As the pH is lowered, thus decreasing the charge, greater interaction of the heated molecules can occur.

The fact that some residual stabilizing power has always remained after heating suggests that the aggregate itself may have some ability to stabilize α_s -casein.

There are specific ionic effects, as shown by the action of imidazole cations (pH 6.2) and calcium ions in bringing about visible aggregation of the heated κ -casein solutions. Calcium by its divalent charge could enhance the association between κ -casein molecules and thus make more extensive the cross-bonding brought about by heat, as well as contribute to neutralization of charge, probably a role of the imidazole ions also. Calcium ions must also be involved directly in the heating process, for κ -casein that has been inactivated (loss of stabilizing power) by heating without calcium ions is not precipitated when calcium ions are subsequently added. A similar difference is observed when β -lactoglobulin is heated (7). When low concentrations (0.005 M at pH 7.5) of CaCl_2 are present, heating leads to precipitation of the β -lactoglobulin (7). On the other hand, when β -lactoglobulin is heated without CaCl_2 present, on subsequent addition of CaCl_2 very much larger concentrations are required for precipitation (9).

The precipitates obtained by heating κ -casein in the presence of CaCl_2 are largely soluble in 6.6 M urea and are almost completely soluble when mercaptoethanol is added. On dialysis some increase in solubility (to 60%) remains, with a parallel increase in stabilizing ability.

The heating of κ -casein treated with mercaptoethanol or related compounds may lead to disulfide cross-bonds. This suggestion is supported by the results of heating the re-

duced and alkylated κ -casein containing no sulfhydryl groups after reduction as potential sources of disulfide bonds. This κ -casein derivative was not influenced by mercaptoethanol when heated. The disulfide bonds if formed when κ -casein itself is heated must be of special type, since the effect of heat is not reversed by additional higher concentrations of reducing agent. This resistance to reversal may be due to the rigidity imparted by the other types of cross-bonds, indicated by the heating of the alkylated κ -casein monomer. That the heated κ -casein has remained aggregated is apparent from the gel electrophoresis and the ultracentrifuge runs. Loss of stabilizing power was not reversed by addition of urea (6.2 M), followed by dialysis. A similar influence of pH, salts, and reducing agents on the interaction of the sulfhydryl-, disulfide-containing β -lactoglobulin, measured by the opacity and viscosity of the heated solutions, has been shown (8).

Mercaptoethanol and dithiothreitol, because of their effectiveness at very low concentrations, are probably capable of initiating chain reactions among the disulfide bonds of the κ -casein. Dithiothreitol shows some effect at a concentration less than 5×10^{-5} M. The concentration of potential sulfhydryl groups in 0.25% κ -casein is about 25×10^{-5} M.

Skimmilk to which 0.03 M mercaptoethanol was added flocculated within 10 min when heated at 100 C. The skimmilk without the mercaptoethanol was stable indefinitely at this temperature. A specific contribution of κ -casein to this phenomenon cannot be assigned in this complex system and particularly with other disulfide-containing proteins like β -lactoglobulin present. Further information is desirable on the influence of the α_s - κ -casein association on the lability of the latter to heat.

There have been few reports on the influence of heat on κ -casein. Alais et al. (1) have reported on the release of glycopeptide when κ -casein is heated at 120 C. Actually, when the periods of heating were only 10 min no glycopeptide was released; hence, glycopeptide release will not be a factor in the present experiments, since the heating was considerably milder (100 C for 5 min).

Hayes et al. (3) reported that a 5% concentration of κ -casein containing 0.5 M CaCl_2 did not form a gel or precipitate even on gentle boiling. These results are contrary to the instability observed in the present studies with 0.25% κ -casein containing as little as 0.0048 M CaCl_2 . A 5% concentration of κ -casein containing 0.48 CaCl_2 , pH 6.9, remained stable when heated at 100 C for 5 min, thus confirming

the report of Hayes et al. Considerable of the α_s -casein stabilizing power of this κ -casein solution was lost, but not to the degree occurring in 0.25% solutions. Usually, concentrated protein solutions are less stable than dilute solutions when heated, due to greater opportunity for cross reaction; hence, the behavior of 5% κ -casein solutions is puzzling. Further studies will be needed to relate the contrasting behavior in concentrated and dilute systems.

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