

It has been found helpful in studies of the caseins to characterize the calcium-sensitive casein by its quantitative precipitation with calcium chloride (6), and the kappa-casein by its ability to quantitatively stabilize the calcium-sensitive casein so that it does not precipitate with calcium chloride (5). The latter test, which has not been reported in detail, is performed with calcium-sensitive (α_s) casein (0.3%), at pH 6.7, with varying amounts of kappa-casein, with a 0.020 *M* concentration of calcium chloride. The test is set up in 15-ml. centrifuge tubes; total volume of the test mixture is 10.0 ml. A 2% solution of the α_s -casein at pH 7.8 is used; subsequent addition of calcium chloride brings the pH of the mixture to the desired pH of 6.7. The solution of α_s -casein (30 to 34 mg.) is added to the tubes, followed by the required volumes of water, and amounts of kappa-casein such that the kappa/ α_s ratios vary from about 0.03 to 0.12. Finally, 2.0 ml. of 0.1 *M* calcium chloride is added and the mixture stirred with a spatula. The test mixtures are kept at 30° C. for 15 min., then centrifuged at about 3,000 \times G for 5 min. Samples of the supernatant solutions are withdrawn, appropriate dilutions made, and 1 drop of 0.5 *M* NaOH added to clarify the dilutions. The casein in solution is determined from the light absorption at 280 $m\mu$. A factor of 1.0 is used for convenience for converting light absorption (optical density) to milligrams of protein per milliliter. The amount of kappa-casein used in the test is deducted from the casein in solution and the results expressed as per cent of α_s -casein in solution. Solutions of α_s -casein for the test are stored at 7° C. and are not kept more than three days, since the soluble portion at zero kappa concentration tends to increase. A stabilization test with an aged α_s -casein solution parallels that obtained with a fresh solution, but is at a higher level. A typical curve for kappa-casein by this test procedure is shown by the curve No. 1 (○—○) in Figure 1-A, where the per cent α_s -casein soluble is plotted against the ratio of kappa/ α_s in the test mixtures. Choice of a method for preparing kappa-casein of maximum stabilizing power has been guided by the use of this stabilization test.

This test has also been useful in investigating the influence of various treatments on kappa-casein. One of the treatments investigated was the influence of heat (90° C. for 15 min.). On investigating various preparations of kappa-casein it was found that some were

heat-stable, that is, after heating they retained their ability to stabilize the calcium-sensitive casein, whereas others were heat-labile. The latter were adequate stabilizers before heating,

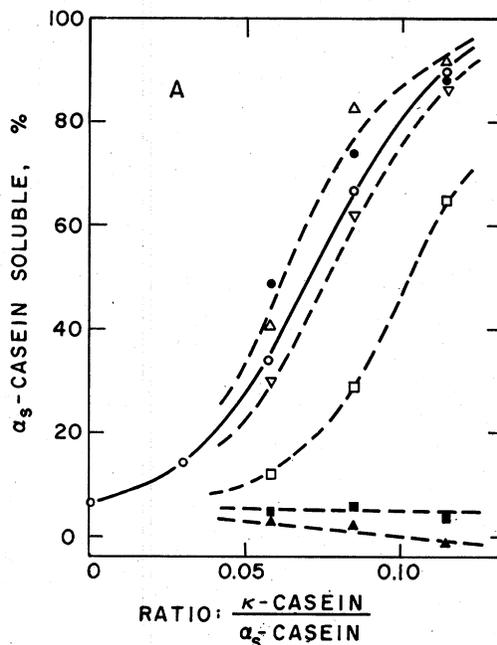


FIG. 1. Stabilization of calcium-sensitive (α_s) casein by kappa-casein. Influence of heat and chymotrypsin treatment on kappa-casein. Details of the test are described in the text. Results with kappa-casein prepared by the method of McKenzie and Wake (3) and subsequently ultracentrifuged to remove the heavy fraction (results by Dr. M. P. Thompson have shown that the heavy fraction has very little stabilizing ability). Stabilization tests with the kappa-casein subjected to various treatments are designated by the following curves: (1) ○—○ Kappa-casein as is (1.0% stock solution at pH 7.0 used). (2) △—△ Kappa-casein heated at 90° C. for 15 min. (7). (3) ●—● Kappa-casein plus chymotrypsin (10.0 ml. 1.0% kappa-casein, pH 7.0 and 0.3 ml. (3.5 γ) of chymotrypsin, at 25-27° C.), tested for stabilization immediately. Same as curve drawn for 2. (4) ▽—▽ Same as (3), but tested after chymotrypsin had acted for 60 min. (5) ■—■ Same as (4), but heated at 90° C. for 15 min. before tested. (6) □—□ Same as (3), but tested after chymotrypsin had acted for 120 min. (7) ▲—▲ Same as (6), but heated at 90° C. for 15 min. before tested.

but after heating had lost much or all of this property.

The explanation for this difference in the effect of heat on kappa-casein is not apparent yet. Chemical manipulation during the preparation of the kappa-casein might produce the heat-labile form, or enzymes in milk might produce the heat-labile form. The protease in milk is a possibility, since this enzyme is precipitated almost quantitatively with the casein in the preparation of acid-precipitated casein (4). This protease is of the trypsin-chymotrypsin type, most active at pH 8.5. The enzymes trypsin and chymotrypsin are considerably less specific in their action on casein than are pepsin and rennin (2). Chymotrypsin was available, so the effect of this enzyme on the stabilizing activity of kappa-casein has been investigated. The effect of heat on the partially enzyme-altered kappa-casein also has been studied. The results with two preparations of kappa-casein are given in Figures 1 and 2.

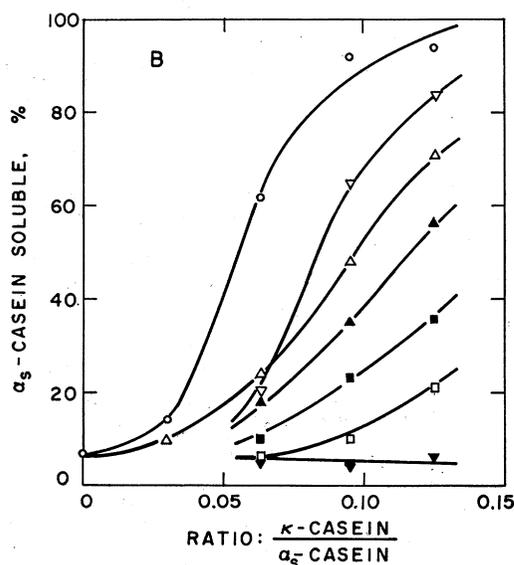


FIG. 2. Results with kappa-casein prepared by the method of McKenzie and Wake (3). This casein had not been treated to remove lipides and the solution was slightly turbid. (1) \circ — \circ Kappa-casein as is. (2) \triangle — \triangle Kappa-casein heated at 90° C. for 15 min. (3) ∇ — ∇ Kappa-casein plus chymotrypsin (same conditions as for A-3). Stabilization test after chymotrypsin had acted for 60 min., no heat. (4) \blacktriangle — \blacktriangle Kappa-casein plus chymotrypsin. Immediately after addition of chymotrypsin, mixture was heated at 90° C. for 15 min. (5) \blacksquare — \blacksquare Similar to (4), but not heated until chymotrypsin had acted for 15 min. (6) \square — \square Similar to (4), but not heated until chymotrypsin had acted for 30 min. (7) \blacktriangledown — \blacktriangledown Similar to (4), but not heated until chymotrypsin had acted for 60 min.

The experiments described utilize amounts of chymotrypsin (ratio of kappa-casein to chymotrypsin 28,500 to 1) that have only a limited action on casein. Calcium caseinate is clotted by chymotrypsin (2) and the present studies have shown that kappa-casein can be clotted with chymotrypsin (500:1 at pH 6.8) with or without calcium chloride present. The kappa-casein, inactivated by successive action of chymotrypsin and heat as described in the present experiments, is clotted by calcium chloride at concentrations of 0.005 M, but this clot dissolves at concentrations of 0.025 to 0.050 M (this has varied with different preparations) calcium chloride. This clot can also be dissolved with sodium chloride. Furthermore, this inactive kappa-casein, with a concentration of calcium chloride in which it is soluble, will clot when acted on by rennin.

Figure 1 shows the stabilization by a preparation of kappa-casein that is not affected by heat alone (Δ — Δ) (compare with \circ — \circ ; the apparent increase in stabilization by heating is probably not significant). When acted on by chymotrypsin for 60 min. there is a slight decrease in stabilization (∇ — ∇), and after 120 min. a considerable decrease (\square — \square). Both of these solutions lose their stabilization ability when heated at 90° C. (the negative stabilization slope indicates that some of the kappa-casein is precipitated with the calcium-sensitive casein).

In Figure 2 a preparation of kappa-casein is illustrated (\circ — \circ) that has some heat-lability without chymotrypsin treatment (\triangle — \triangle). Treatment with chymotrypsin for increasing periods (0, 15, 30, and 60 min.), followed by heat, leads to increasing inactivation of the kappa-casein. At 60 min., stabilization ability is completely lost (\blacktriangledown — \blacktriangledown), whereas treatment by chymotrypsin alone has

caused

The relation of these results to the properties of kappa-casein in milk, and the properties of kappa-casein in the isolated form, can only be guessed. It does suggest, however, as a subject for further study, that the naturally occurring protease in milk might be the cause of the variable heat-lability of various preparations of kappa-casein. The protease in milk may be of the chymotrypsin type, for it has been found that free tyrosine was released by its action (1). Furthermore, the protease in milk should be studied to see if it might be responsible for some of the adverse changes that occur in the storage of high-temperature, short-time sterilized concentrated milks.

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TECHNICAL NOTES

REFERENCES

- (1) HARPER, W. J., ROBERTSON, J. A., JR., AND GOULD, I. A. Observations on Milk Protease. *J. Dairy Sci.*, 43: 1850. 1960.
- (2) MATTENHEIMER, H., AND NITSCHMANN, H. Das Lab und Seine Wirkung auf das Casein der Milch. VIII. Die Abspaltung von Nicht-Protein-Stickstoff (NPN) aus Casein durch Verschiedene Proteolytische Fermente, Verglichen mit der Abspaltung durch Lab. *Helv. Chim. Acta*, 38: 687. 1955.
- (3) MCKENZIE, H. A., AND WAKE, R. G. An Improved Method for the Isolation of Kappa-Casein. *Biochim. et Biophys. Acta*, 47: 240. 1961.
- (4) WARNER, R. C., AND POLIS, E. On the Presence of a Proteolytic Enzyme in Casein. *J. Am. Chem. Soc.*, 67: 529. 1945.
- (5) ZITTELE, C. A. Column Chromatography of Casein on the Adsorbent Diethylaminoethyl (DEAE)-Cellulose. *J. Dairy Sci.*, 43: 855. 1960.
- (6) ZITTELE, C. A., CERBULIS, J., PEPPER, L., AND DELLAMONICA, E. S. Preparation of Calcium-Sensitive α -Casein. *J. Dairy Sci.*, 42: 1897. 1959.
- (7) ZITTELE, C. A., DELLAMONICA, E. S., AND CUSTER, J. H. Precipitation of Calcium Caseinate by Heat and Subsequent Reversal. *J. Dairy Sci.*, 39: 1651. 1956.