

1274

**The Stabilizing Fraction of  $\alpha$ -Casein**

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## INTRODUCTION

Berridge (1) has reviewed the theories of the clotting of casein. One of the theories discussed is the protective colloid theory, developed by Linderstrøm-Lang (2, 3) and by Holter (4), and supported by Nitschmann and Lehmann (5), and by recent data of Waugh and von Hippel (6) and of Wake (7). It is possible that the protective or stabilizing substance is the  $\kappa$ -casein fraction (6, 7).

In the present studies, calcium-sensitive  $\alpha$ -caseins, similar to  $\alpha$ -paracasein in their precipitation with  $\text{CaCl}_2$ , have been prepared<sup>2, 3</sup> using modifications of the urea method of Hipp *et al.* (8). It was found that the amount of soluble nitrogen and phosphorus released by the enzyme rennin was correlated with the calcium sensitivity of the  $\alpha$ -casein. This finding supports the protective colloid theory (2-4).

## EXPERIMENTAL

### *Rennin, Highly Purified*

The rennin was obtained through the courtesy of Dr. R. A. Sullivan, National Dairy Research Laboratories, L. I., N. Y. This rennin had the same specific activity as crystalline rennin.

### *Preparations of $\alpha$ -Casein*

Each preparation of casein is characterized by its precipitation with calcium ions. A preparation is termed "calcium-insensitive" when a 1% solution is not precipitated by 10 mmoles  $\text{CaCl}_2$ /l. at pH 7. The "calcium-sensitive" preparations, on the other hand, precipitate to the extent of 90% or more with the same concentration of  $\text{CaCl}_2$ . The extent of precipitation of the sensitive preparations with  $\text{CaCl}_2$  is similar to that of paracaseins.

### *Calcium-Insensitive $\alpha$ -Casein*

Calcium-insensitive  $\alpha$ -casein was prepared by the urea-solubility method of Hipp *et al.* (8). The  $\alpha$ -casein was washed with water and then dissolved in dilute NaOH

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<sup>1</sup> Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

TABLE I  
Action of Rennin on Casein: N and P in Solution at pH 4.7  
Per cent

Casein	Soluble before rennin		Soluble after rennin		Net change in soluble by rennin		Reaction with CaCl <sub>2</sub>
	N	P	N	P	N	P	
$\alpha$ -Casein (H1)	0.69	4.56	3.44	6.75	2.75	2.19	Calcium-insensitive
$\alpha$ -Casein (H2)	0.43	0.76	3.89	3.02	3.46	2.26	Calcium-insensitive
$\alpha$ -Casein (C1)	0.26	0.40	1.47	1.76	1.21	1.36	Medium-sensitive
$\alpha$ -Casein (C2)	0.38	1.02	1.02	1.92	0.64	0.90	Calcium-sensitive
$\alpha$ -Casein (Z1s)	0.32	0.51	1.12	1.55	0.80	1.04	Calcium-sensitive
$\alpha$ -Casein (Z1i)	1.80	0.52	8.15	4.40	6.35	3.88	Calcium-insensitive
Whole casein	0.35	0.91	4.81	3.56	4.46	2.65	Calcium-insensitive

and precipitated at the isoelectric point, pH 4.7, with HCl. Then the  $\alpha$ -casein was washed with water, ethanol, acetone, and ether. Preparations made by this procedure are marked *H* in Table I. This preparation is better named "whole  $\alpha$ -casein" since it contains components that are calcium-sensitive and calcium-insensitive. The term "calcium-insensitive" as used above is relative.

#### *Calcium-Sensitive $\alpha$ -Casein*

Several preparations were made as follows:<sup>2</sup> Acid-precipitated whole casein was dissolved in 6.6 *M* urea, and  $\alpha$ -casein was precipitated by diluting the solution with water to 4.6 *M* urea concentration (8). The mixture was left for 30 min. and then decanted [Hipp *et al.* (8) used centrifugation]. The whole procedure was repeated with the sediment once more. Then the  $\alpha$ -casein was stirred with 4.7 *M* urea, left for 30-40 min., and decanted. The washing with 4.7 *M* urea was repeated five times. Further purification and washings were carried out as described for the calcium-insensitive  $\alpha$ -casein. This procedure, unfortunately, has not always given calcium-sensitive  $\alpha$ -casein. Preparations by this method are marked *C* in Table I.

A procedure has been developed,<sup>3</sup> however, that regularly gives calcium-sensitive  $\alpha$ -casein in good yield. The regular urea-solubility method of Hipp *et al.* (8) was used with a simple modification. The crude  $\alpha$ -casein obtained from the first precipitation was dissolved in 6.6 *M* urea containing double the concentration of NaCl (31.8 g./1500 ml. instead of 15.9). The other steps remain the same. The product is designated *Z1s* in Table I. The portion remaining in solution with the double concentration of NaCl was precipitated with additional water. It is calcium-insensitive and is designated *Z1i* in Table I.

#### *Reaction of Rennin on Casein*

Two per cent casein solution was prepared by dissolving 10 g.  $\alpha$ -casein in a mixture of 56 ml. of 0.1 *N* NaOH and 440 ml. of distilled water. Then the solution was finally adjusted to pH 6.4 with a small amount of 0.1 *N* HCl or NaOH. Five milligrams en-

<sup>2</sup> J. Cerbulis, unpublished studies, 1957.

<sup>3</sup> C. A. Zittle, E. S. DellaMonica, J. Cerbulis, and L. Pepper, unpublished studies, 1958.

zyme was dissolved in 5 ml. water and added to the casein solution at 30°C. The reaction was run for a period of 5 min. at 30°C. The enzyme reaction was stopped by keeping the flask in a boiling water bath for 15 min. Then the flask was cooled quickly to room temperature and adjusted to pH 4.7 with HCl and centrifuged. The supernatant solution was analyzed for nitrogen and phosphorus.

#### *Analytical Methods*

The nitrogen contents of the proteins were determined using the Koch and McMeekin method (9). The phosphorus contents of the proteins were determined using the Koch and McMeekin technique (9) for digesting the samples, and a modified Sumner method (10) for the color development.

#### RESULTS AND DISCUSSION

The quantitative data are summarized in Table I. Results show that the largest amount of soluble N and P is released from the calcium-insensitive  $\alpha$ -casein when it is treated with rennin. The calcium-sensitive  $\alpha$ -casein gave the lowest amount of soluble N and P, and the medium-sensitive  $\alpha$ -casein gave the result between both extremes. Similar data were obtained when the enzyme pepsin was used instead of rennin. The released soluble N and P is correlated with the calcium sensitivity of  $\alpha$ -casein, presumably because the calcium-insensitive  $\alpha$ -casein contains more of the protective fraction than the calcium-sensitive  $\alpha$ -casein (6, 7). This protective fraction of the  $\alpha$ -casein complex is the primary target of rennin action (6, 7).

#### SUMMARY

The quantitative relation of soluble N and P released from  $\alpha$ -casein, treated with the enzyme rennin, has been studied. The amounts of soluble N and P are correlated with the calcium-sensitivity of the  $\alpha$ -casein. The calcium-insensitive  $\alpha$ -casein contains a larger amount of the protective fraction than does the calcium-sensitive  $\alpha$ -casein. These findings support the Linderstrøm-Lang (2, 3) and Holter (4) protective colloid theory of the clotting of casein by rennin.

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