

## Action of Rennin on $\kappa$ -Casein

### Abstract

The action of rennin on  $\kappa$ -casein was studied as a function of time using several methods to measure activity. The first indication of rennin cleavage of  $\kappa$ -casein is precipitability in .1 M acetate buffer at pH 5.2 and 5 C. A longer exposure to rennin is required to alter  $\kappa$ -casein so that it forms a precipitate with calcium ions and loses its ability to stabilize  $\alpha_s$ -casein. The least sensitive indication of rennin activity is measurement of nitrogen soluble in 2% trichloroacetic acid. Electrophoresis experiments showed that these methods detect various stages in the conversion of  $\kappa$ -casein to para- $\kappa$ -casein.

### Introduction

When rennin (EC 3.4.4.3) clots milk, the specific substrate hydrolyzed is  $\kappa$ -casein (10). Recent reviews have described the action of rennin on milk (11) and on  $\kappa$ -casein (7). Rennin is believed to attack  $\kappa$ -casein at pH 6.7 by cleaving one specific peptide bond, forming an insoluble para- $\kappa$ -casein and a soluble macro-peptide. The presence of  $\text{Ca}^{2+}$  is not essential for precipitation of para- $\kappa$ -casein (3, 5). Lawrence and Creamer (5) assayed rennin activity by measuring the turbidity that occurs prior to the precipitation of para- $\kappa$ -casein. By omitting  $\text{Ca}^{2+}$  in their reaction mixture, they felt that complications from the interaction of  $\text{Ca}^{2+}$  and para- $\kappa$ -casein would be avoided.

The action of rennin on  $\kappa$ -casein was measured at pH 6.7 with no added  $\text{Ca}^{2+}$ . I observed that  $\kappa$ -casein hydrolyzed by rennin precipitates on addition of .1 M acetate buffer (pH 5.2) at 5 C whereas  $\kappa$ -casein remains soluble under these conditions. Because this appeared to be a sensitive method of determin-

ing rennin activity, I examined the reaction and compared it to other methods of testing rennin activity.

### Materials and Methods

Kappa-casein and  $\alpha_s$ -casein were prepared by the method of Zittle and Custer (12). Polyacrylamide gel electrophoresis of  $\kappa$ -casein at pH 8.6 showed no contamination with other caseins. The conversion of  $\kappa$ -casein to para- $\kappa$ -casein was examined by polyacrylamide gel electrophoresis at pH 3.0 by the method of Peterson and Kopfler (8). Crystalline rennin (81 units per mg) was purchased from Calbiochem<sup>2</sup>.

Protein was determined by ultraviolet absorption at 290 nm with .1 N NaOH to dissolve the proteins. The extinction coefficient of  $\kappa$ -casein in .1 N NaOH at 290 nm is identical to that at 280 nm at pH 6.7. Protein concentration was expressed as absorbance.

Solubility of  $\kappa$ -casein was determined from pH 3.3 to 5.4 with acetate buffers. The acetate buffers were made by combining .2 M sodium acetate and .2 M acetic acid to the desired pH. Kappa-casein was dissolved in water, adjusted to pH 6.7 with .1 M NaOH, diluted to a concentration of 10 mg/ml, and cooled to 5 C. One milliliter of .2 M acetate buffer (5 C) was added to 1 ml of the  $\kappa$ -casein solution. The pH of the acetate buffer was not changed by addition of  $\kappa$ -casein. The mixture was then centrifuged 10 min at 5 C at 2,000  $\times$  g and the protein concentration in the supernatant determined. Similar experiments were performed after rennin had acted on  $\kappa$ -casein for designated times.

Rennin activity was measured in a reaction mixture containing 10 mg per ml of  $\kappa$ -casein adjusted to pH 6.7 with .1 N NaOH and rennin (.1  $\mu\text{g}$  per ml  $\kappa$ -casein solution). The solution was incubated at 30 C for various periods. Rennin activity was evaluated in several ways:

1) A 1 ml aliquot of the reaction mixture was cooled to 5 C, and 1 ml of .2 M acetate buffer (pH 5.2) was added. The mixture was centrifuged at 2,000  $\times$  g for 10 min at 5 C and the amount of protein in the precipitate determined. Under these conditions  $\kappa$ -casein is soluble; the formation of a precipitate occurs only if rennin has acted.

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<sup>1</sup> Agricultural Research Service, United States Department of Agriculture.

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

2) A 1 ml aliquot of the reaction mixture was added to 1 ml of .1 M  $\text{CaCl}_2$  (pH 6.7). The mixture was centrifuged at  $2,000 \times g$  at room temperature. The amount of protein in the precipitate was determined.

3) An aliquot of the  $\kappa$ -casein reaction mixture was heated at 80 C for 10 min to inactivate the rennin. Kappa-casein was then tested for its ability to stabilize  $\alpha_s$ -casein against precipitation by  $\text{Ca}^{2+}$ . Stabilization curves were obtained by the method of Zittle and Custer (12).

4) Trichloroacetic acid (TCA)-soluble nitrogen is liberated during the action of rennin (1). An equal volume of 4% TCA was added to the assay mixture, and the solution was filtered. The nitrogen was then determined on the filtrate by the Kjeldahl method (2).

5) The time for precipitation of  $\kappa$ -casein following the addition of rennin was noted.

### Results

*Precipitate formation by acetate buffer.* When the ratio of rennin to  $\kappa$ -casein is low (1:100,000), no evidence of hydrolysis is visible for several hours; turbidity followed by precipitation is not evident for 20 h. When  $\kappa$ -casein is precipitated at 5 C with acetate buffers (pH 3.3 to 5.4), maximum precipitation occurs at pH 4.5 as shown in Fig. 1. However, rennin produces changes in the solubility properties of  $\kappa$ -casein (Fig. 1). The solubility curves shift towards the alkaline side as the reaction proceeds, suggesting an increase in the isoelectric point of the proteins. Because the

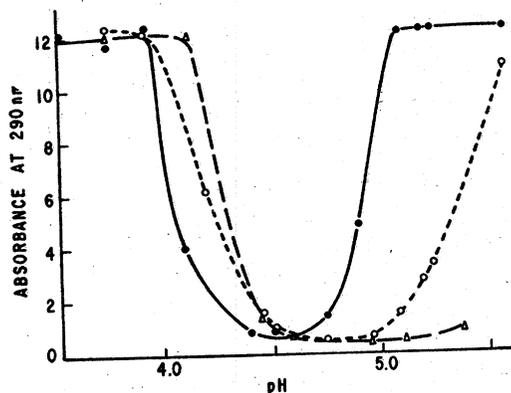


FIG. 1. Solubility of  $\kappa$ -casein treated with rennin as a function of pH. Procedure is described under "Materials and Methods". One percent  $\kappa$ -casein was incubated with rennin (.1  $\mu\text{g}/\text{ml}$ ) at 30 C for zero time (●-●-●); 30 min (○-○-○); 75 min (Δ-Δ-Δ).

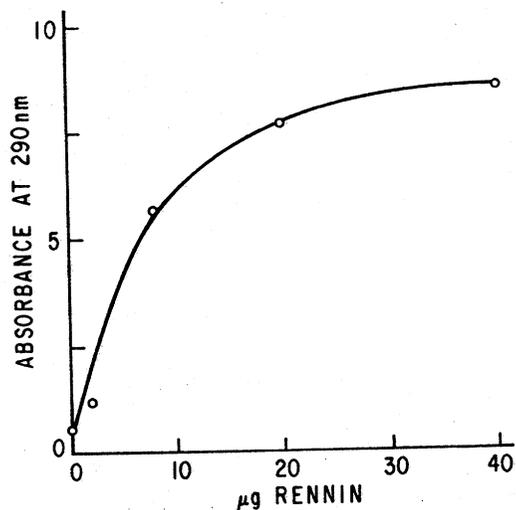


FIG. 2. The effect of rennin on  $\kappa$ -casein as measured by the amount of precipitate formed by the addition of acetate buffer at pH 5.2 and 5 C. One milliliter of 1%  $\kappa$ -casein was incubated with varying amounts of rennin at 30 C for 1 min at pH 6.7.

macropeptide split off from  $\kappa$ -casein contains the acidic portion of the molecule, a decreased negative charge on  $\kappa$ -casein should occur following hydrolysis by rennin. The effect is most

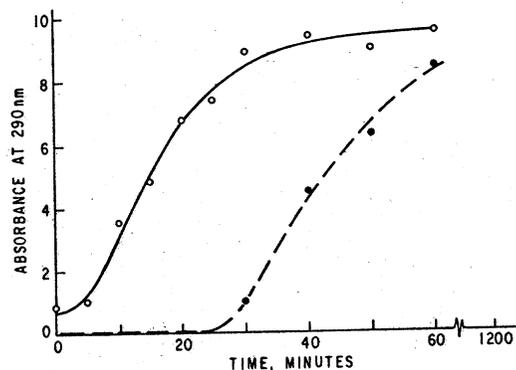


FIG. 3. Rennin activity vs time as measured by the appearance of precipitated protein. The reaction mixture contained 1%  $\kappa$ -casein and .1  $\mu\text{g}$  rennin/ml at pH 6.7 and 30 C. One milliliter aliquots were removed at the indicated times and the amount of precipitated protein determined. In one experiment, the protein was precipitated by the addition of 1 ml of .2 M acetate buffer at pH 5.2 and 5 C (○-○-○). In the second experiment, the protein was precipitated by the addition of 1 ml of .1 M  $\text{CaCl}_2$  (●-●-●). Protein in the precipitate was determined. The time of spontaneous precipitation is indicated by the arrow.

evident at pH 5.2. At this pH the solubility of  $\kappa$ -casein exceeds 10 mg/ml. However, the solubility of  $\kappa$ -casein decreases following hydrolysis by rennin (Fig. 1). Therefore, precipitation at pH 5.2 and 5 C was investigated as it seemed to be a sensitive method of assaying the effect of rennin on  $\kappa$ -casein. Fig. 2 shows the effect of rennin concentration on the precipitate formation at pH 5.2. The amount of protein that is precipitated by acetate buffer increases as the concentration of rennin is increased.

**Precipitation by calcium ions.** The action of rennin on  $\kappa$ -casein can be measured by the amount of precipitate formed when  $\text{CaCl}_2$  is added. Fig. 3 compares the amount of precipitate formed as a function of time of exposure to rennin using  $\text{CaCl}_2$  or acetate buffer to precipitate the proteins. Maximum precipitation by acetate buffer (pH 5.2, 5 C) occurs after 30 min exposure while complete precipitation by  $\text{Ca}^{2+}$  requires 60 min of rennin action. Spontaneous precipitation, which occurs without the addition of  $\text{CaCl}_2$  or acetate buffer, takes considerably longer (approximately 20 h).

**Stabilization of  $\alpha_s$ -casein by  $\kappa$ -casein treated with rennin.** Kappa-casein stabilizes  $\alpha_s$ -casein in the presence of  $\text{Ca}^{2+}$  (10). Without  $\kappa$ -casein the  $\alpha_s$ -casein precipitates. Kappa-casein was treated with rennin at pH 6.7, and at various times two aliquots were removed. One aliquot was tested for the formation of a precipitate with acetate buffer (pH 5.2), and the results are shown in Fig. 4a. A second aliquot was heated to 80 C for 10 min to inactivate the rennin and was then tested for its ability to sta-

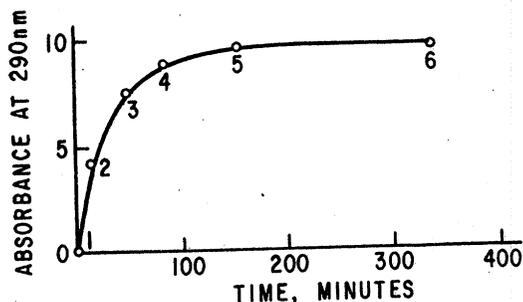


FIG. 4a. Rennin activity vs time as measured by the appearance of precipitated protein. The reaction mixture contained 1%  $\kappa$ -casein and .05  $\mu\text{g}$  rennin/ml at pH 6.7 and 30 C. One ml aliquots were removed at the indicated times and cooled to 5 C. The proteins were precipitated by the addition of 1 ml of .2 M acetate buffer (pH 5.2 and 5 C). After centrifugation, the protein in the precipitate was determined.

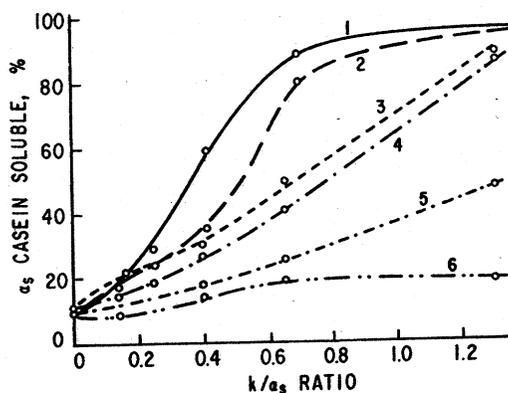


FIG. 4b. The effect of  $\kappa$ -casein treated with rennin on the stabilization of  $\alpha_s$ -casein in the presence of  $\text{Ca}^{2+}$ . Aliquots were removed at the times indicated in Fig. 4a, heated at 80 C for 10 min to inactivate the rennin, then tested for their ability to stabilize  $\alpha_s$ -casein. The sample numbers in Fig. 4a correspond to the numbers on the curves. Stabilization curves were run according to the procedure of Zittle and Custer (12). There was 1.25 mg/ml  $\alpha_s$ -casein. Kappa-casein was added to obtain the  $\kappa/\alpha_s$  ratio indicated on the graph. The  $\text{CaCl}_2$  concentration was .01 M. The solutions were incubated for 15 min at 30 C and then centrifuged for 5 min at  $3,000 \times g$ . The supernatant protein was determined and expressed as percent soluble casein. No correction was made for the  $\kappa$ -casein hydrolyzed by rennin.

bilize  $\alpha_s$ -casein in the presence of  $\text{Ca}^{2+}$ . As in Fig. 4b, when  $\kappa$ -casein was hydrolyzed by rennin, the ability of  $\kappa$ -casein to stabilize  $\alpha_s$ -casein was reduced. This effect occurs early in the reaction and seems to parallel precipitation by acetate buffer at 5 C.

**Liberation of TCA-soluble nitrogen by rennin.** The action of rennin on  $\kappa$ -casein can be followed by estimation of the nitrogen soluble in 2% TCA. Fig. 5 compares liberation of 2% TCA-soluble nitrogen with formation of a precipitate by acetate buffer. Two rennin concentrations were used, .1  $\mu\text{g}$  per ml and 1.0  $\mu\text{g}$  per ml. For .1  $\mu\text{g}$  per ml of rennin, no changes were visible in the  $\kappa$ -casein solution. When the rennin concentration was increased tenfold, the solution became milky in appearance after 152 min and soon precipitated. Both reactions show that less than 9% of the TCA-soluble nitrogen is liberated when 90% of the protein can be precipitated by acetate buffer. MacKinnlay and Wake (7) have shown that the macropeptide represents 30% of the nitrogen of  $\kappa$ -casein. Therefore, precipitation by acetate buffer occurs when only one-third of the macro-

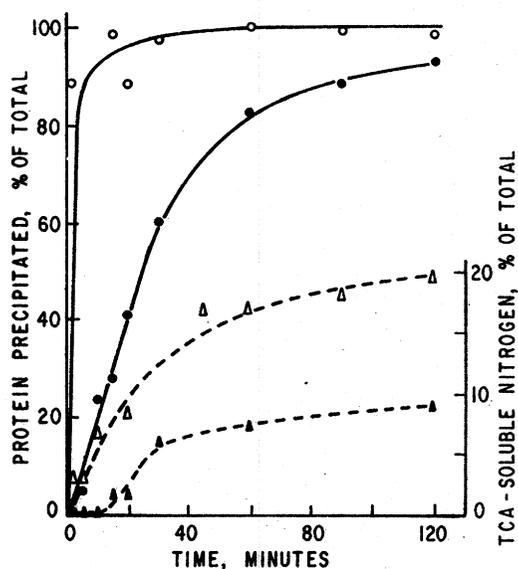


FIG. 5. The action of rennin (1  $\mu\text{g}/\text{ml}$  and .1  $\mu\text{g}/\text{ml}$ ) on a 1% solution of  $\kappa$ -casein (pH 6.7, 30 C) vs time. In one experiment, 1 ml aliquots were precipitated by .2 M acetate buffer at pH 5.2 and 5 C. The precipitated protein was measured; rennin concentration was 1  $\mu\text{g}/\text{ml}$  (○—○—○) and 0.1  $\mu\text{g}/\text{ml}$  (●—●—●). In the second experiment, supernatant nitrogen was measured on a 1 ml aliquot following the addition of 1 ml 4% TCA; rennin concentration was 1  $\mu\text{g}/\text{ml}$  ( $\Delta$ — $\Delta$ — $\Delta$ ) and .1  $\mu\text{g}$  ( $\blacktriangle$ — $\blacktriangle$ — $\blacktriangle$ ).

peptide has been released.

**Gel electrophoresis.** Aliquots of  $\kappa$ -casein which had been incubated with rennin were lyophilized and subjected to electrophoresis. Fig. 6 shows the electrophoretic patterns, which are typical of acid gels. Sample 1 shows  $\kappa$ -casein. Samples 2 and 3 show the effect of incubating 10 mg of  $\kappa$ -casein with .1  $\mu\text{g}$  of rennin for 30 and 75 min. There was no evidence of turbidity in these solutions. Samples 4 and 5 show the effect of increasing the rennin concentration tenfold to 1  $\mu\text{g}$  with incubation

TABLE 1. Rennin activity: time required to complete half of the reaction.

Treatment of sample	Time <sup>a</sup>
Precipitation by acetate buffer, pH 5.2 and 5 C	100
Precipitation by $\text{Ca}^{2+}$	271
Stabilization of $\alpha_s$ -casein	252
Liberation of TCA <sup>b</sup> -soluble nitrogen	1,600

<sup>a</sup> The precipitation by acetate buffer was 100 and the other time values are relative to this.

<sup>b</sup> Trichloroacetic acid.

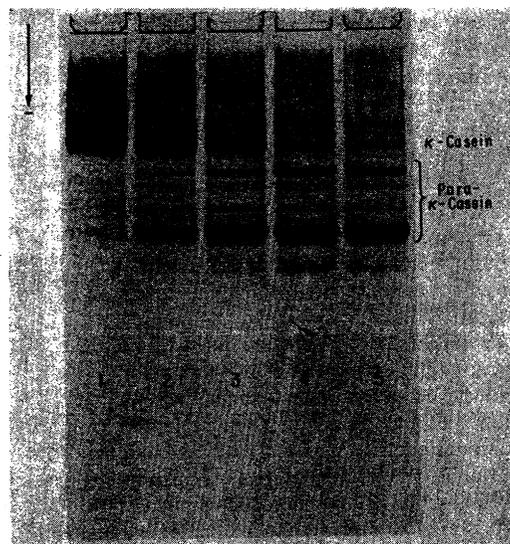


FIG. 6. Polyacrylamide-gel electrophoresis run at pH 3.0. One ml  $\kappa$ -casein (10 mg) was incubated with rennin. At a specified time, the samples were frozen and lyophilized. (1)  $\kappa$ -casein; (2)  $\kappa$ -casein, .1  $\mu\text{g}$  rennin, 30 min; (3)  $\kappa$ -casein, .1  $\mu\text{g}$  rennin, 75 min; (4)  $\kappa$ -casein, 1.0  $\mu\text{g}$  rennin, 15 min; and (5)  $\kappa$ -casein, 1.0  $\mu\text{g}$  rennin, 30 min.

times of 15 and 30 min. These solutions were very turbid. Sample 5 shows predominantly para- $\kappa$ -casein. However,  $\kappa$ -casein was in all the samples to some extent.

**Comparison of experiments.** For comparison of the experiments, times were computed at which half of the reaction was completed. A rough estimate of half-times could be obtained by examination of the graphs. The results are summarized in Table 1. In general, the experiments fall into three categories of half-times of the reaction. Precipitation by acetate buffer gives the first indication of rennin action. Precipitation by calcium ions occurs at approximately the same time that  $\kappa$ -casein has lost its ability to stabilize  $\alpha_s$ -casein in the presence of calcium ions. The least sensitive indications of rennin activity is the amount of nitrogen soluble in 2% TCA.

## Discussion

These experiments are based on marked changes in the solubility properties of  $\kappa$ -casein in the region of its isoelectric point when hydrolyzed by rennin. On this observation, the effect of rennin on  $\kappa$ -casein was assayed by measuring the protein precipitated by acetate buffer at pH 5.2 and 5 C. Precipitation of the reactants at pH 5.2 could be observed before

precipitation by calcium ions or before TCA-soluble nitrogen was liberated. Therefore, this method may have value as a sensitive assay for rennin activity. The assay method has some disadvantages of clotting methods. Like clotting the assay measures two parameters: the proteolytic action of rennin, followed by the precipitation of protein. It is, therefore, important for the investigator to know which reaction is affected when the conditions of assay are altered.

Enzyme reactions can be measured either by the appearance of product or the disappearance of substrate. The products obtained when rennin hydrolyzes  $\kappa$ -casein are para- $\kappa$ -casein and a macropeptide. The liberation of TCA-soluble nitrogen measures macropeptide formation while precipitation or clotting is due to formation of an insoluble para- $\kappa$ -casein. These two reactions have been studied extensively and have been reviewed by Lindquist (6) and Foltmann (4).

The presence of substrate, native  $\kappa$ -casein, can be shown in several ways. Kappa-casein is soluble at pH 5.2 at 5 C in .1 M acetate buffer, is soluble in the presence of .05 M  $\text{CaCl}_2$ , and stabilizes  $\alpha_s$ -casein in the presence of calcium ions. The disappearance of  $\kappa$ -casein can be measured by these parameters. My experiments show that  $\kappa$ -casein disappears in 1 to 2 h, yet product formation, as measured by TCA-soluble nitrogen, is low. The experiments indicate that disappearance of  $\kappa$ -casein occurs before the final product is obtained.

A possible explanation for these results is that an intermediate is formed in the conversion of  $\kappa$ -casein to para- $\kappa$ -casein. If the precipitate formed by  $\text{Ca}^{2+}$  represents an intermediate, one experiment confuses the problem. Despite all solubility criteria showing that  $\kappa$ -casein has disappeared,  $\kappa$ -casein is evident by polyacrylamide electrophoresis experiments. One explanation can be offered. The gel electrophoresis was run in the presence of urea and mercaptoethanol. Under these conditions  $\kappa$ -casein is a monomer. In aqueous solutions,  $\kappa$ -casein is a high molecular weight polymer (9). Perhaps  $\kappa$ -casein loses its distinguishing characteristics when rennin has acted upon the exterior of the polymer. The  $\kappa$ -casein on the inside of the aggregate remains intact, but its properties are masked. Therefore, the substrate could be considered colloidal  $\kappa$ -casein rather than monomeric  $\kappa$ -casein.

Another explanation is that the para- $\kappa$ -casein has different solubility characteristics with  $\kappa$ -casein and that unhydrolyzed  $\kappa$ -casein inter-

acts with para- $\kappa$ -casein, keeping it in solution. The precipitability of the hydrolyzed  $\kappa$ -casein, therefore, may depend on the ratio of para- $\kappa$ -casein to  $\kappa$ -casein in a highly reacting system.

Wheelock et al. (11) suggested that the  $K_m$  for rennin action on casein depends on the carbohydrate composition of the molecule. His results show that carbohydrate-free fraction of  $\kappa$ -casein is hydrolyzed preferentially because of its lower  $K_m$  value. Further experimentation is needed to determine whether there is a significant difference in the action of rennin as measured by the above assay on  $\kappa$ -casein with and without carbohydrate.

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