Factors Affecting Stability of Rennin 1, 2

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Abstract

The effects of temperature, pH, ionic strength, and ionic species on the stability of rennin were studied. Stability was determined by measuring decreases in clotting activity during 96 hr of incubation. Above pH 6.0, activity losses increased with pH, and were highly temperature dependent. They were accompanied by a precipitation of protein and appeared to be the result of pH denaturation. Maximum stability was maintained from pH 5.0 to 6.0. A region of instability was observed between pH 3.0 and 4.9 in buffers of 0.03 ionic strength. An instability maximum at pH 3.8 resulted in a loss of 35% of initial activity at 30°C over 96 hr. When the ionic strength was increased from 0.03 to 1.0 with NaCl, with other conditions constant, activity losses increased from 35 to 70%. Under the same conditions of pH, ionic strength, and temperature, rennin was more stable in sodium citrate, sodium lactate, and ammonium sulfate than in sodium chloride or potassium chloride. Below pH 4.5, activity losses were accompanied by an increase in the amount of Ruhemann’s purple formed by the action of ninhydrin on the rennin mixture. This suggested that activity losses were due in part to self-digestion.

Cheese rennet is a highly standardized extract of the abomasum of young calves and contains the milk clotting enzyme rennin. Purification and crystallization of rennin (1, 10) have made possible studies of the biochemical nature of this enzyme.

Rennin stability is affected by heat (5, 15, 16), ultrasonic vibration (18), and alkali (3, 11, 12). Foltman (6) found that solutions of crystalline rennin had maximum stability between pH 5.5 and 6.0, with moderate stability at pH 2. Between pH 3 and 4 and above pH 6.5, the enzyme was unstable. Above pH 6.5, inactivation of rennin was more temperature dependent than between pH 3 and 4. The latter zone of inactivation corresponds to the pH at which rennin exhibits optimum proteolytic activity on bovine serum albumin, hemoglobin, and casein (2, 6, 9). It was reported (4) that rennin split into four electrophoretic peaks during boundary electrophoresis in buffers of ionic strength 0.033 at pH 6.8. Foltman (7) subsequently fractionated crystalline rennin into one inactive and three active components. The active components were designated Rennins A, B, and C, on the basis of specific activity. Further studies (8, 9) demonstrated the proteolytic degradation of A rennin into C rennin at pH 3.5, with a significant reduction in activity. Thus, it would appear that the activity loss between pH 3 and 4 might be due to autolysis.

Tsugo and Yamauchi (17) reported that blood serum inhibited the milk-clotting activity of rennin. Although the specific component of blood serum responsible for inhibition was not reported, it was inactivated by heat and certain chemicals. Reports from industry suggest that rennin activity may be affected by agents and conditions commonly employed in extraction and purification of rennet extract.

The purpose of this study was to investigate factors affecting the stability and activity of rennin. Further progress in rennin research and in the economic utilization of veils (calf stomachs) will require accurate information on conditions under which this enzyme is stable.

Experimental Procedures

Crystallization of rennin. Rennin was crystallized from commercial rennet extract 5 by a procedure previously described (4), except that 0.1 M disodium phosphate rather than 0.1 M sodium hydroxide was used to neutralize the potassium alum to pH 6.3. This modification resulted in a much greater yield of purified rennin.

Rennin activity. Rennin activity was determined by comparing the activity of each unknown solution with that of a standard rennet

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1 Received for publication November 14, 1966.
2 Published with the approval of the Director, Wisconsin Agricultural Experiment Station.
3 This study was supported in part by a research grant from Dairyland Food Laboratories, Inc., Waukesha, Wisconsin.
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extract that has been described, and assigned an activity value of 100 rennin units (RU) per milliliter (4, 13).

**Measurement of rennin stability.** Crystalline rennin was dissolved in dilute acetic acid and immediately dialyzed against cold 0.1 M phosphate buffer at pH 6.3. It was then dialyzed exhaustively against cold distilled water, and lyophilized. The dry amorphous rennin was suspended in distilled water to make a stock suspension containing approximately 525 rennin units per milliliter. Two milliliters of the stock suspension were added to 12 ml of an appropriate buffer solution at a desired temperature, ionic strength, and pH. Stability was measured by determining loss of milk-clotting activity during 96 hr of incubation at 2, 10, 20, and 30 C.

**Preparation of buffer solutions.** Stability studies were carried out between pH 2.5 and 6.8, at ionic strengths of 0.03 and 1. The following buffer solutions were used: glycine-HCl from pH 2.5 to 3.0, lactic acid-sodium lactate from pH 3.0 to 4.0, acetic acid-sodium acetate from pH 4.0 to 5.2, cacodylic acid-sodium cacodylate from pH 5.2 to 6.0, and sodium acid phosphate-disodium phosphate from pH 6.0 to 6.8. Sodium chloride was added to the above buffers to increase the net ionic strength from 0.03 to 1. Potassium chloride and ammonium sulfate were added in like manner to lactic acid-sodium lactate buffer at pH 3.8, to study the effects of specific ions on rennin stability. Also, citric acid–sodium citrate and lactic acid–sodium lactate buffers were prepared to yield ionic strengths of 1 at pH 3.8 for studies involving the effect of specific ions on rennin stability.

**Results**

**Effect of temperature and pH.** Figure 1 shows the percentage of rennin activity lost during 96 hr of incubation at 2, 10, 20, and 30 C, at various pH values between 2.5 and 6.8 and at an ionic strength of 0.03. Above pH 6.0, activity losses increased with pH and were highly temperature dependent. They were accompanied by a precipitation of protein and appeared to be the result of pH denaturation. Maximum stability was observed from pH 5.0 to pH 6.0. In agreement with findings of Foltman (6), a region of instability was noted between pH 3.0 and pH 4.9. Loss of activity in this region, in contrast to that observed above pH 6.0, was not accompanied by a precipitation of protein, and it was also less temperature dependent than at pH 6.8. Rennin appeared to be moderately stable below pH 3.0.

**Ionic strength.** The effect of ionic strength contributed by sodium chloride on the stability of rennin activity is shown in Figure 2. Activity losses below pH 4.5 increased sharply with increasing concentration of sodium chloride. The isoelectric point of rennin is approximately pH 4.5 (10, 14). Below that pH, rennin has a net positive charge, suggesting the possibility...
of chloride ion binding. If ion binding were related to activity loss, one might expect a recovery of activity if the inactivated rennin should be brought to a pH above its isoelectric point.

Solutions of rennin, as shown in Table 1, were prepared in the following buffers and incubated at 30 C: (1) glycine-HCl at pH 2.55, ionic strength 0.03, (2) glycine-HCl at pH 2.55, ionic strength 1.0 (0.03 from buffer, 0.97 from NaCl). Both solutions were tested for rennin activity after 0 and 72 hr. Solution Two was then divided into three portions (2A, 2B, 2C), and dialyzed 24 hr at 3 C against (2A) glycine-HCl buffer at pH 2.55, ionic strength 0.03; (2B) redistilled water; (2C) cacodylate buffer at pH 6, ionic strength 0.03. Volume changes during dialysis were noted, so that corrections could be made for dilution upon subsequent measurement of rennin activity.

Data in Table 1 show that exhaustive dialysis, under the three conditions of this experiment, failed to restore any of the rennin activity lost during previous incubation in the presence of sodium chloride.

Specific ion effect. Since sodium chloride was especially destructive to rennin below its isoelectric point, the possibility of other salts having a similar effect was investigated. A comparison is shown in Figure 3 of rennin activity lost during incubation at pH 3.8 for 96 hr at 30 C, in the presence of the anions chloride, sulfate, citrate, and lactate and the cations ammonium, sodium, and potassium.

Activity loss in control Sample A, containing only lactate and sodium ions at an ionic strength 0.03, was 35%. This agreed with results previously obtained, as illustrated in Figure 1. When the ionic strength was increased from 0.03 to 1.0 with sodium chloride (B), a loss of 70% was observed. This also corresponds to losses shown in Figure 2, under the same conditions. Substitution of potassium chloride for sodium chloride resulted in a similar loss in rennin activity (C). When sulfate ions were substituted for chloride ions, and ammonium ions for sodium and potassium ions (D), activity losses at pH 3.8 were markedly reduced. Losses under these conditions were comparable to those sustained at low (0.03) ionic strength. Likewise, the activity loss was the same as the control when citrate buffer was used at an ionic strength of 1, with all the ionic strength being contributed by the buffer (E). In a solution of ionic strength 1.0, in which all the ions were contributed by sodium and lactate (F), a loss of only 17% of the initial activity was observed.

These results suggest that chloride ions were...
more destructive to rennin activity at pH 3.8 than any of the ions studied. It would appear that sodium lactate at high ionic strength increased the stability of rennin over that of the control.

*Nature and rate of activity loss.* The possibility of proteolytic degradation accompanying activity losses was studied by following changes in the ninhydrin reaction of rennin solutions at pH 3.8 during incubation at 30°C. Eighty milligrams of rennin were dissolved in each of two 40-ml solutions of sodium lactate buffer. The first solution contained only the buffer at an ionic strength of 0.03. The second contained the same concentration of buffer, but the ionic strength had been increased to 1.0 by addition of sodium chloride. Aliquots from each solution were removed periodically for analysis, up to 120 hr. One milliliter of an appropriate dilution of the sample, plus 1 ml ninhydrin reagent (95 g KH₂PO₄, 43 g Na₂HPO₄, 5 g triketohydridene hydrate, and 3 g fructose per liter) were placed in a covered test tube and immersed in a boiling-water bath for 15 min. After cooling, 5 ml of diluent (2 g KIO₃ in 600 ml H₂O plus 400 ml 95% ETOH) were added to the colored solution. Figure 4 shows a plot of reaction time vs. absorbancy at 570 μ, and percentage of activity lost. The increase in absorbancy during incubation, which accompanied losses of activity, suggested that proteolytic breakdown was the cause of instability. This provides further support for the proposal that rennin is capable of digesting itself under these conditions (6).

*Effect of competitive substrate.* Since loss of rennin activity at pH 3.8 was accompanied by proteolytic degradation, an experiment was designed to study the effect of a competitive protein substrate on activity losses in rennin solutions; the question being, Would rennin hydrolyze the competitive protein, and degrade itself at a slower rate? Hemoglobin was selected for the experiment, because it was known to be readily hydrolyzed by rennin between pH 3.0 and 4.0 (2).

Five milligrams rennin were dissolved in 25 ml of each of the following sodium lactate buffers at pH 3.8: 1) \( \Gamma/2 = 0.03 \) buffer + 0.97 NaCl, 2) \( \Gamma/2 = 0.03 \) buffer + 0.97 NaCl + 5 mg he-

![Graph showing the increase in ability to react with ninhydrin during inactivation of rennin at pH 3.8 (30°C).](image-url)
moglobin, 3) $\Gamma/2 = 0.03 + 0.97\ NaCl + 25\ mg$ hemoglobin, 4) $\Gamma/2 = 0.03$, 5) $\Gamma/2 = 0.03 + 5\ mg$ hemoglobin, and 6) $\Gamma/2 = 1$, contributed by the buffer. The samples were incubated at 25 C, and aliquots removed for activity tests at 8-hr intervals for 40 hr. Results, shown in Figure 5, indicate that the hemoglobin did not retard activity losses of the rennin.

Discussion

This study demonstrated that rennin is relatively stable between pH 5.0 and 6.0, and unstable above pH 6.0 and between pH 3.0 and 4.9. This is in agreement with findings of Foltman (6). Activity losses above pH 6.0, accompanied by precipitation of protein from solution, appeared to be highly temperature dependent and were attributed to pH denaturation. Activity losses between pH 3.0 and 4.9 were less temperature dependent than those above pH 6.0, and no protein precipitation was evident. This suggested that activity losses in these two regions were due to different phenomena. Evidence of proteolysis which accompanied activity losses between pH 3.0 and 4.9 supported the proposal that instability in this region was due to autolysis.

Increasing the ionic strength from 0.03 to unity with sodium chloride was particularly damaging to rennin below pH 4.5. Stability tests in the presence of a number of anions and cations demonstrated that chloride was responsible for this rapid deterioration of activity. Further evidence was found that high ionic strength contributed by lactate ions actually gave a protective effect to rennin and reduced rate of activity loss to less than that in control samples with an ionic strength of 0.03.

It was noted that significant activity losses in some high ionic strength buffers occurred at pH values near or below the isoelectric point of rennin. A logical assumption was that anions were being bound to the positively charged rennin molecules. If activity losses were due to simple anion binding, adjustment of the pH to values above the isoelectric point should release the bound ions and restore activity. However, this did not occur. Incubation of rennin at pH 3.8 and 30 C resulted in proteolysis, as indicated by the increasing concentration of Ruhemann’s purple formed by the action of ninhydrin on the rennin solution. Proteolysis was evident at an ionic strength of 0.03, but was more pronounced when the ionic strength was increased to unity with sodium chloride. Sodium ions were vindicated by parallel stability tests, using combinations of ions. These same tests showed that chloride ions were responsible for accelerating activity losses. This indicated that in some way these ions seem to hasten autolysis of rennin.

Prorennin is the inactive precursor of rennin, and in commercial rennet manufacture is extracted from calf stomach tissue with sodium chloride solutions. It is subsequently activated with hydrochloric acid at pH values as low as 4.6. Activation is allowed to continue until there is no further increase in activity. This may require up to 36 hr. After activation, the extract is neutralized to pH 5.7. It was previously reported (13) that activation of prorennin was greatly affected by pH and sodium chloride concentration. Maximum activation was realized when the pH was not allowed to fall below 5.0. Activation below pH 5.0 was rapid, but gave reduced yields (13). This study demonstrated that commercial activation losses that occur below pH 5.0 might be attributed to autolysis of rennin in the presence of a high concentration of chloride ions.

References

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