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Studies on Rennin

III. On the Solubility of Rennin

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Rennin crystals show a slow solubility rate. They are very soluble in distilled water (more than 8 %) and very slightly soluble in concentrated salt solutions (about 0.02 % in 1 N NaCl, pH 5.5). Amorphous precipitates prepared of crystallised rennin fulfil the requirements of the phase rule for a single-component system at the pH of optimum stability. Amorphous rennin shows a solubility minimum in Na-acetate buffers at ionic strength 0.1, pH 5.45. In NaCl solutions, ionic strength 2.5 to 4.85, the log.sol. decreases linearly with ionic strength. The solubility around the isoelectric point (pH 4.6) is small, 0.01 % at ionic strength 0.005, increasing with rising ionic strength.

1. INTRODUCTION

Previous studies on rennin have only contained few remarks on its solubility. Hankinson and Palmer [1] have stated that purified, non-crystallised rennin behaves like a globulin, soluble in a dilute NaCl solution, precipitating at pH 4.5—4.7 (the isoelectric point), insoluble in saturated salt solutions. Berridge [2] has stated that rennin crystals have a slow solubility rate and a very low solubility in salt solutions. Berridge [2] also made a solubility test for homogeneity using an $MgCl_2$/Na-acetate buffer as the liquid phase.

As rennin preparations are most often made using precipitations with NaCl, and as divalent metal ions will interfere in the milk-clotting test, the following experiments are made with NaCl-solutions buffered with Na-acetate.

The crystalline rennin used in these experiments was prepared and recrystallised twice as described earlier (Foltmann [3]). During the experiments, it was very difficult to get exact, reproducible results when working with rennin crystals, as these have a very slow rate of solubility in salt solutions, and as the final phase of the crystallisation also proceeds slowly. The experiments in the sections 3.2, 3.3 and 3.4 were therefore performed using amorphous precipitates of rennin prepared in the following way: Rennin crystals were dissolved by dialysis against distilled water, and by rapid mixing of the rennin solution with the solvent in question, the rennin was precipitated in an amorphous state.

2. EXPERIMENTAL

The rennin activity was assayed and expressed in rennin units (RU) as previously described [3]. The results may be converted to mg rennin after dividing by 140, as the rennin used had an activity of 140 RU/mg dry weight. The optical density of the solutions was determined on a Beckman spectrophotometer DU at 277.5 mμ.

The solubility experiments were made in 2—3 ml centrifuge tubes closed by a rubber stopper. In order to avoid air-bubbles in the tubes during the experiments, the tubes were closed, using a thin syringe needle — through which the air could escape — beside the rubber stopper. However, it was very difficult to expel all the air, and during most of the experiments, air was present in the tubes. Control experiments have shown that the loss of enzymatic activity due to surface inactivation on the air-bubbles in the tubes was negligible.

The tubes were attached to a rectangular grid by means of a rubber band. For experiments with amorphous precipitates, equilibrium between the solid and the liquid phases was ensured by mechanical inversion of the tubes (3 inversions per min) during 18—20 h. For experiments with the crystalline form, the inversions were continued for up to 10 days. The experiments at 2°C were performed in a refrigerator in which the temperature was controlled to ± 1°.

The solid phase was separated from the liquid phase by means of centrifugation in the refrigerator. A possible rise of temperature during the centrifugation could not be observed. For experiments at 25°C, equilibration took place in a water thermostat (± 0.1°C). Centrifugation took place at room temperature, 23°C ± 2°.

As the precipitates of amorphous rennin were formed by mixing rennin solutions with a solvent, the equilibrium was in most cases arrived at from the supersaturated side. However, by salting-out in concentrated salt solutions, where rennin in the amorphous state is more soluble at 2° than at room temperature, the equilibrium was arrived at from both unsaturated and supersaturated solutions.

The unsaturated conditions were established by mixing the rennin and salt solutions at room temperature, and letting them stand at this temperature for 3 h before the inversion process was started in the refrigerator. The supersaturation was obtained by cooling the rennin and salt solution to 2°C before mixing and inversion.

3. RESULTS

3.1. Solubility of rennin crystals

Exact determinations of solubility of rennin crystals in solutions of NaCl are very difficult to carry out. The solubility rate is very slow. When working with a slight surplus of solid phase, even 8 days of inversion of the tubes in the refrigerator will not be sufficient to establish equilibrium. However, it is observed that rennin crystals are very soluble in distilled water. The most concentrated solution we have analysed has had an activity of 11 000 RU/ml, corresponding to an 8 % solution, pH of the solution 5.5. This solution was made by dialysis of the crystals against distilled water. During the process of dialysis, semi-dissolved crystals formed a heavy bottom phase, somewhat like honey; the viscous liquid was not analysed, but it was obviously much more concentrated than the final 8 % solution. However it shall be added, that by electrodialysis of the solution pH was displaced to 4.6 and the rennin was precipitated in an amorphous state.

In salt solutions, the solubility of the crystals decreases very rapidly when the salt concentration is raised. In 0.2 N NaCl, the solubility is around 200 RU/ml, in 1 N NaCl 30 RU/ml and in 4 N NaCl 5 RU/ml, corresponding to 1.43, 0.21 and 0.04 mg rennin/ml. The pH of the liquid phase has been 5.5 ± 0.1

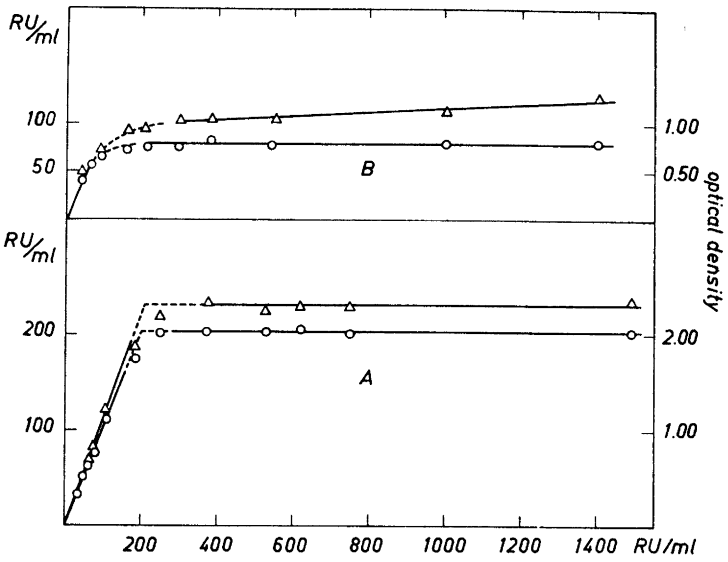

Fig. 1. Solubility test for the homogeneity of rennin. Solid phase: Amorphous precipitates of rennin. Liquid phase: A, NaCl-acetate, pH 5.5, ionic strength 4. B, Na-acetate buffer, pH 4.6, ionic strength 0.05. Abscissa: Total rennin activity of the suspensions. Ordinate: (△) optical density of the liquid phase at 277.5 mμ. (○) rennin activity of the liquid phase. All determinations made at 2°C.

during all these determinations. Contrary to precipitates of amorphous rennin, the rennin crystals seem to be more soluble at 25°C than at 2°C.

In the experiments on the solubility rate of rennin crystals, it was noted that the solubility rate did not only depend on the total amount of the solid phase. Also of importance was whether or not the crystals had been used in earlier solubility experiments. If the crystals had been "extracted" once, the rate of solubility would be much slower in a second experiment, almost as if a superficial layer of rennin molecules was more loosely bound in the crystals than the deeper placed molecules. A constant ratio between the rennin activity and the optical density of the liquid phase suggested that no fractionation took place owing to repeated "extraction" of the crystals.

3.2. Solubility test for homogeneity of rennin (Fig. 1)

Solubility tests for the homogeneity of rennin were made at the pH of optimum stability (pH 5.5) and at a pH near the isoelectric point (pH 4.6). All experiments in this section were made at 2°C. These experiments were performed using amorphous precipitates of rennin as the solid phase. The precipitates were formed by rapid mixing of dialysed rennin solutions with the solvent.

In experiments made at pH 5.5, 0.4 ml rennin solution was mixed with 1.6 ml of a stock salt solution consisting of 117 g NaCl + 41 g Na-acetate + 45

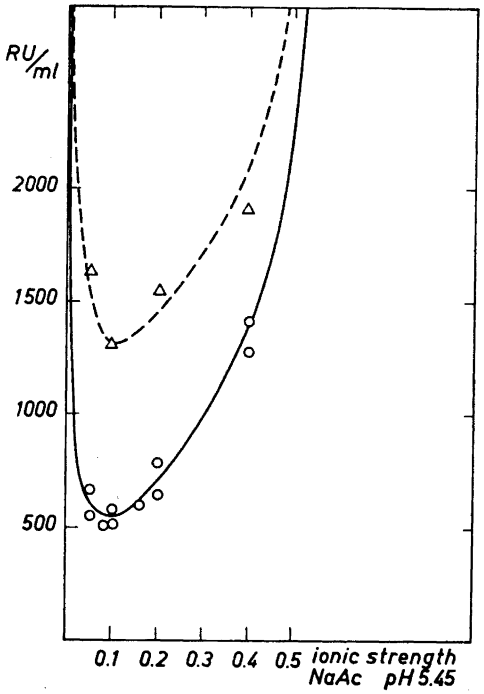

Fig. 2. Solubility of amorphous precipitates of rennin at low salt concentrations. Liquid phase: Na-acetate buffers pH 5.45 ± 0.05. Dotted line, solubility at 25°C. Full line, solubility at 2°C.

ml 1 N acetic acid made up with water to 500 ml. The pH of the solution was 5.5 and the resulting ionic strength of the suspensions was 4.0. The suspensions were equilibrated by 18 h of inversion of the tubes. The activity and the optical density of the supernatants after centrifugation are illustrated in Fig. 1 A. At pH's near the isoelectric point rennin is not quite stable (Foltmann [3]), but as further experiments (section 3.4) were made in this range of pH, the homogeneity was also tested at pH 4.6. In these experiments, 1 ml of rennin solution was mixed with 1 ml Na-acetate buffer pH 4.6, ionic strength 0.1. The activity and optical density of the supernatants after centrifugation are shown in Fig. 1 B.

3.3. Salting-out of rennin (Figs. 2 and 3)

As previously stated (Foltmann [4]), rennin in the amorphous state has a solubility minimum at ionic strength 0.1, pH 5.4. In order to localise this solubility minimum in dilute salt solutions, experiments were made with suspensions of amorphous rennin in solutions of ionic strength from 0.05 to 0.4. To control pH, Na-acetate buffers were used in the liquid phase. When preparing the buffers, the relative concentration of acetic acid was adjusted so as to maintain

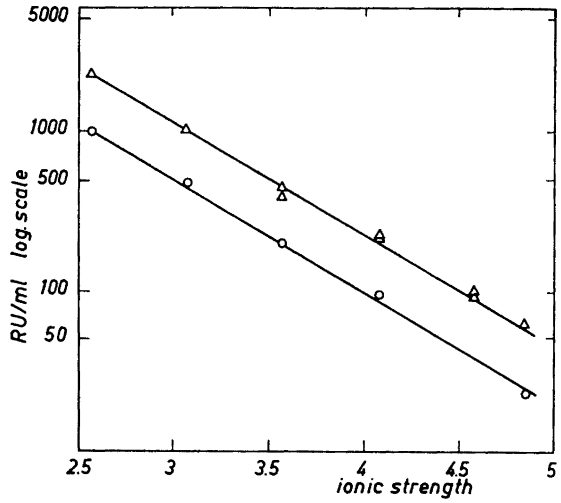

Fig. 3. Salting-out of amorphous rennin by NaCl. pH of the liquid phase 5.3 ± 0.05. Upper line (△) solubility at 2°C. Lower line (○) solubility at 25°C.

the pH at 5.45 ± 0.05. The experiments were made at 2°C and at 25°C. Results are shown in Fig. 2.

In the experiments on salting-out of rennin in concentrated salt solutions, NaCl was dissolved in Na-acetate buffer, pH 5.6, ionic strength 0.1, giving ionic strength of the mixtures from 2.5 to 4.85. The resulting pH, 5.3 ± 0.05. Results are shown in Fig. 3, and it is seen that the enzyme in equilibrium with amorphous precipitates is more soluble in concentrated salt solutions at 2°C than at 25°C.

In Fig. 3, the results are illustrated by log RU/ml, as RU/ml was the quantity observed directly. The logarithm of the solubility decreases linearly with the ionic strength according to Cohn's equation [5], $\log s = B - Ks \cdot \frac{\Gamma}{2}$. After the concentration of soluble rennin was converted from RU/ml to g/litre, the constants of Cohn's equation were calculated by the method of the least squares. The following values were obtained:

$$2°,\ B = 3.00,\ Ks = -0.703$$
$$25°,\ B = 2.67,\ Ks = -0.708$$

As experience has shown that crystallisation often starts at glass surfaces, these experiments were performed in polyethylene tubes, assuming the polyethylene surface to be more inactive than the glass surface. In spite of this, it has not been possible to determine the solubility of amorphous rennin at ionic strengths from 0.4 to 2.5. Within this range, the solutions were so supersaturated with respect to crystals, that crystallisation started immediately. In experiments at ionic strengths 2.5 and 3, a microscopic examination of the precipitate disclosed a few crystals, but the granular, amorphous precipitate

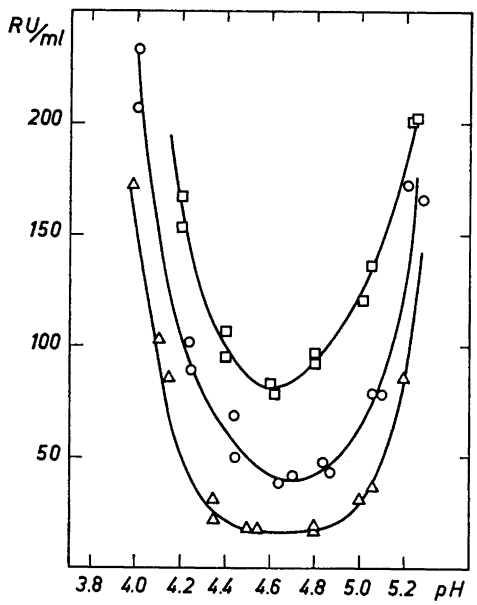

Fig. 4. The influence of pH and ionic strength on the solubility of amorphous precipitates of rennin near the isoelectric point. Liquid phase: Na-acetate buffers, △: ionic strength 0.005; ○: 0.025; □: 0.05.

dominated. The great surplus of the most soluble form will have fixed the concentration of the liquid phase. Experience has also shown that at rennin concentrations below 500 RU/ml, crystallisation starts very slowly, although the solutions are supersaturated with respect to crystals.

3.4. Solubility of rennin at the isoelectric point (Fig. 4)

The experiments were made using acetate buffers; ionic strengths of the mixtures: 0.005, 0.025 and 0.05. In each determination, 1 ml of dialysed rennin solution has been mixed with 1 ml of buffer. The dialysed rennin solution had a pH of 5.5. In experiments at low ionic strength especially, the buffer capacity of the rennin was sufficient to displace the pH of the buffer. The pH of every mixture was therefore checked after equilibrium was obtained, and the final pH was used when plotting the results in Fig. 4. All experiments referred to in Fig. 4 were made at 2°C.

Results from experiments made at 25°C are rather uncertain, due to the instability of the enzyme in this range of pH. However, it seems that under these conditions, the solubility is higher at 25°C than at 2°C. At pH 4.6, ionic strengths 0.025 and 0.05, the solubility at 25°C was determined as 54 and 114 RU/ml.

4. DISCUSSION

The solubility test for homogeneity of the rennin, made with 4 N NaCl-acetate as the liquid phase (Fig. 1 A), nearly fulfilled the requirements of the phase rule for a single-component system, although the point of inflection is not absolutely sharp. When using Na-acetate buffer, ionic strength 0.05, pH 4.6, as the liquid phase (Fig. 1 B), the results do not follow the phase rule for a single-component system. The »point of inflection» is round, and the optical density especially will rise with rising concentration of the solid phase. Here, it must be remembered that the enzyme is not stable at pH 4.6, and the deviations may be ascribed to the beginning of degradation. This experiment cannot be regarded as a test for homogeneity, but as further experiments were made at pH's around the isoelectric point, it was considered expedient to report these results.

At pH near the isoelectric point rennin is rather insoluble (Fig. 4), but the instability below pH 5 greatly complicates a theoretical interpretation of the solubility curves. The second solubility minimum at ionic strength 0.1 (Fig. 2), further complicates a theoretical examination. Because of this second solubility minimum the solubility at ionic strength 0.1 will be diminished, relative to lower ionic strength, on raising pH from 4.6 to 5.4. This means that the rising branches of the solubility curves, above pH 4.6, will intersect between pH 5.2 and pH 5.4. The point of intersection has not yet been fixed.

At pH 5.3—pH 5.5, the solubility of amorphous rennin is greater at 25°C than at 2°C, this when the ionic strength is below 0.4, whilst the opposite is the case at ionic strengths above 2.5. However, it has not been possible to determine the point of intersection of the curves owing to crystallisation of the rennin.

In Figs. 2, 3 and 4, the results of the experiments are only illustrated by means of rennin activity curves, this for purposes of clarity. However, the ratio between the milk-clotting activity and the optical density at 277.5 mμ has been checked in all experiments. As could be expected (Fig. 1B), this ratio was found not to be constant in the experiments around the isoelectric point (Fig. 4). In the other experiments made at 2°C, the ratio was constant ± 5 %. However, the ratio decreased up to 30 % in the least active supernatants from experiments at 25°C. As a corresponding increase in the same ratio was not found after dissolution of the remaining solid phase, the decrease of the enzymatic activity of the liquid phase was probably due to beginning inactivation and not to fractionation.

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