HETEROGENEITY OF CRYSSTALLINE RENNIN 1, 2

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SUMMARY

Block-shaped crystals were prepared from a concentrated solution of purified rennin by dialyzing it against Berridge's salting-in buffer. The purification procedure was relatively simple, and proved successful in each of several attempts to obtain rennin pure enough to crystallize. A single boundary in electrophoresis represented over 96% of the protein when the dissolved crystals were run at pH 6.8 in sodium phosphate buffer at an ionic strength of 0.2. However, at least four components were evident in the same buffer at 0.033 ionic strength. These results show that rennin preparations can not be considered homogeneous on the basis of their crystalline form or their electrophoretic behavior in phosphate buffers of high ionic strength.

Several European workers (1, 4, 10, 13) have crystallized rennin in the form of rectangular plates or cubes. Electrophoretic examination of the crystals dissolved in 0.1 M phosphate buffer led Alais (1) to conclude that his preparation was electrophoretically homogeneous. Schwander et al. (25) had found earlier that Berridge's (5) crystalline rennin behaved uniformly in paper electrophoresis, but showed some evidence of heterogeneity in sedimentation and diffusion experiments. Berridge (8) felt that this heterogeneity resulted from using an old sample of crystals which might have sustained some decomposition, or that the enzyme was partially inactivated while running the experiments at pH 7.0. By use of the constant solvent solubility test (11), he had shown earlier that, under one set of conditions, rennin activity was proportional to nitrogen in solution, and both were independent of the amount of solid phase present (5).

Berridge (7) suggested that the method of crystallization described by Berridge and Woodward (10) might not work with all rennet extracts. He (9) attributed the failure of such rennin preparations to crystallize to the presence of contaminating impurities which defied removal, but which could be detected electrophoretically.

Hostettler and Stein (18) concluded that the nature of the starting material as well as the method of purification had a marked influence on the properties of crystalline rennin.

Needle-shaped crystalline rennin was prepared by Hankinson (16) and DeBaun et al. (14) from rennet extract available in the United States. However, electrophoretic patterns published by DeBaun et al. (14) indicated considerable heterogeneity in phosphate buffers at ionic strengths of 0.1 and 0.2. Oeda and Kasai (24) also produced needle-shaped crystalline rennin, for which they reported electrophoretic homogeneity in phosphate buffer at an ionic strength of 0.14.

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It was the purpose of this work to prepare crystalline rennin from rennet extract available in the United States, and to study it under conditions of electrophoresis likely to give greater resolution than had been previously reported.

**EXPERIMENTAL METHODS**

*Rennin activity.* Rennin activity was measured by comparing the milk-clotting ability of unknown rennin solutions with that of a standard-strength rennet extract received monthly from a commercial rennet manufacturer. Each fresh standard was in turn checked against the standard of the previous month, using a substrate in which 60 g. low-heat nonfat dry milk (NDM) was dissolved in 500 ml. of 0.01 \(M\) calcium chloride. The same lot of NDM was used to prepare all substrates which, after reconstitution, were allowed to equilibrate for 20 hr. at 2° C. before use. Testing was carried out at 30° C. on 25-ml. portions of substrate in 125-ml. wide-mouthed bottles. One ml. of diluted enzyme was added, and the clotting time measured in revolutions (16 r.p.m.) on the apparatus described by Sommer and Matsen. The first appearance of visible flecks on the moving glass surface was taken as the end-point. The standard rennet extract, arbitrarily given a value of 100 R.U/ml, had an activity such that one milliliter of a 1:250 dilution produced visible clotting of the substrate in 100 ± 5 revolutions. Variation in the response of different lots of substrate to rennin accounted for some variation in clotting time. Consequently, whenever rennin solutions were tested, the activity of the standard was measured simultaneously on the same substrate.

Dilutions of unknown rennin solutions were made with distilled water, and adjusted so they gave coagulation times within ten revolutions of a 1:250 dilution of the standard.

Rennin activities were calculated as:

\[
R.U/ml = \frac{T_s - C_s}{C_u} \times \frac{T_u}{C_u}
\]

Where

- *R.U.* = rennin units
- *T\(_s\)* = coagulation time of standard
- *T\(_u\)* = coagulation time of unknown
- *C\(_s\)* = concentration of unknown
- *C\(_u\)* = concentration of standard

*Nitrogen determinations.* Nitrogen content of rennin fractions was measured by a micro-Kjeldahl procedure.

*Purification and crystallization of rennin.* Rennin was concentrated and purified from Chr. Hansen’s rennet extract by a modification of the procedure of DeBaun *et al.* The original extract contained no added color or flavoring, and only 0.1% sodium benzoate as a preservative. The strength of this rennet had not been standardized, and most of the lots received had greater activity than commercial cheese rennet.

Rennet standard supplied by Chr. Hansen’s Laboratory, Inc., Milwaukee, Wis.
The purification and crystallization procedure is outlined below:

1. Rennet extract at room temperature was adjusted to pH 5.0 with 3 N HCl, and saturated with sodium chloride. A precipitate formed (Precipitate I) which was separated from the supernatant by centrifuging for 45 min. at 5,000 × gravity (max.) in an International refrigerated centrifuge with a No. 845 angle head.

2. Precipitate I was dissolved in distilled water, and diluted to one-half the original volume. The solution was adjusted to pH 6.3 with 0.1 N NaOH and filtered.

3. Steps 1 and 2 were repeated until four salt precipitations had been made (Precipitates I, II, III, and IV). The dissolved Precipitate IV was diluted to the same volume as the solution of Precipitate III.

4. Potassium alum (1.0 g. per 1000 ml. rennin solution), dissolved in a small amount of water, was added to the solution of Precipitate IV, and immediately neutralized to pH 6.3 with 0.1 N NaOH. An aluminum hydroxide gel formed which was removed by centrifuging for 15 min. at 5,000 × gravity (max.). The alum supernatant was retained.

5. Rennin in the alum supernatant was precipitated by adjusting to pH 4.6 with 0.1 N HCl and saturating with sodium chloride, and separated by centrifuging in a refrigerated centrifuge for 45 min. at 5,000 × gravity (max.), (Precipitate V.). The supernatant was discarded.

6. Precipitate V was dissolved in a minimum of 0.05 M sodium phosphate buffer at pH 6.8 and dialyzed at 2 °C. against Berridge’s (5) salting-in buffer (50 g. MgCl₂ · 6 H₂O plus 15 g. CH₃COONa · 3 H₂O per liter, adjusted to pH 5.4 with 10 N H₂SO₄).

7. Crystals formed after a few days and continued to deposit for about 4 wk. Crystal formation was materially hastened by adding a few seed crystals from a previous batch.

8. The crystals were washed twice in the crystallizing buffer and twice at pH 5.6 in 0.05 M acetate buffer containing 0.3% sodium chloride.

RESULTS

Analyses of fractions during the purification and crystallization of a representative batch of rennin are given (Table 1).

The data in Table 1 show a 20-fold increase in specific activity of the crystals over the original rennet. Other preparations showed increases of 20- to 23-fold, depending on the purity of the original extract. Attempts to further increase the specific activity by recrystallization have not been successful.

Figure 1 shows a photomicrograph of crystalline rennin. The crystals are similar in appearance to those described by Berridge (4, 5), and actually have considerable depth. Many of them are as deep as they are wide, resembling blocks more than plates.
TABLE 1

Analyses of fractions during purification and crystallization of rennin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Activity (R.U./ml)</th>
<th>Total activity (R.U.)</th>
<th>Activity recovered (%)</th>
<th>Nitrogen (mg/ml)</th>
<th>Specific activity (R.U./mg N.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original extract</td>
<td>3,870</td>
<td>100</td>
<td>387,000</td>
<td>100</td>
<td>5.01</td>
<td>20</td>
</tr>
<tr>
<td>Solution of Precipitate I</td>
<td>1,960</td>
<td>150</td>
<td>294,000</td>
<td>76</td>
<td>0.96</td>
<td>156</td>
</tr>
<tr>
<td>Precipitate II</td>
<td>955</td>
<td>263</td>
<td>251,200</td>
<td>65</td>
<td>1.07</td>
<td>246</td>
</tr>
<tr>
<td>Precipitate III</td>
<td>550</td>
<td>394</td>
<td>216,700</td>
<td>56</td>
<td>1.48</td>
<td>266</td>
</tr>
<tr>
<td>Precipitate IV</td>
<td>477</td>
<td>459</td>
<td>218,900</td>
<td>57</td>
<td>1.56</td>
<td>294</td>
</tr>
<tr>
<td>Alum supernatant</td>
<td>499</td>
<td>265</td>
<td>132,200</td>
<td>34</td>
<td>0.78</td>
<td>340</td>
</tr>
<tr>
<td>Solution of Precipitate V</td>
<td>72</td>
<td>1,718</td>
<td>123,700</td>
<td>32</td>
<td>4.81</td>
<td>357</td>
</tr>
<tr>
<td>Crystals</td>
<td>62,270</td>
<td></td>
<td>401</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A portion of the crystals was dissolved in 0.2 N acetic acid, dialyzed against 0.05 M phosphate buffer at pH 6.8, and recrystallized by dialyzing at 2°C against Beridge's (5) buffer. The specific activity of the recrystallized rennin was 399 R.U./mg N.

Electrophoresis of crystalline rennin. Electrophoresis was carried out in 0.1 and 0.017 M sodium phosphate buffer at pH 6.8. The ionic strengths were 0.2 and 0.033, with specific conductivities at 0°C of $4.8 \times 10^{-8}$ and $1 \times 10^{-3}$ ohms$^{-1}$·cm$^{-1}$, respectively. At 0.2 ionic strength the buffer approximated that employed by Alais (1) in his electrophoretic analysis of crystalline rennin. An ionic strength of 0.033 was then used to achieve greater resolution by providing a higher ratio of protein concentration to ionic strength, and by enabling the use of a higher field strength in the electrophoresis cell.

Figure 2 shows electrophoretic patterns of rennin at a protein concentration of 0.6% in sodium phosphate buffer at an ionic strength of 0.2. The ascending pattern shows evidence of a small amount of material apart from the leading boundary. Using an assumed refractive increment of $dn/dc = 1.9 \times 10^{-3}$ ($c =$ gram per 100 ml.), and counting the Rayleigh fringes intercepted by the

Fig. 1. Crystalline rennin ×180.
CRYSTALLINE RENNIN

Fig. 2. Electrophoretic patterns of crystalline rennin dissolved to a protein concentration of 0.6% in sodium phosphate buffer at pH 6.8 and 0.2 ionic strength. The field strength was 4.34 volts/cm, with a current of 16 ma. (A) initial boundary, (B) 118 min., (C) 311 min. Horizontal, the amount of material represented by the trailing boundary was calculated to be about 3.8%.

Figure 3 illustrates an electrophoretic pattern of the same material at a protein concentration of 0.7% in pH 6.8 sodium phosphate buffer at an ionic strength of 0.033. This figure shows the presence of at least four, and possibly five, electrophoretically distinguishable components in the ascending pattern.

Fig. 3. Electrophoretic patterns of crystalline rennin dissolved to a protein concentration of 0.7% in sodium phosphate buffer at pH 6.8 and 0.033 ionic strength. The field strength was 10.46 volts/cm, with a current of 8 ma. (A) initial boundary, (B) 127 min.
These results have led to the conclusion that rennin preparations can not be considered homogeneous on the basis of their crystalline form or their electrophoretic behavior in phosphate buffers of high ionic strength.

Crystalline rennin supplied by Foltmann (15) gave similar results when run under the same conditions of electrophoresis.

**DISCUSSION**

The effect of reducing the ionic strength on improving the electrophoretic resolution of rennin is similar to that reported by Cann (12), in his studies on the resolution of ovalbumin in electrophoresis. This effect may be attributed to the higher field strength which can be used with a buffer of low conductivity, and to an increase in the ratio of protein concentration to ionic strength. In the absence of diffusion, resolution of components in electrophoresis is proportional to the product of field strength and time. Since boundary spreading by diffusion is proportional to the square root of time, it follows that resolution in ideal electrophoresis is proportional to field strength times the square root of time (3). Consequently, increased field strength gives greater resolution, since more rapid movement of the boundaries allows less time for diffusion.

Alberty (2) points out that in the electrophoresis of proteins descending peaks are usually, but not always (20), broader and shorter than the corresponding ascending peaks. This is due to deviation from ideality, which becomes more pronounced as the ratio of protein concentration to ionic strength is increased. Longsworth (20) showed that these boundary characteristics resulted from differences in conductivity and pH between the leading and trailing edges of the boundaries. When the conductivity effect predominates, the field strength along the leading edge of the ascending boundary is less than along the trailing edge. Therefore, the molecules along the trailing edge move more rapidly than those on the leading edge and the boundary is sharpened. A broadening of the peak occurs on the descending side, where the field strength is greater along the leading than on the trailing edge of the boundary. The pH effect usually opposes the conductivity effect above the iso-electric point of the protein, but in most instances it does not predominate (2).

The results of this work point to the interesting possibility that either: (a) more than one electrophoretically distinguishable component in crystalline rennin possesses milk-clotting activity, or (b) further fractionation should achieve a considerably greater increase in specific activity. Since at least four electrophoretic components were observed, both (a) and (b) may be correct.

There are several known instances of more than one electrophoretic form of an active enzyme. Examples are enolase (21) and chymotrypsin (22). Of these, the case of chymotrypsin is particularly relevant. Jacobsen (19) found that $\pi$- and $\delta$-chymotrypsin appeared to be different stages in the activation of a single pro-enzyme, chymotrypsinogen. It was later shown that these two active enzymes possessed different electrophoretic mobilities, and that crystalline $\alpha$-chymotrypsin (23) represented an electrophoretically heterogeneous mixture (22).
ACKNOWLEDGMENT

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REFERENCES

(7) BERRIDGE, N. J. Personal communication. 1953.