ACTA CHEMICA SCANDINAVICA 12 (1958) 343—346

On the Purification of Prorennin with a Note on the Recrystallisation of Rennin

BENT FOLTMANN

Chr. Hansen's Laboratory Ltd., Copenhagen, Denmark

Prorennin was extracted from dried calf-stomachs, using a solution of 2 % $NaHCO_3$. After clarification with $Al_2(SO_4)_3$, neutralisation with Na_2HPO_4 and reprecipitation with NaCl, a purified preparation was obtained. N-Determinations and paperelectrophoresis indicated that at least 50 % of the protein was prorennin. Paperelectrophoresis was carried out with crystalline rennin for purposes of comparison, and it was found that the isoelectric point of prorennin is about 0.3 pH unit higher than that of rennin. Crystallisation and recrystallisation of rennin are described.

Few investigations have been published concerning prorennin, the inactive precursor of the milk-clotting enzyme rennin. Only one attempt has been made to purify the pro-enzyme (Kleiner and Tauber[1]). As the preparation by Kleiner and Tauber actually appears to be rather impure, experiments have been carried out to produce a high-grade prorennin preparation.

METHODS AND RESULTS

Determination of the milk-clotting activity of samples from the preparation was carried out as a relative test, using fresh skim-milk and a sample of liquid rennet as basis for comparison. The activity is expressed in rennin units (RU) according to Berridge [2]. The eluates from the paperelectrophoresis experiments called for a very sensitive test. This determination was carried out with 5 ml skim-milk and 5 ml acetate buffer pH 4.9 (Ege and Menck-Thygesen [3]). In this determination, the activity is expressed directly as the inverse of the time of coagulation. The activity is illustrated in relation to each separate experiment.

Activation. The activation of prorennin was carried out at pH 2 for 15 min for samples of the preparation (*cf.* Ege and Lundsteen [4]). The eluates from the paperelectrophoresis only had a very slight buffer capacity, and as the determination of the milk-clotting activity of these samples was carried out in a milk/acetate mixture, it was most convenient to activate the eluates in acetate buffer pH 4.6 during 20 h before testing (Holwerda [5]).

Table 1. Purification of prorennin.

	ml	RU/ml		mg N/ml	RU per mg N after activation	per cent activity recov.
		preformed activity	after activation			
Raw extract (pH 8.4)	2 000	0	220	2.75	80	100
Addition of 100 ml 1/3 M $Al_2(SO_4)_3$. Precipitate discarded *	1 600	0	208	1.85	112	75
Addition of 80 ml 1/3 M $Al_2(SO_4)_3$ and 80 ml 1 M Na_2HPO_4. Precipitate discarded *	1 500	0	184	1.62	114	63
1 350 ml saturated with NaCl. Left to stand overnight. Precipitate redissolved in phosphate buffer 1/20 M, pH 6.3	220	6.6	690	1.35	510	34
215 ml saturated with NaCl. Left to stand overnight. Precipitate redissolved in 100 ml phosphate buffer 1/20 M, pH 6.3	110	12	1 350			
110 ml dialysed ** during 7 h against 2 × 2 l distilled water	145	9	1 000	1.77	560	33

* During the clarification operation pH fluctuated between 6.1 and 6.9.

** The dialysis was carried out in a rotating bag (Visking Cellulose) at 2°C during 2 h against 2 l of distilled water, and 5 h against a further 2 l of distilled water. (The dialysis was not prolonged in order to prevent activation during the experiment. The final product was not, therefore, free of salt. The N-contents of the freeze-dried preparation was 84 mg N/g.)

Prorennin. After preliminary experiments, the following procedure was adopted: 7 dried calf-stomachs, (taken from a charge proved to have a high prorennin/rennin value), were finely cut, and extracted with 3 000 ml of 2 % $NaHCO_3$ at room temperature whilst stirring continuously. After 2 h, the tissue was separated by means of centrifugation, and the muddy raw extract was purified as described in Table 1. After freeze-drying 135 ml of the purified dialyzate, 2.7 g of powder were obtained. The preformed activity of this powder was 3.6 RU/mg N, the activity after activation 500 RU/mg N.

Crystalline rennin was prepared from rennet after four precipitations with NaCl (Berridge [6], Alais [7]). The fourth precipitate was dissolved in distilled water to give a solution of 2 000 RU per ml. The NaCl enclosed in the wet precipitate produced a concentration in the final solution of approximately 1 N

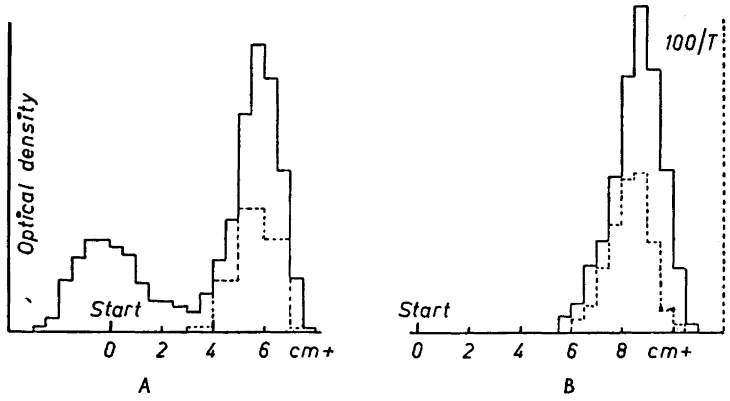

Fig. 1. Paperelectrophoresis of purified prorennin (A) and crystalline rennin (B). The samples are run 7 h in phosphate buffer, pH 6.0, ionic strength 0.1. Field strength 7 V/cm, room temperature. Full line: Colour after staining with bromophenol blue. The density is determined at 595 mμ after elution. Dotted line: The milk-clotting activity of the eluates illustrated as the inverse time of coagulation (in case of prorennin after activation).

NaCl. pH was regulated to 5.4 with 0.5 M phosphate buffer, pH 5.9, (5—10 ml were added per 100 ml solution).

From this solution, 1/2—2/3 of the active material crystallised in rectangular plates after 3—5 weeks in a refrigerator. The crystals were separated by means of centrifugation, and after gently washing with distilled water (2°C) they were dissolved in distilled water to give a solution of 3 000—5 000 RU per ml. Recrystallisation took place after addition of NaCl to a final concentration of 1 N, pH 5.4. When adding NaCl, a precipitate of amorphous rennin was noticed between 0.1—0.3 N NaCl. The precipitate dissolved when the salt concentration was further raised. The yield of the recrystallisation was 90 %. The activity of 10 preparations varied from 880 RU/mg N to 1 000 RU/mg N.

Paperelectrophoresis was carried out according to Kunkel and Tiselius [8]. Experiments were conducted with two spots (0.025 ml) in parallel. One strip was stained with bromophenol blue, and the colour was eluted with 0.1 equimolar hydrocarbonate/carbonate buffer. From the other strip, the enzyme was eluted with buffer, (phosphate buffer 0.05 M, pH 6.3 in the case of rennin, acetate buffer 0.1 M, pH 4.6 in the case of prorennin).

Fig. 1 illustrates two experiments with paperelectrophoresis at pH 6.0 (phosphate buffer, ionic strength 0.1) of purified prorennin and crystalline rennin. The experiment shows a clear difference in mobility of the two proteins. Further electrophoresis experiments at pH's ranging from 5.5 to 7.5 indicate by extrapolation that the isoelectric point of prorennin is about 0.3 pH-unit higher than that of rennin, or approximately 5.0 (Schwander, Zahler and Nitschmann [9], Hankinson [10]).

DISCUSSION

Kleiner and Tauber stated that 1 ml of a solution of 50 mg prorennin preparation in 100 ml of water, after activation, would clot 10 ml reconstituted skim-milk in 11 min at 40°C. As one RU is defined as the rennin activity which clots 10 ml of reconstituted skim-milk in 100 sec at 30°C, it may be surmised that Kleiner and Tauber's preparation, after activation, has had a rennin activity of about 0.3 RU per mg dry substance, (N-contents were not stated). Concerning the rennin activity before activation, a relative rennin activity of less than 5 % would not have been noticed, using the method described by Kleiner and Tauber.

The prorennin preparation resulting from the present work was not fully free of milk-clotting activity, but as experience has shown that purified prorennin easily activates during the process, the milk-clotting activity may be due to rennin formed during the fractionation. Meanwhile, the pre-formed rennin activity is less than 1 % of the total activity.

Assuming that the N-content of prorennin and rennin is of the same order of magnitude, the potential activity of the prorennin preparation corresponds to a prorennin content of approx. 50 %. The milk-clotting activity at the sharp main peak of the paperelectrophoresis indicates that this peak represents the prorennin. The area under this peak, compared with the total area, corresponds fairly well to the above-mentioned 50 %-content of prorennin in the preparation.

As the curve of the milk-clotting activity in the eletrophoresis of crystalline rennin conforms to that of the stained protein, the crystalline rennin should be regarded as electro-homogeneous under the conditions present.

The recrystallisation of rennin described above seems more convenient, and gives a better yield than that previously used by Berridge and Alais. After completion of this work, attention has been drawn to a paper read by Berridge before the International Circle of Research Workers in Dairying (Bern 1957). This paper mentions a recrystallisation similar to that described above.

REFERENCES

1. Kleiner, J. S. and Tauber, H. *J. Biol. Chem.* **96** (1932) 755.
2. Berridge, N. J. *Biochem. J.* **39** (1945) 179.
3. Ege, R. and Menck-Thygesen, P. *Biochem. Z.* **264** (1933) 13.
4. Ege, R. and Lundsteen, E. *Biochem. Z.* **268** (1934) 164.
5. Holwerda, B. J. *Biochem. Z.* **134** (1923) 381.
6. Berridge, N. J. *J. Dairy Research* **20** (1953) 255.
7. Alais, C. *Lait* **36** (1956) 26.
8. Kunkel, H. G. and Tiselius, A. *J. Gen. Physiol.* **35** (1951) 89.
9. Schwander, H., Zahler, P. and Nitschmann, H. *Helv. Chim. Acta* **35** (1952) 553.
10. Hankinson, C. L. *J. Dairy Sci.* **26** (1943) 53.

Received November 26, 1957.