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Purification of Rennin from Commercial Rennet by Sephadex G-75 Gel Chromatography*

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Introduction

The purity of the rennet, especially with respect to its contamination with pepsin, is of essential importance in understanding the biochemistry of the coagulation of milk by pure rennin. Many investigations have been conducted on this general problem from which erroneous conclusions may have been drawn because of the mixed nature of the enzymes in the rennin preparation used.

Several attempts to purify the rennet have been reported. Fenger[4], Luler and Bauer[8], Tauler and Kleiner[12] reported the method of purification of rennin from calf stomach mucosa. However, Holter[6] has pointed out that the sensitivity of the method used by these workers for measuring proteolytic activity is not sufficiently great to justify their conclusion that their purest rennin is devoid of pepsin.

The method of purification of rennin by column chromatography has been reported by Schober[10] with CM-cellulose and Foltmann[10] with DEAE-cellulose. More recently Yoshino et al[13] reported the purification of rennin from commercial rennet by DEAE-cellulose chromatography. The objective of this investigation was to get the purified rennin by more simplified procedure. This investigation has shown that a commercial rennet can be purified by Sephadex G-75 gel chromatography.

Materials and Method

1. The method of purification of rennin

Ten grams of Hansen's commercial rennet powder was dissolved in 0.1 M phosphate buffer at pH 6.0. One hundred milliliters of rennet solution a

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reddish brown liquid, were adjusted to pH 4.5 with 2N HCl. After standing for seven hours at 4°C, it was filtrated with Toyo Roshi NO 2 filter paper. Then it was neutralized to pH 6.0 with 2 N NaOH and dialyzed against phosphate buffer.

Method of salting-out: Sodium chloride to the rennet filtrate until salting-out (30g NaCl per 100ml phosphate buffer) and allowed to stand overnight at 4°C. After twice salting-out with NaCl, the precipitate was collected by 20 min. of centrifugation at 2,500rpm. Then, it was dissolved in phosphate buffer and dialyzed against phosphate buffer at 4°C.

Sephadex G-75 gel chromatography: Gel filtration on Sephadex G-75 (Pharmacia, Upsala, Sweden) was carried out with 0.1M phosphate buffer at pH 6.0 as the eluting buffer. The Sephadex was washed with water to remove fine particles. After establishing equilibrium with the buffer, it was packed at room temperature in a Sephadex chromatography column (60cm x 2cm diameter). A flow rate of 2ml/min. was maintained by positioning the level of fluid in the buffer reservoir about 15cm above the top of the column. The samples in 10ml volumes were applied to the gel column.

All the experiments were done at room temperature (20°C). The eluate was collected in 10ml fractions by use of a fraction collector. The absorption of materials at 280nm was detected with a Hitachi 101 Spectrophotometer. Blue dextran 2,000 with a average molecular weight 2×10^6 to the mark of the void volumn of the column was used. Yellow dextran with a molecular weight 2×10^4 and Vitamine B_{12} with a molecular weight 1,357, used as a low molecular weight substance, were eluted with Blue dextran.

2. Determination of molecular weight by thin layer gel filtration (TLG)[9]

One of the most striking properties of Sephadex gels chromatographic materials is their capacity for separating substances according to molecular size.

Over a considerable range, the elution volume is approximately a linear function of the logarithm of the molecular weight. This method can be also used for determination of molecular weight of proteins. TLG apparatus can often be adapted for this purpose. With this method the molecular weight of rennin can be determined by Pharmacia TLG apparatus.

From the calibration curve, produced by chromatography of proteins of known molecular weight (Cytochrome C, Albumin, Oboalbumin, Myoglobin), the molecular weight of the rennin can be estimated.

The gel prepared by Sephadex G-200 superfine is spread at the thickness of 0.8mm on the plate. Before a run is started, the plate should be equilibrated by allowing the phosphate buffer to flow through the gel. After equilibration, the sample (5μl) is applied with a micropipet as a round spot 2–3mm in diameter. The best resolution in layer gel filtration is usually

obtained at flow rates not exceeding 1–2cm/hour. After completion of a run, the separated substances are transferred from the gel layer by absorption into a superimposed filter paper (Watman 3MM). After about one minute the sheet is removed, dried, stained with Lissamine Green, and washed with 7% acetic acid.

3. Determination of clotting activity

Clotting activity was determined by the same method as Yoshino et al[13]. Throughout the preparations the activity of the various fractions were determined by measuring the time required for 1 ml. of the diluted enzyme to clot 10ml. of standard substrate at 30°C.

The standard substrate was made by dissolving 12g of dried skim milk powder in 100ml 0.01M $CaCl_2$ solution. All the minor details of the techniques of measurement e.g. time of keeping milk at 30°C before adding rennin, method of determining end point, etc., were rigidly standarized. The end point was observed by allowing a thin film of the milk to flow from a glass rod down the side of the test tube containing the milk. When clotting occurred, the almost invisible film broke into a number of white particles. The activity of the solution was then obtained from the formula,

$$\text{activity} = \frac{1}{t \cdot m}$$

$$\text{specific activity} = \frac{1}{D \cdot t \cdot m}$$

where t = clotting time in sec.,
m = rennin solution (ml)
and D = optical density in 280nm.

4. Polyacrylamide gel electrophoresis (PGE)

Polyacrylamide gel electrophoresis was performed according to the method of Davis[3]), in pH 8.5 Tris-Glycin buffer. The sample (200–300μg) equilibrated with its buffer was inserted into the gel column, and PGE was run for about 90 min. at 3 mA per column at room temperature. When the Bromephenol blue was moved at the regular distance, the current was stopped. The gel was stained with Amide Black 10B for 20 min., then washed with water and fixed 7% acetic acid.

5. Determination of proteolytic activity[7])

Determination of proteolytic activity on the purified rennin and originate dialyzed rennet were carried out as follws.

One hundred milliliters of reaction mixture containing 1% crude κ-casein prepared by the sulfuric acid technique of Zittle[14] in 0.1M phosphate

buffer at pH 6.0 were incubated for 30 min. at 30°C. Two milliliters of the enzyme solution respectively were added to aliquot of each case in solution and after a given time 10 ml of casein solution was pipettied from reaction mixture to test tube and quickly 10 ml of 6% trichroloacetic acid (TCA) solution was added. After standing for about one hour, the mixture was filtered and non-protein nitrogen (NPN) was determined by the Cu-Folin method.

Results and Discussion

The recent extensive application of electrophoresis and chromatography for the characterization and fractionation of proteins has shown that a number of enzymes which were previously considered to be pure are actually mixtures of closely related compounds. Thus, it has also found that rennet contains other substances.

Sephadex gel filtration has been found to offer some possibilities for fractionation and examination of enzymes. It is relatively rapid, simple and reliable and can be used to follow changes in the smaller-sized proteins which can not normally be accomplished by such procedures as the ultracentrifuge, light scattering and polarization of fluorescence. Furthermore, by the Sephadex gel filtration technique, the enzymes can be examined in their natural environment and the necessity for detailed and often lengthy sample preparation is avoided.

The result in Figure 1 shows the fractionation, obtained using a column of Sephadex G-75 at room temperature (20°C), of a rennet solution prepared from Hansen's rennet. Fraction A is eluted in the void volume of the Sephadex G-75 column and, therefore, has a molecular weight of 50,000 or more. Whereas, smaller molecular (fraction B) penetrate the gel particles and is less than 50,000. Fraction B has clotting activity and proteolytic activity. Fraction C, D and E are smaller substances than the fraction A and B.

The fraction E with the smallest moleculr weight contains much foreign materials. In this experiments, it would be simple to separate the fraction E from the rennet solution either by slating-out or Sephadex G-75 gel filtration.

To check the extent to which dissociation occurred, composition of the fraction were investigated by means of polyacrylamide gel electrophoresis, a sensitive method for detecting minor components.

The electrophoretic patterns of fraction A, B, C, D and E eluted on Sephadex G-75 are shown in Figure 2. According to the PGE patterns, fraction A and C showed a number of bands on the polyacrylamide gel. It is assumed that fraction A and C are composed with heterogeneous components. Fraction B showed a single spreading band. On the other hand,

components of fraction D and E, which have smaller molecular weight than fraction A, B and C as shown in Figure 1, went through the polyacrylamide gel pores.

About 100% of the total milk clotting was recovered in fraction B. By the comparative studies on purified rennin prepared with Sephadex G-75 and DEAE-cellulose, the result in Figure 3 shows that rennin prepared with Sephadex G-75 had an electrophoretic composition similar to that prepared with DEAE-cellulose. Therefore, the rennin prepared with Sephadex G-75 is identical substance electrophoretically to that prepared with DEAE-cellulose.

The difference of proteolytic activity and clotting activity of both rennins are small as shown in Table 1 and the results in Figure 4 and 5 shows that the rennin and pepsin are eluted at the same position. But, significant difference between proteolysis of κ-casein by fraction B and pepsin are shown in Figure 6. It is supposed, therefore, that purified rennin prepared with Sephadex G-75 does not contain pepsin. Yoshino et al[13] reported that pepsin contained a little in rennet, and it comprised only one to two percent of the total activity of the rennet, and it had no effect on proteolytic activity of the rennet.

The molecular weight of rennin has been determined by many workers.[9,11] In gel filtration, Andrews[1] used in his experiments with columns of ager gel and found the molecular weight of rennin to be about 30,000. In later experiments Andrews[2] used Sephadex G-75, and determined the molecular weight of rennin to be about 31,000. By thin layer gel filtration with the Pharmacia TLG-apparatus Figure 7, 8 indicated that molecular weight of rennin prepared by Sephadex G-75 is about 29,000.

Summary

Hansen's rennet was fractionated on Sephadex G-75 with 0.1 M phosphate buffer at pH 6.0 and 20°C.

Fraction A, eluted in void volume, did not exhibit clotting activity. After the elution of fraction A, fraction B was eluted and it was found to have clotting activity together with proteolytic activity. It was found that the difficulty in separation of fraction A and B could be solved by lowering the flow rate of the elution buffer. Thus the flow rate was decreased from 5ml/min. to 2ml/min. In contrast, fraction C, D and E showed no activity.

By the use of polyacrylamide gel patterns, clotting activity tests and proteolytic activity tests, purified rennin prepared on Sephadex G-75 was found to be comparable to that prepared on DEAE cellulose. Quite similar properties in gel patterns, proteolysis and clotting activity were noted.

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References

1. Andrews, P., 1962. Estimation of molecular weights of proteins by gel filtration. Nature, **196**: 36–39.
2. Andrews, P., 1964. Estimation of molecular weights of proteins by Sephadex gel filtration. Biochem. J., **91**: 222–233.
3. Davis, B. J., 1964. Disc electrophoresis. Ann. N. Y. Acad. Sci., **121**: 404.
4. Fenger, F., 1923. A comparison between the chemical and physiological characteristics of pepsin and rennin. J. Am. Chem. Soc., **45**: 249–255.
5. Foltmann, B., 1960. Chromatographic fractionation of rennin. Acta Chem. Scand. **14**: 2059–2061.
6. Holter, H., 1932. Über die Labwirkung. Biochem. Z. **255**: 160–188.
7. Kato, I., K. Mikawa and T. Yasui, 1970. Action of rennin on casein. (1) Effect of salts on the primary phase. Memoirs of the Faculty of Hokkaido University, **7**: 477–481.
8. Lüers, H. and Bader, J., 1927. Über die Reinigung des Chymosins. Bochem. Z., **190**: 122–142.
9. Morris, C., 1964. Thin layer chromatography of proteins on Sephadex G-100 and G-200. J. Chromatogr., **16**: 167–175.
10. Schober, R., N. Himberger and I. Prinz, 1960. Charakterisierung von Labpräparaten verschiedener Reinheitsgrade durch Immuneelektrophorese. Milchwissensch. **15**: 561–564.
11. Schwander, H., Zahler, P. and H. Nitschmann, 1952. Analytische Untersuchungen an kristallisiertem Lab. Helv. Chim. Acta, **35**: 553–560.
12. Tauber, H. and S. Kleiner, 1932. The purification of rennin and its separation from pepsin. J. Biol. Chem. **96**: 745–753.
13. Yoshino, U., N. Nakatani, Y. Tokoro and K. Yamaguchi, 1966. Purification of rennet by DEAE-cellulose chromatography. Nippon Nogeikagaku Kaishi, **40**: 52–57.
14. Zittle, C. A. and J. H. Custer, 1963. Purification and some of the properties of α_s-casein and κ-casein. J. Dairy Sci., **46**: 1183–1188.

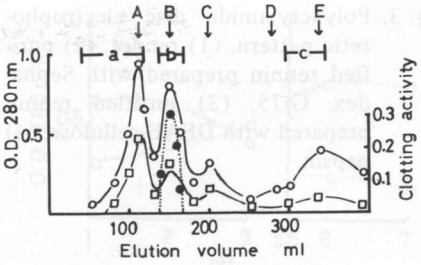

Fig. 1. Sephadex G-75 gel filtration pattern for rennet and twice salting-out rennet. A, B, C, D and E show the fraction number and a, b, and c show eluted positions of blue dextran (M.W.2×10^6), yellow dextran (M.W.2×10^4) and Vitamine B_{12} (M.W.1,357) respectively. ○ – ○ Rennet, □ – □ Twice salting-out rennet ● – ● Clotting activity.

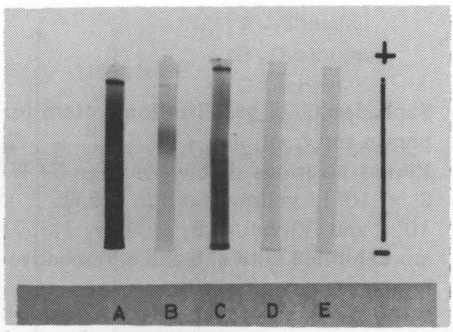

Fig. 2. Polyacrylamide disc electrophoretic pattern. A, B, C. D and E are fractions number of Sephadex G-75 gel filtration.

Table 1. Specific activity of rennet, fraction B and purified rennin prepared with DEAE-cellulose.

Enzyme	O.D. 280nm	Enzyme ml per 10ml skimmilk	Clotting time (sec.)	Specific activity 1/sec·ml·O.D.	Ratio
Rennet	0.405	0.2	80	0.311	100
Fraction B	0.073	0.3	180	0.260	235
Rennin (prepared with DEAE-cellulose)	0.070	0.3	210	0.210	223

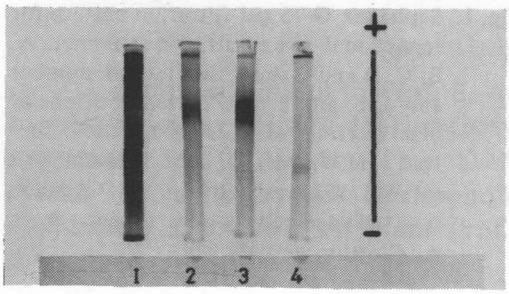

Fig. 3. Polyacrylamide disc electrophoretic pattern. (1) rennet, (2) purified rennin prepared with Sephadex G-75, (3) purified rennin prepared with DEAE-cellulose, (4) pepsin.

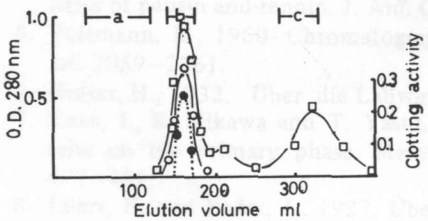

Fig. 4. Sephadex G-75 gel filtration pattern for pepsin and fraction B.
Eluted positions of blue dextran (M.W. 2×10^6), yellow dextran (M.W. 2×10^4) and Vitamine B_{12} (M.W. 1,357) are exhibited with a, b and c, respectively.
□ — □ Pepsin, ○ — ○ Fraction B
● ····· ● Clotting activity

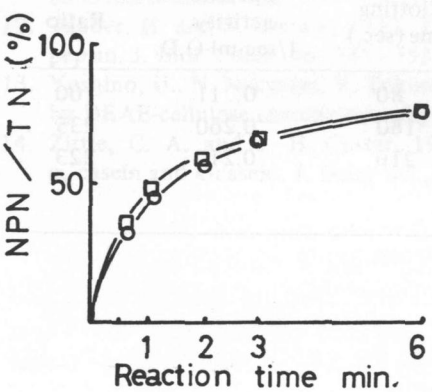

Fig. 5. Proteolytic action of purified rennin prepared with Sephadex G-75 and DEAE-cellulose.
○ — ○ Rennin (Sephadex G-75)
□ — □ Rennin (DEAE-cellulose)

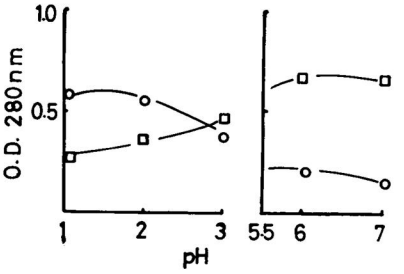

Fig. 6. Proteolytic action of pepsin and fraction B at different pH.
○ — ○ Repsin,
□ —□ Fraction B

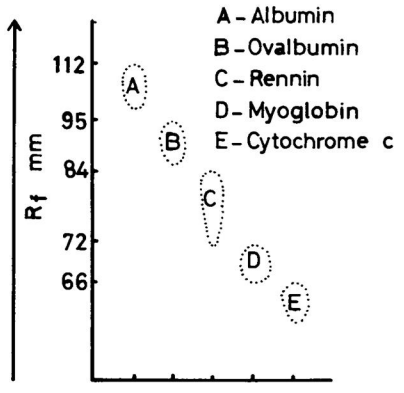

Fig. 7. Thin layer chromatogram of albumin, ovalbumin, rennin, myoglobin and cytochrome C.
Cytochorme C (M.W. 12,400), Myoglobin (M.W. 17,500), Ovalbumin (M.W. 46,000), Albumin (M.W. 66,000)

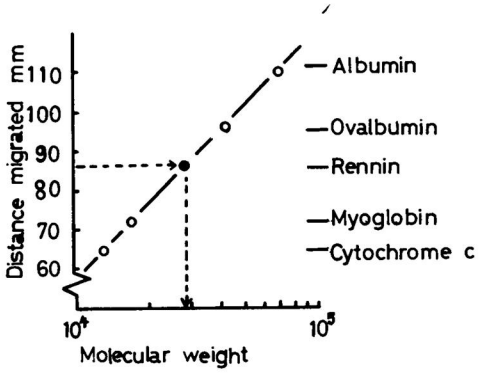

Fig. 8. Molecular weight determination for rennin by thin layer chromatography.
Relationship between rate of migration and molecular weight (on a logarithmic scale) for globular proteins in thin layer gel filtration of Sephadex G-200 superfine.
→ rate of migration and molecular weight of rennin.