Fractionation and Isolation of the Multiple Forms of Prorennin (Prochymosin)

By N. ASATO and A. G. RAND, JR.

*Departments of Animal Science and Food and Resource Chemistry,
University of Rhode Island, Kingston, R. I. 02881, U.S.A.*

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The heterogeneity of prorennin was studied by chromatography on DEAE-cellulose and microgranular DEAE-cellulose columns, as well as by polyacrylamide-gel electrophoresis. Prorennin prepared by alum treatment, salting-out and chromatography was resolved into three components by a compound gradient of sodium phosphate on microgranular DEAE-cellulose. Polyacrylamide-gel electrophoresis confirmed the chromatographic results, but crystalline rennin was shown to consist of four bands. When prorennin was isolated directly by chromatography, four zymogen components were resolved on microgranular DEAE-cellulose with a modified compound gradient of sodium phosphate. Polyacrylamide-gel electrophoresis confirmed the existence of four multiple forms of prorennin as well as homogeneity of the chromatographic fractions.

The heterogeneity of gastric zymogens has been established by chromatographic and electrophoretic studies of extracts of pig stomach (Lee & Ryle, 1967), human gastric mucosa (Turner *et al.*, 1970), chicken stomachs (Donta & Van Vunakis, 1970) and dogfish stomachs (Merrett *et al.*, 1969). Four pepsinogens have been separated from bovine fundic mucosa by chromatography on hydroxyapatite at pH 7.3 (Meitner & Kassell, 1971).

Similarly prorennin (prochymosin), the zymogen of the enzyme rennin (chymosin; EC 3.4.4.3), has been shown to be chromatographically heterogeneous (Foltmann, 1962). Foltmann (1962) partially resolved prorennin into two components, designated prorennin A and B, by chromatography on DEAE-cellulose with a linear gradient of sodium phosphate. The activation of each prorennin component resulted in the formation of a different rennin.

Bundy *et al.* (1964) isolated prorennin by a different procedure. No evidence for heterogeneity of prorennin was found by chromatography on DEAE-cellulose with both a NaCl gradient in tris buffer, pH 7.5, and a linear gradient of sodium phosphate, pH 5.8 (Bundy *et al.*, 1967). Shukuri (1970) also reported that prorennin was homogeneous when subjected to chromatography on DEAE-cellulose and electrophoresis in starch-urea gel. However, multiple forms of prorennin and rennin with proteolytic activity have been demonstrated by polyacrylamide-gel electrophoresis (Asato & Rand, 1971).

In the present paper we describe a method for the fractionation and isolation of four prorennin components by chromatography on DEAE-cellulose, as the initial approach to an investigation of the origin of the individual rennin components. Confirmation

of prorennin heterogeneity and the homogeneity of individual zymogen components by polyacrylamide-gel electrophoresis is included.

Experimental

Materials

Low-heat non-fat dried milk was obtained from Land O'Lakes Co., Minneapolis, Minn., U.S.A., and stored at -20°C . DEAE-cellulose (lot no. 4) was from Matheson Coleman and Bell, Norwood, Ohio, U.S.A. Whatman microgranular DEAE-cellulose (lot no. DE 32/11) was from Reeve Angel, Clifton, N.J., U.S.A. Fresh salted calf stomachs were obtained from a local abattoir, dried in a hood at 40°C with forced air, and stored in polyethylene bags at -20°C until used. Crystalline rennin was prepared by the method of Ernstrom (1968). Pentex rennin (IX crystallized, lot no. 11) was obtained from Miles Laboratories Inc. Kankakee, Ill., U.S.A. Distilled water was used throughout.

Methods

Determination of prorennin. The definition of a rennin unit and the procedure for the assay of rennin and prorennin activity used in this study have been reported (Rand & Ernstrom, 1964). Prorennin activity was measured as the difference in milk-clotting activity before and after activation at pH 2.0, and expressed as prorennin units/ml.

DEAE-cellulose chromatography. DEAE-cellulose (50g) (Matheson Coleman and Bell) and 25g of Whatman microgranular DEAE-cellulose were swollen with acid and alkali treatment according to

the manufacturer's instructions (Whatman Technical Bulletin 1E2) and then carefully equilibrated with 0.05M-sodium phosphate buffer, pH5.9. The exchanger was gently stirred with frequent changes of buffer until there was no drift in pH. The suspensions of equilibrated DEAE-cellulose and equilibrated microgranular DEAE-cellulose were poured into columns (2.5cm diam.) at room temperature. The columns were cooled and washed with 2 litres of cold 0.05M-sodium phosphate buffer, pH5.9, in a cold room at 2°C. The final column heights were 40cm for the conventional DEAE-cellulose column and 26cm for the microgranular DEAE-cellulose column. A sample of freeze-dried prorennin was dissolved in 0.01M-sodium phosphate buffer, pH6.1, and applied to the column. Elution was carried out initially with the combination of stepwise and linear gradients of sodium phosphate described by Foltmann (1962). Subsequently, compound gradient elution was also employed with an increasing phosphate buffer concentration, by utilizing the Varigrad designed by Peterson & Sober (1959). All phosphate buffers employed in column chromatography were prepared at room temperature by the dilution of stock solutions of 0.5M-sodium monohydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and 0.5M-sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), mixed 1:9, v/v (Foltmann, 1962). The flow rate was maintained with a peristaltic pump and three fractions were collected/h by a volumeter. The DEAE-cellulose column (2.5cm $\times$ 40cm) and the microgranular DEAE-cellulose (2.5cm $\times$ 26cm) were regenerated by washing with 300ml of 0.3M-NaOH with a slow flow rate to avoid shrinkage. Columns were again equilibrated with 0.05M-phosphate buffer to a constant value of pH5.9.

Analysis of protein. The concentration of protein in effluents from the chromatographic column was determined by measuring E_{278} with a Cary 15 spectrophotometer. Nitrogen content during purification was determined by a semi-micro-Kjeldahl procedure (Hiller *et al.*, 1948).

Polyacrylamide-gel electrophoresis. Electrophoresis of prorennin and rennin was conducted with polyacrylamide gels at pH7.1 in a vertical cell as previously described (Asato & Rand, 1971).

Results

Purification of prorennin: method (1)

Isolation. Prorennin was purified by a modification of the procedure of Rand & Ernstrom (1964) as previously described (Asato & Rand, 1971). The last two fractions in the purification process (Table 1) were prepared by DEAE-cellulose-column chromatography. Stepwise elution of prorennin was conducted first (1 $\times$ DEAE-cellulose), followed by a linear gradient procedure (2 $\times$ DEAE-cellulose), steps which have been described by Foltmann (1962). The analysis of fractions obtained during prorennin purification are presented in Table 1, and illustrate that the specific activities resulting from the second chromatographic step are comparable with values previously reported (Rand & Ernstrom, 1964).

The electrophoresis of prorennin, purified by either one or both of the chromatographic steps, on a polyacrylamide gel is shown in Plate 1. The results presented are typical for several preparations of 2 $\times$ DEAE-cellulose-purified prorennin and demonstrate that this preparation generally consists of three protein components, two main bands and one minor

Table 1. *Analysis of fractions obtained during extraction and purification of prorennin by method (1)*

Prorennin was extracted from dry calf stomachs with 0.1 M-sodium borate soln. and the extract was clarified with aluminium potassium sulphate. Prorennin was precipitated from the alum supernatant with NaCl, redissolved in a minimum of 0.01 M-sodium phosphate buffer, pH6.3, and dialysed (Rand & Ernstrom, 1964; Asato & Rand, 1971). Prorennin was further purified by DEAE-cellulose chromatography, as described by Foltmann (1962). Activities are expressed in prorennin units (see the Experimental section).

Fraction	Volume (ml)	Activity (units/ml)	Total activity (units)	Activity recovered (%)	Nitrogen (mg/ml)	Specific activity (units/mg of N)
Original extract	630	81.3	51219	100.0	2.747	29.6
Alum supernatant	650	73.7	47905	93.5	2.186	33.7
Solution of NaCl ppt.	140	246.2	34468	67.3	3.677	66.9
Filtrate of dialysis residue	150	203.2	30480	59.5	1.560	130.2
1 $\times$ DEAE-cellulose	900	23.8	21420	41.8	0.099*	240.4
2 $\times$ DEAE-cellulose	1100	18.0	19800	38.7	0.045†	400.0

* Determined on a 3ml sample.

† Determined on an 8ml sample.

EXPLANATION OF PLATE I

Polyacrylamide-gel-electrophoresis patterns of prorennin purified by method (1) and rennin stained with Coomassie Blue

Samples: nos. 1-5, 2×DEAE-cellulose-purified prorennin (10mg/ml); no. 6, 1×DEAE-cellulose-purified prorennin (10mg/ml); nos. 7-8, rennin (5mg/ml); nos. 9-10, rennin (10mg/ml).

EXPLANATION OF PLATE 2

Polyacrylamide-gel-electrophoresis of prorennin fractions

(a) Electrophoretic pattern showing the location of zymogen and enzyme components with proteolytic activity for prorennin, rennin and the individual prorennin components obtained by method (1). Samples: nos. 1 and 3, $2 \times$ DEAE-cellulose-purified prorennin, purified by method (1); no. 2, partially purified prorennin prepared by method (2); no. 4, rennin; no. 5, fraction (I) in Fig. 2 (5 mg/ml); no. 6, fraction (II) in Fig. 2 (5 mg/ml); no. 7, fraction (III) in Fig. 2 (5 mg/ml); no. 8, fraction (I) in Fig. 2 (10 mg/ml); no. 9, fraction (II) in Fig. 2 (10 mg/ml); no. 10, fraction (III) in Fig. 2 (10 mg/ml). (b) Gel electrophoresis of the multiple forms of prorennin. Samples: no. 1, $2 \times$ DEAE-cellulose-purified prorennin purified by method (1); no. 2, partially purified prorennin prepared by method (2); no. 3, DEAE-cellulose-purified prorennin prepared by method (2); no. 4, fraction (I) in Fig. 4; no. 5, fraction (II) in Fig. 4; no. 6, fraction (III) in Fig. 4; no. 7, fraction (IV) in Fig. 4; no. 8, crystalline rennin; no. 9, Pentex rennin; no. 10, same as no. 3.

band. The prorennin prepared by only stepwise elution from DEAE-cellulose contains the three prorennin components, and two bands with a mobility exceeding that of rennin, which are eliminated by the second chromatographic step. The electrophoretic pattern for crystalline rennin also shows three protein components, two main bands and one minor band.

Fractionation. Attempts to fractionate prorennin into the components noted in polyacrylamide-gel electrophoresis were unsuccessful with linear gradient elution on conventional DEAE-cellulose. A single broad peak was consistently obtained. Rechromatography of $1 \times$ DEAE-cellulose-purified prorennin was done on a microgranular DEAE-cellulose column with linear phosphate gradient elution, as shown in Fig. 1. Prorennin was resolved into one inactive and two active components. The smaller prorennin peak appeared to correspond to component A, and the main peak to component B, designated by Foltmann (1962). The fractions under each peak were pooled, dialysed against 0.2 M-disodium phosphate, pH 7.5, at 2°C and freeze-dried. The analysis of each component by polyacrylamide-gel electrophoresis demonstrated that prorennin A and B were electrophoretically distinct. Prorennin B contained some contamination from A, whereas prorennin A appeared to be a mixture of A and another prorennin component that moved ahead of prorennin A and corresponded to the minor band shown in Plate 1.

Fig. 1. Rechromatography of prorennin purified by method (1) on microgranular DEAE-cellulose

A sample (400 mg) of prorennin was applied in 15 ml of 0.01 M-sodium phosphate buffer, pH 6.1. A linear phosphate gradient was developed by mixing 600 ml of 0.1 M-sodium phosphate buffer, pH 5.8, and 600 ml of 0.2 M-sodium phosphate buffer, pH 5.7. The flow rate was 21 ml/h and three fractions/h were collected. —, E_{278} (total protein); ○, potential milk-clotting activity.

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Although electrophoretically distinct prorennin components appeared to exist, only two could be resolved by chromatography with a linear gradient of phosphate. A compound gradient (Peterson & Sober, 1959) was developed to achieve further resolution of prorennin. The rechromatography of $1 \times$ DEAE-cellulose-purified prorennin on microgranular DEAE-cellulose, when eluted with an increasing concentration of phosphate and utilizing the Varigrad, is shown in Fig. 2. Three distinct prorennin peaks were obtained, as indicated by E_{278} and the potential rennin activity. Each prorennin fraction was dialysed against 0.2 M-disodium phosphate, pH 7.5, at 2°C, freeze-dried and analysed by electrophoresis on polyacrylamide gel as shown in Plate 2(a). Components with actual or potential proteolytic activity were detected on the casein substrate incorporated as part of the gel. The relative position of migration for each individual prorennin component was consistent with the order of elution from the DEAE-cellulose column. Electrophoretic analysis of whole prorennin samples demonstrated that purification of the zymogen by method (1) resulted in preparations that contained only three bands with potential activity. However, crystalline rennin was consistently resolved into four active components, as shown in Plate 2(a).

Fig. 2. Chromatographic fractionation on microgranular DEAE-cellulose of prorennin purified by method (1)

A sample (200 mg) of prorennin was applied in 10 ml of 0.01 M-sodium phosphate buffer, pH 6.1. Compound gradient elution was done with a Varigrad, which contained 250 ml of sodium phosphate buffer (pH 5.8–5.7) in each of the nine cylinder chambers at concentrations of 0.10, 0.13, 0.10, 0.16, 0.10, 0.18, 0.10, 0.18 and 0.18 M respectively. The flow rate was 45 ml/h and 15 ml fractions were collected. —, E_{278} (total protein); ○, potential milk-clotting activity. The effluents under peaks (I), (II) and (III) were pooled as indicated.

Purification of prorennin: method (2)

Isolation. To establish the possible existence of separate prorennin components as precursors for each individual rennin, the purification procedure for method (1) was simplified as follows.

(1) Prorennin was extracted from dry calf stomachs with 0.1 M-sodium borate soln. as previously described (Rand & Ernstrom, 1964; Asato & Rand, 1971).

(2) The extract was dialysed in a rotating dialyser against three lots of 12 litres of 0.2M-disodium phosphate, pH 7.5, for 2, 4 and 14 h. The suspension was centrifuged at 10000g in a Sorvall RC-2B refrigerated centrifuge at 2°C for 30 min. The supernatant was filtered through Eaton Dikeman filter paper no. 615 and freeze-dried. Electrophoretic analysis of this crude prorennin extract demonstrated that the zymogen was a complex of four active components, as shown with sample no. 2 in Plate 2(a) and (b).

(3) The freeze-dried prorennin extract was purified by stepwise elution from a column of conventional DEAE-cellulose as shown in Fig. 3. The pooled fractions of prorennin activity were dialysed against three lots of 8 litres of 0.2M-disodium phosphate, pH 7.5, as described above, and freeze-dried.

The analysis of fractions obtained during the extraction and purification of prorennin by method (2) are summarized in Table 2.

Fractionation. Prorennin purified by method (2) was rechromatographed on microgranular DEAE-cellulose as shown in Fig. 4. Four prorennin peaks were obtained with a modified compound gradient, as indicated by E_{278} and potential milk-clotting activity. Each chromatographic fraction of prorennin was dialysed against three lots of 8 litres of 0.2M-disodium phosphate, pH 7.5, as previously described, and freeze-dried separately.

The isolated chromatographic components and whole prorennin prepared by method (1) and method (2) were analysed and compared by polyacrylamide-gel electrophoresis together with crystalline rennin, as shown in Plate 2(b). Again, the electrophoretic

mobility of the isolated prorennin components was compatible with the order of elution from the DEAE-cellulose column (Fig. 4). Both the crude prorennin extract and DEAE-cellulose-purified prorennin prepared by method (2) consisted of four components, whereas the prorennin purified by DEAE-cellulose in method (1) contained three components. In addition to the four prorennin components, the crude prorennin extract contained another component with

Fig. 3. Chromatographic purification of prorennin by method (2)

Freeze-dried prorennin extract (2.0g) was applied to a column of conventional DEAE-cellulose in 100ml of 0.01 M-sodium phosphate buffer, pH 6.1. Stepwise elution was carried out with 500ml of 0.05 M-sodium phosphate buffer, pH 5.9, 500ml of 0.1 M-sodium phosphate buffer, pH 5.8, and 800ml of 0.2 M-sodium phosphate buffer, pH 5.7. The buffers were changed at the points indicated by the vertical arrows. The flow rate was 30ml/h and three fractions/h were collected. —, E_{278} (total protein); o, potential milk-clotting activity. Fractions containing prorennin activity were pooled as indicated.

Table 2. Analysis of fractions obtained during extraction and purification of prorennin by method (2)

Activities are expressed in prorennin units (see the Experimental section).

Fraction	Volume (ml)	Activity (units/ml)	Total activity (units)	Activity recovered (%)	Nitrogen (mg/ml)	Specific activity (units/ml of N)
Original extract	560	61.1	34216	100.0	2.194	27.8
Filtrate of dialysis residue	590	50.4	29736	86.9	0.754	66.8
Effluent of 1 × DEAE-cellulose	800	28.6	22880	66.9	0.150*	190.7

* Determined on a 3ml sample.

Fig. 4. Chromatographic fractionation on microgranular DEAE-cellulose of prorennin purified by method (2)

A sample (200mg) of prorennin was applied in 20ml of 0.01M-sodium phosphate buffer, pH 6.1. Elution was done with a compound gradient of increasing phosphate buffer concentration, by utilizing the Varigrad. The concentrations of 250ml of phosphate buffer (pH 5.8–5.7) in the cylinder chambers (one to nine inclusive) were 0.10, 0.11, 0.10, 0.16, 0.10, 0.18, 0.10, 0.18 and 0.18M respectively. The flow rate was 30ml/h and three fractions/h were collected. —, E_{278} (total protein); o, potential milk-clotting activity. The fractions under each peak were pooled separately as indicated.

proteolytic activity, presumably bovine pepsinogen, which moved together with the front. However, this fast-moving proteolytic component was not included in DEAE-cellulose-purified prorennin. Crystalline rennin was again resolved into four active components.

Discussion

Although the heterogeneity of prorennin, originally demonstrated by Foltmann (1962) with DEAE-cellulose-column chromatography, was confirmed by gel electrophoresis (Asato & Rand, 1971), the chromatographic separation of prorennin components has not been substantiated (Bundy *et al.*, 1967; Shukuri, 1970). Several attempts in this study to resolve prorennin with linear phosphate gradients at pH 5.8 and 6.8, as well as salt gradients and different sources of conventional DEAE-cellulose, were also unsuccessful. When chromatography was done on microgranular DEAE-cellulose, the heterogeneity of prorennin was clearly established with definite resolution of two components (Fig. 1). This difference in

resolution of prorennin between different preparations of ion-exchanger is understandable, since the chromatographic properties of commercial preparations of DEAE-cellulose or DEAE-Sephadex vary substantially (Himmelhoch & Peterson, 1966; Brown *et al.*, 1969).

Further attempts to obtain the prorennin components as homogeneous compounds by linear gradient elution proved unsuccessful. However, complete separation of three individual prorennin components was achieved with a compound gradient. The separation of prorennin B, prorennin A and a third minor component was more successful with 1×DEAE-cellulose-purified prorennin than with 2×DEAE-cellulose-purified prorennin. This was probably due to some loss of the third minor component during the second chromatography step. It was also essential to include any trailing prorennin components during pooling after the first stepwise chromatographic purification on DEAE-cellulose, to ensure recovery of the additional prorennin component.

Electrophoretic analysis of 2×DEAE-cellulose-purified prorennin prepared by method (1) clearly showed three protein bands, whereas crystalline rennin consisted of four enzyme bands. Foltmann (1962) resolved rennin into three components by chromatography on DEAE-cellulose and established that the activation of prorennin A and B resulted in the formation of rennin A and B. Foltmann (1966) assumed that rennin C was a mixture of rennin C and rennin C₂. The C₂ component was defined as a limited proteolytic product of rennin A. He discussed the possibility that separate zymogens existed for each enzyme.

To examine the possibility that separate prorennin components do exist as precursors for each rennin, the original extract of prorennin was analysed by polyacrylamide-gel electrophoresis. Analysis of the electrophoretic run by detection of the proteolytic activity established that the original prorennin extract contained four prorennin bands and another component with proteolytic activity, presumably bovine pepsinogen, which moved together with the front. Electrophoretic analysis of DEAE-cellulose-purified prorennin prepared by method (2) demonstrated that the four prorennin components were recovered whereas all other proteolytic activity was eliminated. It is essential to exclude pepsin activity from prorennin preparations, since this can catalyse activation of prorennin, even at pH 6.0 (Rand & Ernstrom, 1968).

All four prorennin components demonstrated by electrophoresis were fractionated by chromatography of the DEAE-cellulose-purified prorennin prepared by method (2) on microgranular DEAE-cellulose with compound gradient elution. The isolated chromatographic fractions of prorennin were analysed electrophoretically and the order of mobility was consistent with that of elution from the

microgranular DEAE-cellulose column. Prorennin B and A of Foltmann appeared to correspond to prorennin fractions (II) and (III), and fraction (I) may be a precursor of rennin C. The minor component, fraction (IV), another new prorennin component, was eluted last and was recovered by a careful pooling of the chromatographic fractions.

Foltmann (1962, 1966) designated the chromatographic fractions of rennin as A, B and C in the order of decreasing enzymic activity. Rennin C was eluted first and the rennin A last. In the present study, four prorennin fractions have been separated as shown in Fig. 4 and Plate 2(b). These fractions should be re-named as prorennin A, B, C and D in the order of elution from a microgranular DEAE-cellulose column as well as mobility in gel electrophoresis. This designation would be in agreement with the nomenclature system used for isoenzymes, which are defined as multiple molecular forms of enzymes derived from the same organism (or tissue culture) sharing a catalytic activity (Brewer, 1970). Thus prorennin A has the fastest electrophoretic mobility and prorennin D would be the slowest. Prorennin D was eluted from the chromatographic column first and prorennin A last. The order of elution for the individual prorennin components was consistent with the electrophoretic mobility in polyacrylamide gel and the results obtained by the two methods are easily correlated.

Foltmann (1967, 1970) has proposed a change in nomenclature for prorennin and rennin. His suggestion was to return to the older terms of prochymosin and chymosin to avoid confusion with the enzyme renin (EC 3.4.4.15). This suggestion has merit. Thus the multiple forms of prorennin should be designated as prochymosin A, B, C and D.

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