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Purification and Partial Characterization of Prorennin*

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A procedure employing salting-out, ion-exchange chromatography, and gel filtration has been developed for the purification of prorennin from acetone powders of fresh calf stomach. The purified proenzyme is homogeneous as judged by sedimentation analysis and chromatography. The amino-terminal residue has been identified as alanine. The activation of prorennin is accompanied by the release of peptide material which appears to be derived from the amino-terminal portion of the proenzyme.

Several proenzymes or zymogens, especially those secreted by the pancreas and the gastric mucosa, have been obtained in a highly purified state permitting detailed studies of the processes of activation. The results of these investigations have made significant contributions to an understanding of the relation of chemical structure to biological activity (Green and Neurath, 1954; Neurath, 1957).

Prorennin, the inactive precursor of the milk-clotting enzyme rennin, is one of the few remaining uncharacterized zymogens of the digestive tract. Relatively little concerning this zymogen is to be found in the literature. Studies up to 1958 have been cited by Dixon and Webb (1958). Since that time Foltmann (1960, 1962) has succeeded in partially purifying prorennin.

It is the purpose of this communication to report a procedure for a 15-fold purification of prorennin from defatted calf stomach powders together with a preliminary study of the properties of the proenzyme.

Materials and Methods

Calf stomachs were obtained immediately after slaughter from the Rosen Meat Packing Co., Los Angeles, Calif. The fourth stomach was washed, freed of as much fat and connective tissue as possible, and frozen until used.

Determination of Rennin and Prorennin Activity.—Rennin and prorennin (after activation) were assayed by a milk-clotting method which is essentially that of Ege and Menck-Thygesen (1933) using 5 ml of skim milk and 5 ml of 0.1 M sodium acetate buffer, *p*H 4.9, at 35°. The skim milk was prepared as a 12% (w/v) solution of powdered skim milk (Carnation) in 0.01 M $CaCl_2$. The method was checked against a commercial rennin preparation (Nutritional Biochemicals Corp.) and the reciprocal of the clotting time was found to be a linear function of the amount of rennin-protein added. Rennin activity is expressed as 1/CT per ml,[1] the reciprocal of the clotting time produced by 1 ml of original sample.

The prorennin assay is a two-stage procedure. Prorennin samples were first activated by adding an equal volume of 0.4 M glycine-HCl buffer, *p*H 2.3. Activation was complete in 15 minutes at room temperature. The milk-clotting activity of aliquots of the activated samples was determined as described above. Prorennin activity is expressed as rennin activity after activation, corrected for dilution.

Determination of Protein Concentration.—Protein concentration was determined by measuring absorbancy at 280 mμ in a Beckman Model DU spectrophotometer.

NH_2-*Terminal Amino Acid Determination.*—The procedure of Sanger, as described by Fraenkel-Conrat *et al.* (1955), was used to determine the NH_2-terminal amino acid. Losses owing to destruction during hydrolysis and to chromatographic losses were corrected for in the usual manner.

Ultracentrifugal Analysis.—Sedimentation velocity studies were carried out at 25° in a Spinco Model E analytical ultracentrifuge. The solvent was a sodium phosphate buffer, *p*H 7.10, $\Gamma/2 = 0.154$, having the following composition: 0.1054 M NaCl, 0.0057 M NaH_2PO_4, 0.0144 M Na_2HPO_4.

Results

Purification Procedure.—Frozen calf stomachs were partially thawed, cut into strips of convenient size, and minced in a meat grinder. Minced stomach (400 g) was mixed with 3 liters of acetone at −5°, stirred for 1 hour at this temperature, and then filtered on a Buchner funnel at 5°. This procedure was repeated once with 2 liters of acetone. The filtered material was dried *in vacuo*. The following procedure was adopted for further purification:

Step 1. EXTRACTION AND AMMONIUM SULFATE PRECIPITATION.—The material dried from acetone (150 g)

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[1] Abbreviation used in this work: CT, clotting time.

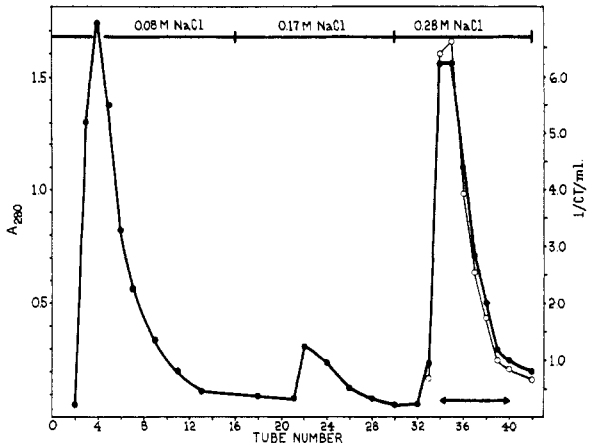

FIG. 1.—Chromatography of prorennin from step 1 on DEAE-cellulose. Stepwise elution with 0.02 M Tris buffers, *p*H 7.5, having NaCl concentrations of 0.08 M, 0.17 M, and 0.28 M. ●, absorbance at 280 mμ; O, milk-clotting activity after activation (1/CT per ml). Fractions within the horizontal arrow were combined.

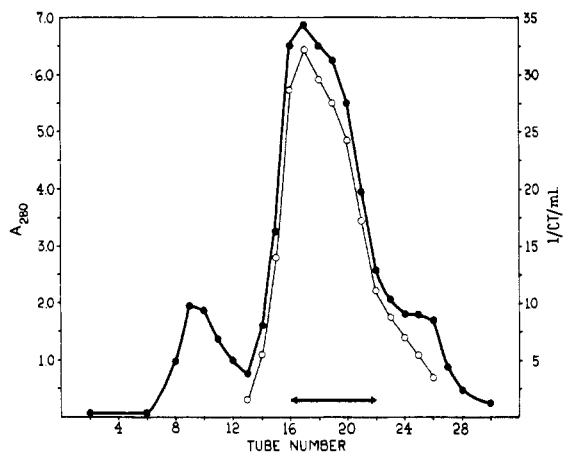

FIG. 2.—Gel filtration of partially purified prorennin on Sephadex G-200. Elution was with 0.05 M Tris–0.1 M NaCl, *p*H 7.5. ●, absorbance at 280 mμ; O, milk-clotting activity after activation (1/CT per ml). Fractions within the horizontal arrow were combined.

was extracted with 3 liters of 0.1 M Tris buffer, *p*H 7.2, with stirring for 0.5 hour at 5°. The preparation was centrifuged and the insoluble material was discarded. Solid ammonium sulfate was added to the supernatant (0.2 g/ml) and the suspension was centrifuged after standing 4 hours at 5°. The precipitate was dissolved in about 150 ml of 0.02 M Tris buffer, *p*H 7.5, dialyzed against 0.001 M Tris buffer, *p*H 7.5, until free of sulfate, and then lyophilized.

STEP 2. CHROMATOGRAPHY ON DEAE-CELLULOSE (FIG. 1).—Equilibrated DEAE-cellulose suspended in 0.02 M Tris–0.08 M NaCl, *p*H 7.5, was poured at 5° into a column (4 cm, i.d.). After the column had settled under gravity, pressure was applied from an air line resulting in a column with a height of 40 cm. Lyophilized material from step 1 (3.5 g) was dissolved in 100 ml of 0.02 M Tris–0.08 M NaCl, *p*H 7.5. The supernatant, after clarification by centrifugation, was applied to the DEAE-cellulose column. The following stepwise elution schedule was used: 0.02 M Tris–0.08 M NaCl, *p*H 7.5; 0.02 M Tris–0.17 M NaCl; 0.02 M Tris–0.28 M NaCl. The flow rate was 90 ml/hour and 70-ml fractions were collected. Assay for prorennin was performed after activation of 0.5-ml aliquots of each fraction. Fractions containing prorennin were combined as indicated in Figure 1, dialyzed against

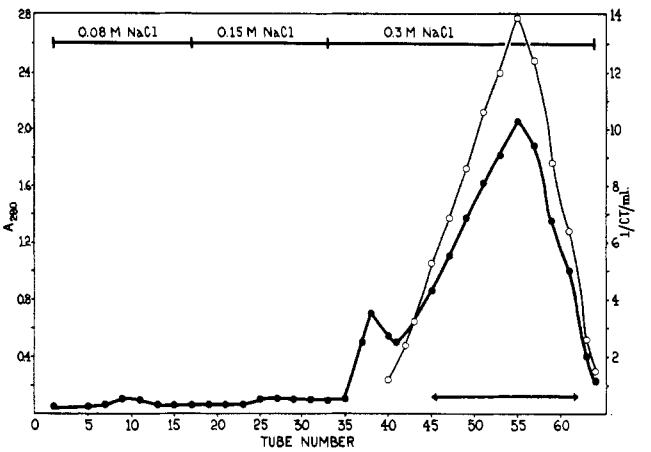

FIG. 3.—Chromatography of partially purified prorennin on DEAE-Sephadex A-50 medium. Stepwise elution with 0.02 M Tris buffers, *p*H 7.5, having NaCl concentrations of 0.08 M, 0.15 M, and 0.3 M. ●, absorbance at 280 mμ; O, milk-clotting activity after activation (1/CT per ml). Fractions within the horizontal arrow were combined.

several changes of 0.001 M Tris, *p*H 7.5, and lyophilized. Ultracentrifugation at this point indicated the presence of about 12% of a 20-25 S component.

STEP 3. GEL FILTRATION (FIG. 2). Sephadex G-200 (Pharmacia, Uppsala, Sweden) was prepared for use by allowing it to swell in an excess of 0.05 M Tris–0.1 M NaCl, *p*H 7.5. After deaeration, it was poured into a column of 2 cm i.d. to a height of 40 cm and washed for 4 hours with 0.05 M Tris–0.1 M NaCl, *p*H 7.5. This step was performed at room temperature. Lyophilized material from step 2 (500 mg) was dissolved in 12 ml of 0.05 M Tris–0.1 M NaCl, *p*H 7.5, and applied to the column. The same buffer was used as the eluent. The flow rate was 30 ml/hr with a hydrostatic pressure of 90 cm, and 5-ml fractions were collected. Prorennin-containing fractions were collected as indicated in Figure 2, dialyzed against several changes of 0.001 M Tris, *p*H 7.5, and lyophilized. Ultracentrifugation revealed the removal of the 20–25 S contaminant by this step.

STEP 4. RECHROMATOGRAPHY ON DEAE-SEPHADEX (FIG. 3).—A suspension of equilibrated DEAE-Sephadex A-50 medium (Pharmacia, Uppsala, Sweden) in 0.02 M Tris–0.08 M NaCl, *p*H 7.5, was poured at room temperature into a 1.4-cm i.d. column to the height of 35 cm. Lyophilized material from step 3 (250 mg) dissolved in 6 ml of 0.02 M Tris–0.08 M NaCl, pH 7.5, was applied to the column. Elution was stepwise with 0.02 M Tris buffers, *p*H 7.5, having NaCl concentrations of 0.08 M, 0.15 M, and 0.3 M. The flow rate was 42 ml/hr and 7-ml fractions were collected. Fractions of the main peak were collected as shown in Figure 3. The combined fractions were dialyzed against several changes of 0.001 M Tris, *p*H 7.5, and lyophilized. The average yield was 200 mg of material having a specific activity of 6.5. This represents a 15-fold purification of the extract of the acetone powder. The specific activity and purification after each step in the procedure are given in Table I.

Properties of Purified Prorennin.—Prorennin prepared as described had only a slight milk-clotting activity prior to activation (1/CT per ml/A_{280} = 0.024) compared with its activity after activation (1/CT per ml/A_{280} = 6.5). The clotting observed prior to activation is possibly due to activation taking place during the assay which is carried out at *p*H 4.7. Prolonged dialysis against water results in some activation, but dialysis against 0.001 M Tris, *p*H 7.5, can be carried out without activation or loss of potential activity.

TABLE I
EXTENT OF PURIFICATION OF PRORENNIN

Fraction	Specific Activity (1/CT per ml/A_{280})	Purification
Extract of Acetone Powder	0.43	1
Step 1	1.55	3.6
Step 2	3.6	8.4
Step 3	4.4	10
Step 4	6.5	15

When a sample of purified prorennin was rechromatographed on DEAE no distinct heterogeneity was observed, as shown in Figure 4. The specific activity was essentially constant across the peak. The results of an ultracentrifugal analysis of purified prorennin are shown in Figure 5. The sedimentation constants at four protein concentrations were extrapolated to give the value $s_{20,w}$ = 3.5 S at infinite dilution. As observed by Foltmann (1960), the sedimentation constant increases with protein concentration, suggesting a concentration-dependent association of prorennin units.

The amino-terminal residue of prorennin was found to be alanine. Dinitrophenylation and hydrolysis yielded 1 mole of DNP-alanine per 47,000 g of protein (with correction for destruction during hydrolysis).

Activation of Prorennin.—Purified prorennin is stable for 24 hours at room temperature at *p*H 7.2. As the *p*H is lowered to 5 spontaneous activation takes place. The velocity of activation increases as the *p*H is lowered to 2. An investigation of the activation of purified prorennin at *p*H 4.4 showed that trichloroacetic acid–soluble material which absorbs in the ultraviolet is liberated during activation, and Figure 6 shows that this follows rather closely the curve describing the appearance of rennin. The data were obtained in the following manner: Purified prorennin (40 mg) was dissolved in 10 ml of water which had been adjusted to *p*H 7.5 by adding ammonium hydroxide. At zero time an equal volume of temperature-equilibrated 0.2 M acetate buffer, *p*H 4.4, was added and the solution was placed in a 30° water bath. At 0.5- or 1-hour intervals 2-ml aliquots were removed and pipetted into 2 ml of 20% trichloroacetic acid. These were placed in a boiling water bath for 3 minutes and in an ice bath for 15 minutes, and were then centrifuged. The supernatants were checked for absorbance at 280 mμ. Simultaneously, 0.1-ml aliquots of the activation mixture were removed, diluted if necessary, and tested for milk-clotting activity.

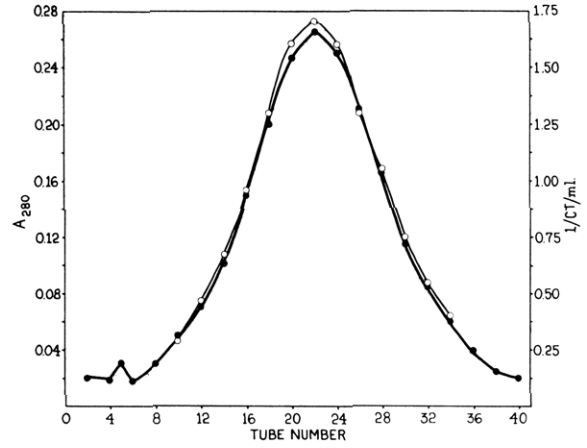

FIG. 4.—Rechromatography of purified prorennin on DEAE-cellulose in 0.02 M Tris, *p*H 7.7. A linear gradient of from 0.2 to 0.4 M NaCl was applied for the elution of protein. ●, absorbance at 280 mμ; O, milk-clotting activity after activation (1/CT per ml). Flow rate: 1 ml/min. Five-ml fractions were collected.

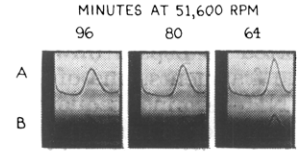

FIG. 5.—Sedimentation of purified prorennin in 0.02 M phosphate–0.1 M NaCl, *p*H 7.10, Γ/2 = 0.154. Bar angle 45°. (A) 12 mg protein per ml; (B) 6 mg protein per ml.

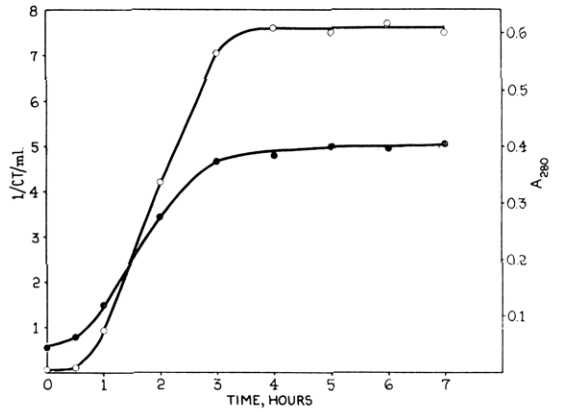

FIG. 6.—Milk-clotting activity and trichloroacetic acid–soluble material as a function of time of activation of prorennin at *p*H 4.4, 30°. O, milk-clotting activity (1/CT per ml); ●, trichloroacetic acid–soluble material (absorbance at 280 mμ).

DISCUSSION

The prorennin isolated by the present procedure is similar with respect to sedimentation constant and NH_2-terminal group to the prorennin prepared by Foltmann (1960, 1962). By way of preliminary purification, Foltmann has employed the extraction of dried calf stomachs with $NaHCO_3$ followed by two clarifications of the extract with Al_2SO_4 and the precipitation of prorennin by saturating twice with NaCl. This procedure yielded a preparation purified 7-fold with a 33% recovery of activity. About 50% of the total protein was prorennin. This material was further purified by chromatography and rechromatography on DEAE-cellulose. No statement regarding the extent of purification after chromatography was given. We believe that the method of purification described here has certain advantages over that used by Foltmann. The preliminary purification, steps 1 and 2, is a simpler operation and results in a greater than 8-fold purification with a 40–50% recovery of activity. It avoids the use of Al_2SO_4, which we have found to result in considerable inactivation of prorennin. Treatment with Al_2SO_4 may remove the high-molecular-weight contaminant which we invariably find to be present after step 2, and of which Foltmann has made no mention. However this contaminant is easily removed by gel filtration on Sephadex G-200 (step 3) with at least 80% recovery of the prorennin. The prorennin preparations obtained by the two methods differ in at least one respect. Foltmann has observed that his chromatographically purified prorennin can be fractionated into two distinct prorennin components by rechromatography on DEAE-cellulose, while the prorennin prepared by the method described here appears to be a single proenzyme, for no distinct heterogeneity was observed on rechromatography (Fig. 4).

From a consideration of the sedimentation constant (3.5 S) and NH_2-terminal amino acid data (1 mole of DNP-alanine per 47,000 g of protein), it seems reasonable that the moleculyr weight of prorennin is between 40,000 and 50,000. The molecular weight of a sample of crystalline rennin has been estimated to be 40,000 (Schwander *et al.*, 1952), and Jirgensons *et al.* (1958) have found glycine to be NH_2-termical in a sample of purified rennin. Thus it seems that as much as 10–15% of the prorennin molcule may be released as peptide(s) during the formation of rennin. The sigmoidal shape of the activation curve in Figure 6, which shows the release of peptide material during activation, is suggestive of an autocatalytic process. However this sigmoidal shape may also be interpreted as indicative of the occurrence of sequential reactions during the activation:

$$\text{prorennin} \longrightarrow \text{intermediate} \longrightarrow \text{rennin}$$

Aronson (1962) has interpreted the activation curves for prothrombin as indicative of the formation of an intermediate in the conversion of prothrombin to thrombin.

In the fragmentation during activation, prorennin appears to resemble pepsinogen. Pepsinogen (mw 42,000) is converted to pepsin (mw 34,000) with the release of peptide fragments, one of which is a pepsin inhibitor. The NH_2-terminal sequence in pepsinogen, pepsin, and the inhibitor is leu-ileu (leu), ileu-gly, and leu-glu, respectively (Van Vunakis and Herriott, 1957). Since the C-terminal group in both pepsinogen and pepsin is alanine, pepsin is derived from the C-terminal portion of pepsinogen while the peptides released are derived from the NH_2-terminal portion of the chain. The nature of the peptide(s) released during the activation of prorennin has not yet been determined, however it is apparent that at least a part of this peptide material is released from the NH_2-terminal portion of the proenzyme.

The further characterization of prorennin, the nature of the peptide material released during activation, and the mechanism of activation are under investigation at the present time.

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Evidence for an Electrophilic Mechanism in Catalysis by Hydrolytic Enzymes*

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Diphenylcarbamyl chloride, diphenylcarbamyl fluoride, methylphenylcarbamyl chloride, and methylphenylcarbamyl fluoride were studied as acid-transferring inhibitors of chymotrypsin, trypsin, acetylcholinesterase, and serum cholinesterase. The fluorides were the better inhibitors in all cases. They are quite potent inhibitors and are the best of the known inhibitors of chymotrypsin and trypsin. The greater activity of the fluorides is evidence that there is an electrophilic component in the enzymic mechanism.

Certain compounds in reaction with proteases and esterases transfer an acid group to the active center yielding an inactive enzyme derivative. These acid-transferring inhibitors include a large number of organophosphates, carbamates, and sulfonates, of which diisopropylfluorophosphate (DFP) is perhaps the best known (Jansen *et al.*, 1949). Dimethylcarbamyl fluoride (Wilson *et al.*, 1961) and methanesulfonyl fluoride (Kitz and Wilson, 1962; Fahrney and Gold, 1963) are examples of fluorine derivatives belonging to the other two classes of compounds.

Acid-transferring inhibitors react with enzymes in a manner analogous to the reactions of substrates (Wilson, 1951). Although there is some disagreement it is generally held that substrates react to form an acyl-enzyme as a reaction intermediate (Wilson *et al.*, 1950). The acyl-enzyme is then rapidly hydrolyzed to complete the catalysis

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_3} E' \underset{+\ P_1}{\xrightarrow[H_2O]{k_4}} E + P_2$$

where E is the enzyme, S the substrate, P_1 the alcohol product, P_2 the acid product, and E′ the acyl-enzyme. The reactions of acid-transferring inhibitors differ in

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