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A PROTEINASE FROM HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

An enzyme has been isolated from human erythrocyte membranes with proteolytic activity for casein, hemoglobin, stroma proteins and certain synthetic substrates. This enzyme may be extracted from membranes by a number of 1 M salt solutions and stored at 4° or frozen for several weeks. A standard assay procedure using Azocoll is defined for this proteinase. Activation is shown to occur when the proteinase is treated with KCNS. Initial purification by column chromatography gives a protein fraction with most of the proteolytic activity in 20% of the total extracted protein. This protein fraction contains 3–5 species by disc electrophoresis, one or two of which have proteolytic activity. Experiments with the ultracentrifuge indicate that the proteolytic activity is a part of, or is bound to, one or more lipoprotein species.

INTRODUCTION

In an effort to improve the storage life of blood-banked whole human blood, attention has been focused on the stabilization of the red cell membrane and on understanding the mechanism of hemolysis *in vitro*. The material presented in this paper developed from the original observation of MORRISON AND NEURATH[1] that the erythrocyte membrane contained proteolytic activity. Interest in this proteolytic enzyme system was enhanced by our observation that the crude extract of MORRISON AND NEURATH[1] was capable of hydrolyzing erythrocyte membranes and isolated membrane proteins. Methods for extraction, concentration and assay for erythrocyte proteolytic activity are presented, as well as observations on stability, substrate specificity and purification experiments.

MATERIALS AND METHODS

Blood bank units (450 ml) of acid–citrate–dextrose blood from young healthy

Abbreviations: TAME, *p*-tosyl-1-arginine methyl ester; BTEE, *n*-benzoyl tyrosine ethyl ester.

male donors were used. Fresh blood is defined as blood used within 18 h of the time it was drawn. Outdated blood was obtained from our blood bank 21 days after drawing. Unless otherwise stated, all blood used in this study was fresh.

Stroma was prepared by a modification of the DODGE[2] procedure. With each wash, the stroma and buffer were mixed in a Dounce tissue grinder and spun at 30 000 × *g* for 15 min. The concentrated stroma was dialyzed overnight against 4 l of cold deionized water.

Extraction studies were based on the methods of MORRISON AND NEURATH[1]. Solutions (1 M) of NaCl, KCNS, KCl, and $MgCl_2$ were mixed in a 3:1 ratio with the stroma suspension, incubated at 4° for various time periods and then spun at 60 000 × *g* for 1 h. The supernatant contained most of the proteinase activity.

Protein content was determined by the biuret method of ITZHAKI AND GILL[3]. Folin assays were done by the method of LOWRY *et al.*[4] and hemoglobin determinations by the method of CROSBY AND FURTH[5].

Natural substrates. Natural substrates included hemoglobin, soluble casein, stroma, stroma sialoprotein prepared according to BLUMENFELD[6] and Cohn Fraction I (ref. 7) prepared from fresh human plasma. A modification of the method of ANSON[8] was used to measure proteolytic activity. The assay mixtures consisted of 30 mg protein substrate in 5.0 ml potassium phosphate buffer (pH 7.4), $I = 0.1$ (ref. 9), and 2.0 ml proteinase extract. Controls contained the substrate protein in 7.0 ml of buffer. The assay mixtures were shaken at 37° for a period of 22 h. Aliquots of 1.0 ml were withdrawn at time zero, 2, 4, 6, 8, 12, and 22 h. Each aliquot was quenched by addition of 3.0 ml 5% trichloroacetic acid, filtered and the filtrate assayed by the Folin reaction[8].

Synthetic substrates. *p*-Tosyl-1-arginine methyl ester (TAME) and *n*-benzoyl tyrosine ethyl ester (BTEE), from Mann Laboratories, were tried as substrates using the standard trypsin and chymotrypsin assays[10]. The hide-bound azo-dye, Azocoll, (Calbiochem Corporation) was used as follows: 25 mg Azocoll were suspended in 4.0 ml potassium phosphate buffer (pH 7.4), 1.0 ml of proteinase extract was added and the mixture shaken at 37° for 1–4 h. The unreacted substrate was filtered off and the absorbance measured at 520 mμ.

Proteinase extracts were concentrated either by dialysis using Aquacide II (Calbiochem) at 4°, or in Amicon ultrafiltration cells with UM-10 membranes, which retain protein but allow water and salts to pass.

A concentrated proteinase sample was placed on a Sephadex G-100 column (25 mm × 750 mm) and eluted with the phosphate buffer containing 0.5 M NaCl, all at 4°.

Disc electrophoresis was performed on the concentrated (4 mg/ml) Sephadex G-100 Peak 4 protein using the Canalco analytical system. The procedure of DAVIS[11] was used for the nonurea system. The 8 M urea system of DEVENUTO *et al.*[12] was also used.

Four tubes were run with each system, one being used for staining with aniline blue black, the other three for elution and proteinase assay.

A Spinco Model E ultracentrifuge equipped with Schlieren optics was used. Preparative ultracentrifugation was done on a Spinco Model L at 4° using a 40 000 rev./min (144 000 × *g*) head for a period of 18 h.

RESULTS

In a typical preparation, one bag (500 ml) of acid–citrate–dextrose blood yields 80 ml of stroma suspension with a protein concentration of about 10 mg/ml. The Dounce tissue grinder must be used if proteinase activity is desired from salt extracts of the final stroma.

Initial studies of proteinase extraction were done according to MORRISON AND NEURATH[1]. The extracted solutions were assayed on casein as shown in Fig. 1 where hours of incubation are plotted against the absorbance at 750 mμ of the Folin assay. All four salt extracts (protein content 0.78–0.90 mg/ml) had activity, but only the KCNS extract was free of a lag period during the first 2 h of the assay.

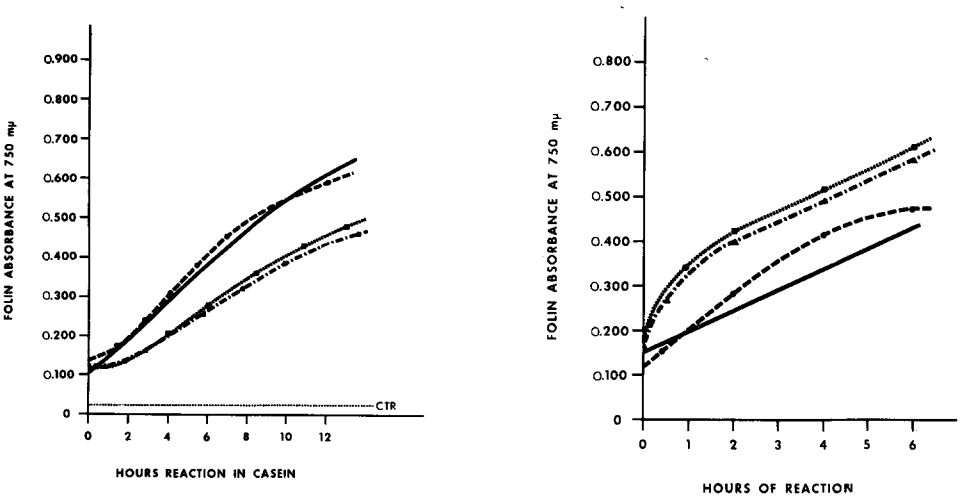

Fig. 1. The hydrolysis of casein by a proteinase extracted from stroma using four different salts. 1 part stroma extracted with 3 parts 1 M salt as follows: ———, KCNS; ●— — —●, $MgCl_2$; ■·····■, NaCl; and ▲—·—▲, KCl. - - - - -, control casein.

Fig. 2. Proteinase activity using as substrates: ———, casein; ●— — —●, hemoglobin; ▲—·—▲, stroma; ■·····■, sialoprotein from stroma.

When stroma aliquots were extracted with 1 M KCNS for 1, 6, 12, 18, 24 and 48 h and the extracts assayed, similar activities were obtained, indicating that the extraction is very rapid. In subsequent studies, 1-h or overnight extracts were used depending on convenience. Most extracts had a protein concentration between 0.5 and 1.0 mg/ml.

In addition to casein, several other protein materials were tested as possible substrates; Fig. 2 shows a 6-h assay of 1 M KCNS-extracted proteinase with the substrates casein, hemoglobin, stroma and solubilized stroma sialoprotein. No activity was obtained using the Cohn Fraction I as substrate. As with casein control samples of the other proteins remained at a constant low level.

Two synthetic substrates were tried. The trypsin-specific substrate TAME was assayed with the proteinase, but no activity was found. Next, the chymotrypsin-specific substrate BTEE was used with the extracted proteinase. Low activity was

obtained with the proteinase, equivalent to 0.261 unit of chymotrypsin per mg of extracted protein.

Assaying with Azocoll was found to be fast, simple and sensitive. Several extracts were assayed for intervals of 0.5–4 h. The released dye was measured by absorption at 520 mμ, and a plot of absorbance *versus* time was linear below 1.500 absorbance units. An assay time of 3 h was found to be convenient for most experiments; however, shorter or longer times were used depending on sample activity.

To facilitate comparison of proteinase activity from different experiments, we have defined units of proteinase activity for the Azocoll assay system. The protein content of each extract was measured by the biuret procedure. Units of activity are defined as:

$$\text{Units} = \frac{A_{520\ \text{m}\mu}}{\text{mg}} \times \frac{1}{f} \times 10$$

where $A_{520\ \text{m}\mu}$ is the measured absorbance of the released azo dye, mg is the mg of extracted protein added to the assay mixture, f is the fraction of 3 h for which the assay was run and the factor $\times$ 10 is used to place all values above unity.

For concentration and storage studies, aliquots of an extract were either stored at 4°, frozen, lyophilized, dialyzed against cold water or reduced to 1/3 volume with Aquacide II. All these operations were done within an 8-h period, then each sample was restored to the same volume and salt strength as the control sample stored at 4°.

From the results shown in Table I, it is apparent that concentration with

TABLE I

RESULTS OF VARIOUS STORAGE AND CONCENTRATION PROCESSES ON THE PROTEINASE ACTIVITY OF 1 M KCNS EXTRACTS

Storage at 4° used as control

Treatment	*Activity (units/mg)*	*% Control value*
4°	20.00	100
Dialyzed	4.67	23.3
Lyophilized	10.67	53.3
Aquacide II	13.30	66.7
Quick frozen	17.78	88.9

Aquacide II produces a 33% loss in activity. Amicon ultrafiltration cells were tried for proteinase concentration. The UM-10 membrane was not stable when KCNS was present in the extract and soon became completely impermeable. When 1 M NaCl was used to extract the proteinase from stroma, the samples could be concentrated up to 10-fold with the UM-10 membrane.

As can be seen from Table I, dialysis should be avoided since only 23% of the activity is recovered after replacement of the lost salt. Lyophilization is also undesirable, since half the activity is lost and redissolving the sample was difficult.

A stroma sample was divided into aliquots which were frozen or lyophilized. Extracts with 1 M KCNS were made at time zero and at 7-day intervals. The activities using Azocoll as substrate remained constant for 42 days. The data show that the stroma may be frozen or lyophilized for later extraction of the proteinase.

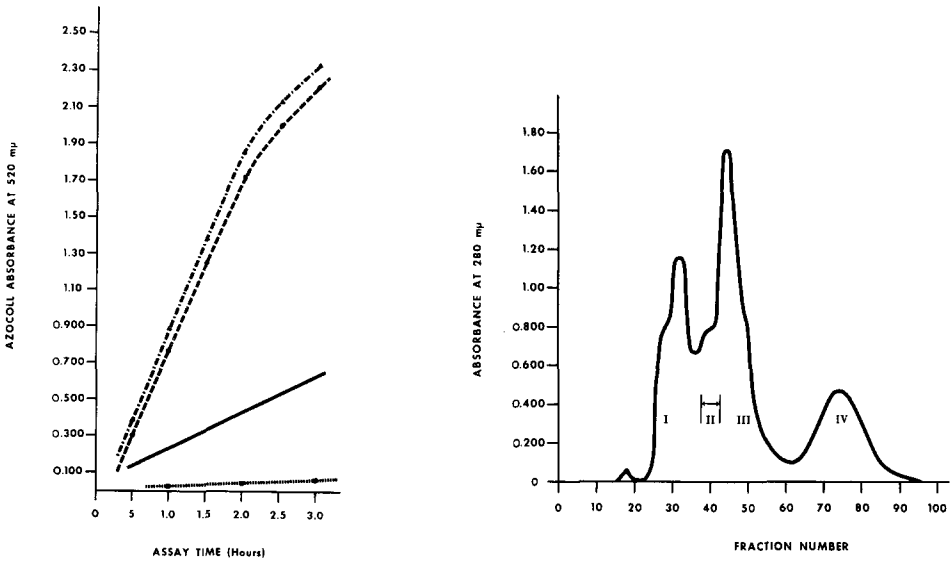

Fig. 3. KCNS activation of stroma proteinase. Extraction with NaCl (———) and KCNS (▲—·—▲), with KCNS added to the NaCl extract during assay (●— — —●). Control Azocoll (■·····■).

Fig. 4. Column chromatography of concentrated proteinase extract on Sephadex G-100. Fraction number (approx. 6 ml each) *versus* protein absorbance at 280 mμ. Main protein fractions labeled I–IV.

NaCl extracts were usually of lower activity than KCNS extracts, although the protein concentration of these extracts was similar. The possibility that KCNS activates the proteinase was tested by dividing a stroma into two aliquots, extracting one with 1 M KCNS and the other with 1 M NaCl. When the NaCl extract was assayed, duplicates were run with and without added KCNS. The results of an Azocoll assay are shown in Fig. 3. The protein concentrations (mg/ml) were 0.45 for the NaCl and 0.48 for the KCNS extract. Proteolytic activity of the NaCl extract was low compared to that of the KCNS extract. However, when 1 M KCNS (1.5 ml) was added to the NaCl assay mixture in place of an equal volume of buffer, the activity approximated that of the KCNS extract. Addition of NaCl to the assay mixture of a KCNS extract did not change its value. Similar activation results were obtained using casein, hemoglobin and stroma as substrates.

Results of chromatography on Sephadex G-100 to separate concentrated proteinase extracts are shown for a typical run in Fig. 4. Four major areas can be seen and are labeled I–IV. Areas I–III obviously represent more than three species. Enzyme activity was associated primarily with Peak IV. In a typical run, Peak IV activity was 20 units/mg while the other pools were below 1 unit/mg protein. Peak IV represented about 20% of the total protein obtained from the column. The contents of Peak IV were concentrated for analysis by disc electrophoresis and ultracentrifugation.

The concentrated proteinase fraction (Peak IV from Sephadex) was analyzed by disc electrophoresis both with and without 8 M urea in the gel system. Fig. 5 shows the schematics of the two methods employed. Unstained replicates of both Tubes A and B were divided into three segments as indicated by the dashed lines. Each gel

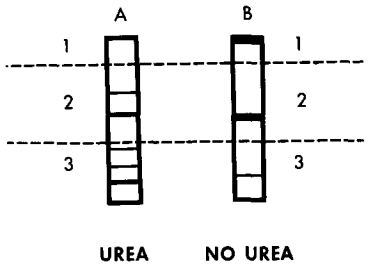

Fig. 5. Disc electrophoresis of proteinase fraction from Sephadex chromatography. Tube A is the urea system and Tube B the nonurea system. Migration is from top to bottom. The three zones used for extraction and assay are shown by dashed lines.

segment (A 1–3 and B 1–3) was extracted with 1 M KCNS overnight and the extractant assayed using Azocoll as the substrate. Proteolytic activity was observed only in sections A 2 and B 1.

Fig. 6 shows the Peak IV ultracentrifuge pattern which resembles that of a lipoprotein in two ways. First, a peak forms at the meniscus but does not move down the cell. Second, the peaks at top and bottom of the cell resemble the floatation patterns seen with lipoproteins in a low-density medium. A slight shoulder can be seen at the meniscus of the first picture which rapidly diffuses. This may be non-lipoprotein which is sedimenting to the bottom of the cell. After the run, a buildup of sedimented material was seen on the cell bottom. Azocoll assays were performed on this sediment, as well as on the top and bottom halves of the supernatant. 10% of the total activity was found in the bottom half of the supernatant, but no activity was seen in the top half or the sediment.

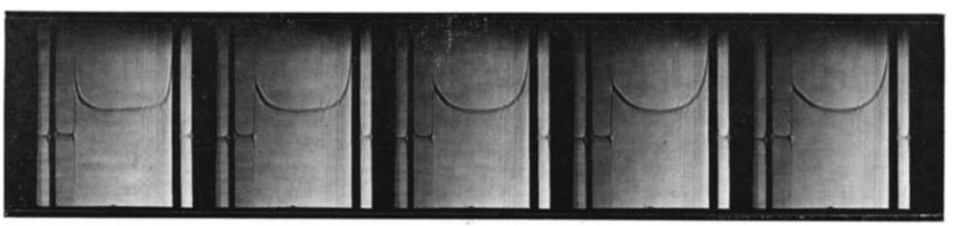

Fig. 6. Ultracentrifuge patterns of protein from Sephadex G-100 Peak IV. Pictures (left to right) taken at 16-min intervals, starting 4 min after reaching top speed of 52 000 rev./min. The bar angle was 50° with a constant temperature of 20°.

In an effort to separate proteinase activity from lipoprotein-like material, 10 ml of sample were spun in the preparative ultracentrifuge for 18 h and the sample divided into nine layers of about 1 ml each, from top to bottom of the tube. Table II shows the results of biuret and Azocoll assays on each of these fractions. This sample was not dialyzed but had been lyophilized and resuspended in water. Therefore, it had a high salt content which raised the density of the solution to 1.196. At this density lipoprotein should have floated to the surface, while non-lipoprotein sedimented. As seen in Table II, protein did tend to concentrate at the top and bottom of the sample. Since it was suspected that the proteinase was a non-lipoprotein component of the total mixture, the bottom layer (No. 9) was rerun in the Model E ultracentrifuge. The

TABLE II

ASSAY ON LAYERS FOLLOWING PREPARATIVE ULTRACENTRIFUGATION OF THE SEPHADEX G-100 PEAK IV

Sample	*No.*	*Biuret protein (mg/ml)*	*Azocoll* $A_{520\ m\mu}$	*Units activity/mg*
Top	1	2.01	—	—
	2	2.26	0.060	2.65
	3	1.55	—	—
	4	1.44	—	—
	5	1.39	0.037	2.57
	6	2.16	—	—
	7	2.34	0.060	2.77
	8	2.39	—	—
Bottom	9	3.55	0.110*	3.10

* Assayed after analysis in the Spinco Model E ultracentrifuge.

results were similar to Fig. 6. Azocoll assays of Fractions 2, 5, 7, and 9 showed that the activity was about equally proportional to the protein concentration throughout the entire sample.

DISCUSSION

MORRISON AND NEURATH[1] reported the presence of proteolytic activity in red cell membranes using various extraction procedures and designated these as Proteinases I, II, and III. These studies have concentrated on isolation and characterization of the salt-extracted Proteinase I, designated simply, proteinase.

It was found that homogenization of the membranes was required prior to salt extraction if proteolytic activity was to be obtained. This may indicate that the proteinase is located in the interior of the membrane.

A broad range of substrates appeared to be acted upon by the extracted proteinase. The potential destructive nature of the proteinase to red cell integrity is seen by the high activity with stroma, stroma protein and hemoglobin. The enzyme does not appear to resemble trypsin but may have some chymotrypsin-like activity.

Column chromatography of an extract isolated the activity in a fraction representing about 20% of the total extracted protein. Disc electrophoresis and preparative ultracentrifugation show that this fraction contains at least three species. From the ultracentrifuge experiments, two or more of these protein species are thought to be lipoprotein in nature. Disc electrophoresis limited proteolytic activity to one or two protein species, while preparative ultracentrifugation distributed the activity uni formly as a function of protein concentration. This could result from a tight binding of proteinase to a group of similar lipoproteins. This binding remains intact during ultracentrifugation but is broken under conditions used during disc electrophoresis. The possibility of a lipoprotein having proteolytic activity is, at this time, unknown.

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