A New Blood-coagulating Protease in Mitochondrial Membranes of Rat Submaxillary Glands

PURIFICATION AND CHARACTERIZATION OF PROTEASE AND ITS BLOOD-COAGULATING ACTIVITY*

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An integral membrane protease was solubilized and purified to homogeneity from rat submaxillary mitochondria. The purified enzyme could coagulate rabbit plasma. The molecular mass of the enzyme is 22 kDa on SDS-polyacrylamide gel electrophoresis under reducing conditions and 24 kDa on gel filtration on a Sephadex G-100 column. Its isoelectric point is 4.2-4.25. Enzyme activity is strongly inhibited by diisopropyl fluorophosphate, soybean trypsin inhibitor, benzamidine, aprotinin, and antipain, suggesting the enzyme as a serine protease. Its pH optimum for activity is 8.5. Zn²⁺ is strongly inhibitory; at 1 mM concentration it produced 72% inhibition. The enzyme is active toward different synthetic substrates (p-nitroanilide derivatives) containing Arg at the P₁ position with blocked NH₂ terminus. Kₐ/Kₘ was highest with the substrate N-Benzoyl-Pro-Arg-pNa (where Bz is benzoyl and pNA is paranitroanilide).

The purified enzyme coagulates rabbit plasma in a dose-dependent manner. Plasma coagulation by the enzyme is completely blocked in the presence of aprotinin or soybean trypsin inhibitor, suggesting that protease activity is required for this coagulation reaction. Antibody raised against the purified enzyme inhibits the plasma coagulation initiated by the enzyme. The enzyme can correct the prolonged clotting time of factor X-deficient human plasma but is unable to convert purified prothrombin. Several properties of the enzyme distinguish it from other reported submaxillary proteases.

Submaxillary tissue is a rich source of several soluble proteases along with some physiologically active peptides (1-8). Some proteases have been correlated with the processing of naturally occurring precursor proteins (9, 10). Kallikrein (2), tonin (3), thiol-activated T-kininogenase (4), RSP-V (5), and esterase B (6) are examples of some well studied soluble proteases in submaxillary tissue. Despite several investigations on a large number of soluble proteases, work on intracellular membrane proteases from this tissue has not been reported. Growing appreciation in the field of intracellular membrane proteases has emerged when it is known that these proteases are involved in several cellular events like peptide processing, posttranslational modifications in the release of biologically active peptides from precursors, removal of signal peptides, and hydrolysis of proteins associated with membrane.

We observed that mitochondrial membrane of rat submaxillary gland contained significant protease activity (11, 12). The enzyme strongly associated with the mitochondrial membrane was solubilized by deoxycholate treatment and purified to homogeneity. Interestingly, it was found that the purified enzyme could coagulate rabbit plasma very effectively. Most of the factors involved in blood coagulation, which comprises a series of cascade reaction, have high molecular masses whereas the molecular mass of the protease tested here was 22 or 24 kDa. This enzyme was also different from the established tissue factors (13) that are known to participate in the extrinsic pathway of blood coagulation. The plasma-coagulating activity of a submaxillary protease has not been reported earlier.

In this communication, we describe the purification of a mitochondrial membrane protease from rat submaxillary gland, characterization of the purified protein, and its ability to coagulate rabbit plasma.

EXPERIMENTAL PROCEDURES

Materials
DEAE-cellulose, sodium deoxycholate, TEMED,¹ acrylamide, Pharmalyte 3-10, molecular weight marker proteins, isoelectric point marker proteins, enzyme inhibitors, synthetic substrates, N,N'-methylenbisacrylamide, bovine fibrinogen, and bovine thrombin were purchased from Sigma. Sephadex G-100 was from Pharmacia (Upsala, Sweden). BAPNA was purchased from Boehringer Mannheim. Human plasmas with deficiencies of coagulation factors were obtained from George King Biomedical. Plasma was supplied in dry ice.

Enzyme Preparation
Preparation of Mitochondrial Fraction from Rat Submaxillary Gland
Sprague-Dawley rats (weighing 170-225 g) of either sex were used. A 10% homogenate of rat submaxillary tissues was made with 10 mM phosphate buffer (pH 7.4) containing 300 mM sucrose and 1 mM EDTA in a motor-driven, Teflon-pestled Potter-Elvehjem homogenizer. The homogenate filtered through a gauze was centrifuged at 12,000 x g for 10 min. The pellet suspended in the same buffer was recentrifuged at 1000 x g for 10 min. The pooled supernatant was centrifuged at 12,000 x g for 30 min, and the pellet was washed three times with homogenizing buffer.

Purification of Mitochondria
Mitochondria were purified from a 12,000 x g pellet on a continuous gradient of sucrose between 1 and 2 M (14).

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¹ The abbreviations used are: TEMED, N,N,N',N'-tetramethylethylenediamine; BAPNA, N-benzoyl-DL-arginine paranitroanilide; BAEED, N-benzoyl-L-arginine ethyl ester; TAME, p-tosyl-L-arginine methyl ester; Bz, benzoyl; r-Boe, t-butoxycarbonyl; SBTI, soybean trypsin inhibitor; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, L-tosyl-aminod-2-phenylethyl chloromethyl ketone; pNA, paranitroanilide; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
Blood-coagulating Protease in Mitochondrial Membrane

**KCI Treatment of Mitochondria**

The purified mitochondria were treated with 10 mM phosphate buffer (pH 7.4) containing 1 mM KCl for 3 h with occasional stirring and then centrifuged at 105,000 x g for 1 h in an ultracentrifuge (Beckman L5-50B). The pellet was resuspended, and the process was repeated.

**Solubilization of Protease**

The pellet obtained after KCl extraction was treated with 0.5% (w/v) deoxycholate in 10 mM phosphate buffer (pH 7.4) containing 0.15 M KCl and kept overnight at 4 °C. The supernatant obtained by centrifuging the mixture at 105,000 x g for 1 h in the ultracentrifuge was used for purification.

**Purification Steps**

The enzyme at different steps in preparation was concentrated using an Amicon, Inc., PM-10 filter. All steps of enzyme preparation were carried out at 4 °C.

**Step 1: Ammonium Sulfate Precipitation**—Solid ammonium sulfate was added to make 45% ammonium sulfate saturation of the solubilized enzyme which, after 15 min, was centrifuged at 15,000 x g for 30 min. The ammonium sulfate saturation of the supernatant was raised to 75%. The precipitate appearing at 45–75% saturation was dissolved in 50 mM Tris-HCl (pH 7.0) and dialyzed for 5 h against 50 mM Tris-HCl buffer (pH 7.0) containing 0.05% deoxycholate with two changes. The precipitate obtained in the experiments when dialyzed was applied directly to the gel and focused at 2000 V until complete.

**Step 2: Sephadex G-100 Gel Chromatography**—The dialyzed material centrifuged at 10,000 x g for 5 min was loaded on a Sephadex G-100 column (1.6 x 80 cm), which was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl and 0.1% deoxycholate.

**Step 3: DEAE-cellulose Chromatography**—The pre fraction was concentrated and dialyzed against 50 mM Tris-HCl buffer containing 0.001% deoxycholate (pH 7.5) for 5 h with two changes. The sample was put on a DEAE-cellulose column (1.2 x 15 cm) equilibrated with the dialyzing buffer. The column was eluted initially with the equilibrating buffer followed by a gradient between 50 mM Tris-HCl buffer and 500 mM Tris-HCl buffer (pH 7.5).

**Step 4: Native PAGE**—The concentrated protease fraction from the ion-exchange column was dialyzed for 4 h against distilled H₂O with two changes containing 0.001% deoxycholate. The sample was then run into gel tubes for electrophoresis (15) in non-denaturing conditions. After electrophoresis, each gel was cut into 2-mm segments. Each segment was taken in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.0, disrupted, and kept for 3 h at 4 °C with occasional stirring. After centrifugation, the activity was assayed in the supernatant. SBTI-sensitive (80% inhibition) protease-containing fractions were concentrated.

**Step 5: HPLC**—The material from step 4 was passed through a gel filtration HPLC column (TSK G2000 SW, 30 x 0.75 cm), which was equilibrated and eluted with 250 mM Tris-HCl buffer, pH 6.7, containing 0.001% deoxycholate.

**SDS-PAGE**

SDS-PAGE was carried out according to Laemmli (15) under reducing conditions in 10% gel. Gels were stained with Coomassie Brilliant Blue R-250.

**Isoelectric Focusing**

This was carried out in an LKB 2117 Multiphor electrophoresis system with a pH gradient of 3–10. The protein sample and PI markers were applied directly to the gel and focused at 2000 V until complete. The gel was stained with Coomassie Brilliant Blue G-250. The pl of the purified enzyme was determined by comparison with the electrophoretic mobility of the PI markers.

**Assay of Enzymes**

**Protease Activity**

**Hydrolisis of BAPNA**—The enzyme activity in all experiments (unless stated) was assayed using BAPNA substrate by the method of Erlanger et al. (16) with slight modifications. The reaction mixture (1.0 ml) contained 90 mM Tris-HCl buffer (pH 8.0), 1 mM BAPNA, and a suitable volume of enzyme preparation. The production of p-nitroaniline was measured at 410 nm in a Pye Unicam spectrophotometer. The enzyme activity was expressed as nanomoles of p-nitroaniline liberated per 30 min. The activity against p-nitroanilide derivative of different synthetic substrates was assayed similarly.

**BAEE Hydrolysis**—The enzyme activity in column eluates during purification was monitored by measuring BAEE hydrolysis (17) at pH 8.0. The activity was measured spectrophotometrically at 233 nm.

**Succinic Dehydrogenase**

The activity was assayed by the method of Singer and Kearney (18) with slight modifications.

**Plasma Coagulation**

Coagulating activity was measured essentially by following the method of Kamiguti et al. (19). Plasma was prepared from citrated blood collected from healthy adult white Belgian rabbits (2.0 ± 0.3 kg). Clotting was studied in the reaction mixture (0.3 ml) containing 0.1 ml of plasma, 8 mM CaCl₂, and a suitable volume of enzyme or saline. Clotting time was recorded after the addition of CaCl₂. In controls, clotting time was measured in the absence of enzyme. Clotting times of human plasmas deficient in clotting factors were determined similarly. Fibrinogen clotting was assayed similarly except that purified fibrinogen (600 µg) was used in place of plasma.

**Kinetic Studies**

Kinetic parameters, Kₘ, V₉₉, and K₉₉, were calculated from Eadie and Holfseth plots, which were done in a computer using “Sigma Plot.”

**Preparation of Polycyalon Antibody**

Antiserum against the purified protease was raised in two rabbits by four successive injections to each. The immunization schedule comprised four subcutaneous injections at days 0, 7, 15, and 35. The first and second injections were given in the thumbspads using 300 and 150 µg of protein, respectively, in Freund's complete adjuvant. The last two injections comprised 100 µg of protein each in incomplete Freund's adjuvant. At the 10th day after the last injection, blood was drawn by cardiac puncture under aseptic conditions. Serum prepared from pooled blood was stored at -70 °C until use. Non-immune serum was prepared from the rabbits before immunization started.

**Partial Purification of Antiserum**

The antiserum and non-immune serum were partially purified by ammonium sulfate (0–45% saturation) precipitation. The precipitate dissolved in 50 mM Tris-HCl, pH 7.5, was dialyzed overnight against the same buffer. Protein was determined by the Bradford method (20).

**Electron Microscopic Study**

The sample was negatively stained with 2% phosphotungstic acid (pH 7.0), dried in air, and observed in a Jeol, Ltd. 100CX transmission electron microscope at an operating voltage of 60 kV.

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**RESULTS**

We observed significant protease activity as evidenced by BAPNA hydrolysis in the mitochondrial fraction (12,000 x g pellet) of rat submaxillary glands. Mitochondria were purified (14) from the pellet on a continuous gradient of sucrose between 1.0 and 2.0 M. The fraction (at about 1.18 g/ml) enriched in succinic dehydrogenase activity (70%) also contained most of the protease activity. The fraction when checked by negative staining under the electron microscope showed 100% purity of the mitochondria. Glucose-6-phosphatase and acid protease activities were not observed in this fraction. The protease associated with the membrane after extraction of this fraction with 1.0 M KCl was solubilized by 0.5% deoxycholate and purified in several steps as mentioned under “Experimental Procedures.”

**Purification**—The results of purification are summarized in Table I. Solubilization of the enzyme by deoxycholate produced a 65% increase in activity, indicating that part of the enzyme was buried inside the membrane. Deoxycholate itself could not alter the activity of the purified enzyme. Gel DEAE-cellulose chromatography (Fig. 1), a small activity appeared just after the start of the gradient whereas major activity eluted later on was further purified. This fraction when electrophoresed on polyacrylamide gel under non-denaturing conditions was resolved into two active fractions: a slower migrating enzyme (A) sensitive to SBTI and a faster migrating enzyme (B) resistant to SBTI (Fig. 2). The enzyme fraction (A) was resolved into six...
Purification of protease from mitochondrial membrane of rat submaxillary gland

Activity toward BAPNA at pH 8.0 is shown below.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein mg</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification fold</th>
<th>Recovery %</th>
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<td>KCl-extracted pellet</td>
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<td>Deoxycholate-soluble extract</td>
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<td>8515</td>
<td>40.8</td>
<td>2.94</td>
<td>164</td>
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<tr>
<td>Ammonium sulfate precipitation</td>
<td>18.4</td>
<td>4212</td>
<td>228.9</td>
<td>16.5</td>
<td>81.5</td>
</tr>
<tr>
<td>Sephadex G-100 precipitation</td>
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<td>3877</td>
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<td>34.9</td>
<td>75.0</td>
</tr>
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<td>2680</td>
<td>812.1</td>
<td>58.75</td>
<td>51.8</td>
</tr>
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<td>2634.3</td>
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<td>3.5</td>
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<td>151.4</td>
<td>7570.0</td>
<td>545.8</td>
<td>2.9</td>
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</table>

The methods followed were as mentioned under "Experimental Procedures." The enzyme activity of each peak was measured by studying BAPNA hydrolysis.

peaks on gel filtration HPLC (Fig. 3), among which only peak B possessed protease activity. The final preparation had a specific activity of 7570 with a 545.8-fold purification over the KCl-extracted pellet (Table I). The results of purification representing the data of a typical experiment were reproduced with different batches of the preparation.

Characterization of Purified Protease—The molecular mass of the enzyme was found to be 22 kDa when determined from its electrophoretic mobility in SDS-PAGE under reducing conditions (Fig. 4). We found that high molarity of phosphate buffer inhibited the activity to a great extent. The purified enzyme (peak B in Fig. 3) when chromatographed on the same gel filtration column in HPLC in 250 mM phosphate buffer, pH 6.7, eluted as a single peak. A single precipitation line produced by cross-reaction of the enzyme with the antisera against the purified enzyme also suggested the apparent homogeneity of the purified protein.
phate, benzamidine, aprotinin, antipain, and SBTI are strong inhibitors. Iodoacetamide, N-ethylmaleimide, and TPCK inhibit the enzyme activity moderately. Other reagents tested (Table II) were without any significant effect. TLCK, an inhibitor of trypsin-like proteases, could not alter the protease activity. Among the metal ions tested (data not shown) Zn$^{2+}$ (1 mm) produces 72% inhibition of the activity. $K'_1$, Na$^+$, Cu$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ have no significant effect. Co$^{2+}$ and Mn$^{2+}$ (2 mm each) inhibit the enzyme activity by 42 and 44%, respectively. Tonin from submaxillary glands is inhibited by Cu$^{2+}$ (21).

The substrate specificity of the enzyme is shown in Table III. The enzyme is active toward different synthetic substrates containing Arg at the P$_1$ position with a blocked COOH-terminal end and also with a blocked NH$_2$-terminal end. No other amino acids including another basic residue, Lys at P$_1$, is cleaved by the enzyme.

The esterase activity of the enzyme was studied using BAEE. TAME, and benzoyl-arginine methyl ester. The enzyme hydrolyzes BAEE but fails to hydrolyze TAME or benzoyl-arginine methyl ester. In controls, trypsin could hydrolyze BAEE and TAME effectively. The insensitivity toward TAME singles out the enzyme from many reported submaxillary proteases that can hydrolyze TAME at varying rates (6).

Table IV describes kinetic analyses of some synthetic substrates that were properly hydrolyzed by the enzyme. $K_v/K_m$ was highest in N-Bz-Pro-Phe-Arg-pNA; the next highest value was obtained in the hydrolysis of N-t-Boc-l-Leu-Gly-Arg-pNA. The enzyme shows similar $K_v/K_m$ values with all other substrates except L-BAPNA and DL-BAPNA. $K_a$ of the enzyme is also maximal in N-Bz-Pro-Phe-Arg-pNA hydrolysis. Other kinetic parameters like $V_{max}$ and $K_m$ are also displayed in Table IV.

**Plasma-coagulating Activity of Enzyme**—The purified enzyme coagulates plasma in a dose-dependent manner (Fig. 6). Significant plasma clotting is observed even with 1 μg of enzyme. We observed that plasma coagulation by the enzyme is completely blocked in the presence of 5 kallikrein-inactivating units of aprotinin or 10 μg of SBTI. But aprotinin (up to 50 kallikrein-inactivating units) or SBTI (up to 50 μg) had no influence on the control plasma-clotting time. To locate the site of enzyme action, studies were carried out with human plasmas deficient in clotting factors. The enzyme corrects the clotting time of plasma deficient in factors X, IX, VII, XI, and XII. The coagulation of factor X-deficient plasma by the enzyme (Fig. 6) shows that the prolonged clotting time of the deficient plasma is shortened to a great extent by the enzyme in a dose-dependent manner.

**Immunological Characterization**—Cross-reaction of the antiserum raised against the purified protease with the enzyme was observed by immunodiffusion. Precipitation was observed up to 0.5 μg of enzyme. The antiserum showed specificity with the enzyme only: it revealed no cross-reaction with trypsin, chymotrypsin, or thrombin. Fig. 7 demonstrates that the plasma-clotting time is lengthened when the enzyme is preincubated with varying amounts of partially purified antibody, illustrating that the antiserum strongly inhibits the plasma coagulation elicited by the enzyme. But plasma coagulation remains unaffected on preincubation of the enzyme with partially purified non-immune serum.

**Table II**

| Inhibitors                  | Inhibition (%) | Enzyme inhibited
|-----------------------------|----------------|------------------|
| SBTI                        | 10.0           | **SBTI**
| Aprotinin                   | 29.4           | **Aprotinin**
| LBTI                        | 95.0           | **LBTI**
| Benazamidine                | 81.6           | **Benazamidine**
| DFP                         | 88.1           | **DFP**
| 0.5 mm                      | 4.4            | DTT
| 1.0 mm                      | 2.7            | DTT
| β-ME                        | 2.1            | DTT
| Iodoacetamide               | 2.0            | DTT
| 1.0 mm                      | 54.3           | DTT
| 5.0 mm                      | 51.2           | DTT
| N-Ethylmaleimide            | 54.0           | DTT
| EDTA                        | 55.1           | DTT
| 0.5 mm                      | 0.9            | DTT
| 1.0 mm                      | 0.4            | DTT
| 1,10-Phenanthroline monohydrate (1.0 mm) | 12.0 | DTT
| pCMB (1.0 mm)               | 18.6           | DTT
| Purinomycin                 | 15.7           | DTT
| 25.0 μg                     | 14.9           | DTT
| 50.0 μg                     | 0.0            | DTT
| Pepstatin A (50.0 μg)       | 18.0           | DTT
| Chymostatin (50.0 μg)       | 18.0           | DTT

**Fig. 5. pH profile of the enzyme.** The buffers used were: O, acetate (pH 5.0–6.0); △, phosphate (pH 6.0–7.5); □, Tris-HCl (pH 7.5–9.5); ○, NaOH glycine (pH 9.5–11.5). Other details are described under "Experimental Procedures."
N-toward RAPNA (I)-BAPNA), which was used in most assays, was defined as 100%. 0.4 µg of enzyme was used in each assay. Suc, succinyl.

TABLE III
Hydrolysis of different synthetic substrates

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<th>Position</th>
<th>NH₂</th>
<th>P₁</th>
<th>P₂</th>
<th>P₃</th>
<th>COOH</th>
<th>Activity</th>
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<td>Arg</td>
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Kinetic constants for hydrolysis of different synthetic substrates

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<tr>
<th>Substrate</th>
<th>Vₘₐₓ</th>
<th>Kₐ</th>
<th>Kₘ</th>
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<td>N-Bz-Pro-Phe-Arg-pNA</td>
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<td>N-Bz-Pro-Val-Arg-pNA</td>
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<td>0.072</td>
<td>1.15</td>
<td>16.0</td>
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<td>N-Bz-Pro-Gly-Arg-pNA</td>
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<td>N-Bz-Pro-Gly-Arg-pNA</td>
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<td>0.024</td>
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<td>Bz-BAPNA</td>
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<td>DL-BAPNA</td>
<td>1.99</td>
<td>0.25</td>
<td>0.71</td>
<td>2.86</td>
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**FIG. 6.** Plasma coagulation by the protease. Clotting time was measured in the presence of varying amounts of the enzyme. ○, rabbit plasma (n = 4); ●, normal human plasma (n = 3); ◯, human plasma deficient in factor X (n = 3). Mean ± S.D. are presented in the data.

We observed that this enzyme (2.5–10 µg) cannot coagulate purified fibrinogen even up to 720 s whereas bovine thrombin (Sigma) clots fibrinogen efficiently (Fig. 8). But the enzyme can produce fibrin clots from the purified fibrinogen in the presence of isolated prothrombin (Fig. 8). The cleavage of prothrombin by the enzyme (Fig. 8, inset) reveals that on preincubation (4 h) of prothrombin with the enzyme, four prominent bands are observed on SDS-PAGE. The band corresponding to the position of prothrombin (72 kDa) decreases as compared with 0-h preincubation with the appearance of bands corresponding to nearly 57, 46, and 36 kDa. Preincubation of the zymogen for a longer period (28 h) produced a protein band at 36 kDa and two smaller cleavage products.

**FIG. 7.** Immunoinhibition study on the plasma coagulation by the enzyme. Varying amounts of partially purified immune sera or non-immune sera (as mentioned) were used. 4 µg of enzyme was used in each assay. Mean ± S.D. (n = 4) are presented.

**FIG. 8.** Fibrinogen clotting by the enzyme in the presence of purified prothrombin and SDS-gel analysis on the cleavage of prothrombin by the enzyme. In fibrinogen clotting, purified prothrombin (80 µg) was incubated with the varying amounts of enzyme (as stated) for 12 min at 37 °C before addition of fibrinogen. In the absence of the enzyme, fibrinogen clotting was not observed (study carried up to 720 s) by prothrombin, ●, enzyme + prothrombin; ○, thrombin. Data represent the average value of three determinants. For SDS-PAGE study, purified prothrombin (100 µg) was incubated with enzyme (8 µg). SDS-PAGE was carried out by the method of Laemmli (15) in non-reducing conditions. 14 µg of protein was put in gel (8.5%) for electrophoresis. A, 0-h incubation; B, 4-h incubation; C, 28-h incubation. Prothrombin was purified from bovine plasma (47).

**TABLE IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Position</th>
<th>Vₘₐₓ</th>
<th>Kₐ</th>
<th>Kₘ</th>
<th>Kₘ/Kₐ</th>
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<tr>
<td>N-Bz-Pro-Phe-Arg-pNA</td>
<td>5.32</td>
<td>0.083</td>
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<td>0.024</td>
<td>0.50</td>
<td>20.46</td>
<td></td>
</tr>
<tr>
<td>Bz-BAPNA</td>
<td>4.68</td>
<td>0.22</td>
<td>1.71</td>
<td>7.89</td>
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</tr>
<tr>
<td>DL-BAPNA</td>
<td>1.99</td>
<td>0.25</td>
<td>0.71</td>
<td>2.86</td>
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**DISCUSSION**

We report here the purification and characterization of a mitochondrial membrane protease from rat submaxillary gland, which shows strong blood-coagulating activity. Requirement of a detergent for solubilization of the enzyme suggests its nature as an integral membrane protein. The enzyme activity

**FIG. 6.** Plasma coagulation by the protease. Clotting time was measured in the presence of varying amounts of the enzyme. ○, rabbit plasma (n = 4); ●, normal human plasma (n = 3); ◯, human plasma deficient in factor X (n = 3). Mean ± S.D. are presented in the data.
Blood-coagulating Protease in Mitochondrial Membrane

Comparison of submaxillary mitochondrial membrane (SMM) protease with different known submaxillary proteases

<table>
<thead>
<tr>
<th>Property</th>
<th>Esterase B*</th>
<th>Trenin*</th>
<th>Salivain*</th>
<th>Glandulin*</th>
<th>BSP-V*</th>
<th>Submaxillary kallikreins*</th>
<th>Protease A*</th>
<th>Protease B*</th>
<th>SMM Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (kDa)</td>
<td>25-27 (dimer)</td>
<td>28</td>
<td>30</td>
<td>23</td>
<td>25 (dimer)</td>
<td>34</td>
<td>28.2</td>
<td>30.4</td>
<td>22</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.45</td>
<td>6.0-6.2</td>
<td>6.0</td>
<td>8.0-9.0</td>
<td>5.3</td>
<td>3.87-4.16</td>
<td>4.2-4.25</td>
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</tr>
<tr>
<td>Optimum pH</td>
<td>9.5</td>
<td>6.8</td>
<td>9.2</td>
<td>ND*</td>
<td>10.0</td>
<td>Inhibition</td>
<td>Inhibition</td>
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<tr>
<td>DFP, PMSF</td>
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<td>No inhibition</td>
<td>Inhibition</td>
<td>No inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
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<tr>
<td>SBTA</td>
<td>Inhibition</td>
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<td>No inhibition</td>
<td>ND</td>
<td>Inhibition</td>
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<td>Inhibition</td>
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<tr>
<td>LBTI</td>
<td>Inhibition</td>
<td>ND</td>
<td>No inhibition</td>
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<td>Inhibition</td>
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<tr>
<td>Aprotinin</td>
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<td>No inhibition</td>
<td>No inhibition</td>
<td>ND</td>
<td>Inhibition</td>
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<tr>
<td>TLCK</td>
<td>Inhibition</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Slight inhibition</td>
<td>No inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td></td>
</tr>
<tr>
<td>Cu*</td>
<td>No inhibition</td>
<td>Inhibition</td>
<td>ND</td>
<td>ND</td>
<td>Slight inhibition</td>
<td>No inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td></td>
</tr>
<tr>
<td>Hydrolysis of TAME</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>ND</td>
<td>Slight inhibition</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Slight inhibition</td>
<td>Not sensitive</td>
<td></td>
</tr>
</tbody>
</table>

* Ref. 6.
*† Ref. 21.
* Ref. 1.
*† Ref. 22.
* Ref. 5.
*† Ref. 45.
*† Ref. 46.
* ND, not detected.
† Diisopropyl fluorophosphate.
‡ Phenylmethylsulfonyl fluoride.
§ Lima bean trypsin inhibitor.

The substrate N-Bz-Phe-Val-Arg-pNA, which is efficiently hydrolyzed by thrombin, is insensitive to factor Xa and tissue kallikrein (29). Our enzyme has effective specificity toward the substrate (Table IV). Escouf and Macfarlane (30) showed that factor Xa, unlike our enzyme, is resistant to SBTA, inhibited strongly by phenylmethylsulfonyl fluoride, and can hydrolyze TAME and is aprotinin-insensitive. All kallikreins hydrolyze various synthetic amino acid esters, but BAPNA is hydrolyzed by plasma kallikrein only (31, 32). Moreover, it is reported that salivary kallikrein does not hydrolyze BAPNA (33), while our enzyme can hydrolyze BAPNA efficiently. Also, the molecular mass of kallikreins is different from that of our enzyme (Table V).

A comparison of the properties of this enzyme with those reported for other known submaxillary proteases (Table V) demonstrates that the protease reported is a new one, hitherto unreported.

The preference of the protease toward some thrombin and factor Xa substrates (Table IV) induced us to study whether the enzyme possesses blood-coagulating activity. Fig. 6 clearly demonstrates the potency of this protease in coagulating plasma effectively. Inability of the enzyme to produce fibrin clots from purified fibrinogen suggests that it is not a thrombin-like enzyme. Tissue factor that initiates blood coagulation via the extrinsic pathway (13) differs from our enzyme in the following points. This factor is localized to plasma membranes of cells of many tissues (34), is a lipoprotein (13), has a larger molecular mass (13), and is also a glycoprotein (35). The peptide activity associated with tissue factor is not required for its clotting action (36, 37), whereas plasma clotting by this enzyme is completely blocked by the protease inhibitors SBTA and aprotinin.

Our study with plasmas deficient in different clotting factors suggests a factor Xa-like activity for this enzyme. Factor Xa is a serine protease with the specific function of activating prothrombin and plays a pivotal role in the blood coagulation cascade system. Prothrombin activation by the enzyme has been demonstrated in our study (Fig. 8).

Prothrombin activation by factor Xa in the presence of factor V, phospholipid, calcium ion (38-40), and by some snake ven-
Blood-coagulating Protease in Mitochondrial Membrane

oms (41, 42) has been investigated by several workers who demonstrated that conversion of prothrombin to thrombin is a multistep reaction. Formation of intermediates has been documented in prothrombin activation by all activating agents. In our system, cleavage of prothrombin led to the formation of several cleaved products (Fig. 8); one (36 kDa) corresponds to the molecular mass of thrombin. The formation of the product (about 46 kDa) in the activation process has not been reported. Further studies are necessary to elucidate the mechanism of prothrombin activation by our enzyme and its proper comparison with other systems.

The ability of this enzyme to activate prothrombin suggests a processing role. It may be that the enzyme functions as a processing enzyme in rat submaxillary mitochondria. The physiological importance of the extramitochondrial role of this enzyme in the blood coagulation process is yet to be elucidated. Different patterns of leakage of mitochondrial enzymes into blood in different pathogenic and abnormal physiological states have been reported (45, 44). It may be that in abnormal physiological states when fibrin formation is enhanced, this enzyme leaches out of mitochondrial membranes into the blood to participate in the coagulation process.

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REFERENCES