Characterization of a membrane protease from rat submaxillary-gland mitochondria that possesses thrombin-like activity

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A membrane protease possessing thrombin-like activity was purified to homogeneity from mitochondria of rat submaxillary gland. The molecular mass of the enzyme was determined to be 45 kDa by SDS/PAGE under reducing conditions and by gel filtration on a Sephadex G-100 column. The enzyme is a glycoprotein and has an isoelectric point of 3.25. Maximum activity was observed at pH 10.5. Inhibition by di-isopropyl fluorophosphate, benzamidine, aprotinin and antipain suggested the enzyme to be a serine protease. Other inhibitors such as EDTA, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor, TosLysCH₂Cl and chymostatin did not alter the activity. The enzyme showed affinity towards different synthetic substrates (*p*-nitroanilide derivatives) containing arginine at the P₁ position. Kinetic studies revealed that k_{cat}/K_m was highest with the substrate *N*-Bz-Phe-Val-Arg-*p*-nitroanilide. The enzyme exhibits

significant plasma-coagulating activity. The coagulation initiated by the enzyme was not altered by concanavalin A, indicating that the carbohydrate moiety of the enzyme is not essential for this reaction. Further, this enzyme can catalyse the formation of fibrin clots from purified fibrinogen, which describes its thrombin-like activity. However, an antibody raised against the purified enzyme inhibited the plasma-clotting as well as fibrinogen-clotting activity of the enzyme. Fibrinogen coagulation by the enzyme was blocked in the presence of aprotinin, a protease inhibitor. Release of fibrinopeptides A and B from bovine fibrinogen by the enzyme has been shown by HPLC analysis. Our studies reveal that the enzyme reported here differs from trypsin, chymotrypsin and other mitochondrial proteases reported so far.

INTRODUCTION

Despite the fact that a large number of soluble proteases from submaxillary gland have been studied [1–7], information on intracellular membrane proteases from this tissue is scarce [8–10]. These enzymes are of interest as they are known to be involved in several cellular functions.

We have previously purified a soya-bean trypsin inhibitor (SBTI)-sensitive membrane protease from rat submaxillary-gland mitochondria by (NH₄)₂SO₄ precipitation, Sephadex G-100 column chromatography, DEAE-cellulose chromatography, native PAGE and gel-filtration HPLC [10]. The enzyme fraction obtained by ion-exchange chromatography was resolved into two active fractions on non-denaturing PAGE, a slower migrating enzyme that was strongly inhibited by SBTI and a faster migrating enzyme that was insensitive to SBTI. The purification and characterization of the former enzyme have been reported [10]. An interesting property of that enzyme is its ability to coagulate plasma; it can activate prothrombin to initiate the coagulation reaction. The SBTI-insensitive protease was then purified and several characteristics reveal its difference from the former. This enzyme also coagulates plasma efficiently, but its site of action is different. It functions as a thrombin-like enzyme that catalyses the formation of fibrin clots from fibrinogen.

The present paper describes the characterization of a purified SBTI-insensitive mitochondrial membrane protease from rat submaxillary gland and its plasma- and fibrinogen-clotting activity.

MATERIALS AND METHODS

Sodium deoxycholate, DEAE-cellulose, *NNN'*-tetramethylethylenediamine, acrylamide, *NN'*-methylenebisacrylamide,

pharmalyte 3–10, enzyme inhibitors, synthetic substrates, marker proteins, concanavalin A (ConA), ConA-Sepharose, bovine fibrinogen, bovine thrombin and hirudin were purchased from Sigma. *N*-Benzoyl-DL-arginine *p*-nitroanilide (BAPNA) was from Boehringer-Mannheim, and Sephadex G-100 from Pharmacia. Methyl α -D-mannopyranoside was obtained from Aldrich.

Enzyme preparation

Preparation of the mitochondrial fractions from rat submaxillary glands, purification of mitochondria, solubilization of the enzyme by sodium deoxycholate and its purification by (NH₄)₂SO₄ precipitation, Sephadex G-100 gel chromatography, DEAE-cellulose chromatography and native PAGE were carried out as described previously [10]. The faster-migrating enzyme that appeared on electrophoresis in non-denaturing conditions [10] was further purified by gel-filtration HPLC.

Gel-filtration HPLC

The enzyme recovered from polyacrylamide gel slices (1 mm each) was assayed in the presence and absence of 50 μ g of SBTI. SBTI-insensitive protease fractions were pooled and concentrated. The enzyme was finally purified in a gel-filtration column (TSK G2000 SW; 0.75 cm $\times$ 30 cm) which was equilibrated and eluted with 250 mM Tris/HCl, pH 6.7. Protein concentration in column effluents was quantified by measuring A_{280} .

Enzyme assay

Protease activity was determined [11] by measuring BAPNA hydrolysis in 1 ml of reaction mixture containing 90 mM Tris/

Abbreviations used: BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; TAME, *p*-tosyl-L-arginine methyl ester; Bz, benzoyl; *t*-Boc, *t*-butoxycarbonyl; Suc, succinyl; SBTI, soya-bean trypsin inhibitor; LBTI, lima-bean trypsin inhibitor; DFP, di-isopropyl fluorophosphate; *p*NA, *p*-nitroanilide; ConA, concanavalin A.

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Figure 1 HPLC of the enzyme fraction from native PAGE

The procedure is described in the Materials and methods section. Flow rate was 0.8 ml/min. Enzyme activity in each peak was measured by studying BAPNA hydrolysis at pH 8.0. Peak B possessed the enzyme activity.

HCl buffer, pH 8.0, 1 mM DL-BAPNA and a suitable amount of the enzyme preparation. Liberated *p*-nitroaniline was quantified by measuring A_{410} in a Pye–Unicam spectrophotometer. Enzyme activity is expressed as nmol of *p*-nitroaniline liberated/30 min. Enzymic hydrolysis of different synthetic substrates [*p*-nitroanilide (*p*-NA) derivatives] was measured similarly.

The esterase activity of the enzyme was studied spectrophotometrically at pH 8.0 [12].

Coagulation studies

The coagulation of plasma as well as of purified fibrinogen was measured as reported previously [10] in a reaction mixture (0.3 ml) containing 0.1 ml of plasma or fibrinogen (600 μ g), 8 mM CaCl_2 and a suitable amount of enzyme or saline. Fibrinogen solution (6 mg/ml) was prepared in 10 mM Tris/HCl-buffered saline, pH 7.4. In controls, clotting time was measured in the absence of enzyme. Plasma was prepared from citrated blood collected from healthy adult Belgian rabbits (1.8 ± 0.4 kg body weight).

SDS/PAGE

SDS/PAGE was performed in a 10% gel under reducing conditions [13]. Protein was stained with Coomassie Brilliant Blue R-250. SDS/PAGE analysis of the cleavage of fibrinogen by the enzyme was studied as follows. Fibrinogen (300 μ g) solution in 10 mM Tris/HCl buffer, pH 7.4, was incubated with the enzyme (1 μ g) or bovine thrombin (0.4 NIH unit) in a volume of 0.15 ml at 37 °C for 3 min or 30 min respectively. The sample was then treated with an equal volume of a mixture containing 5 M urea, 2% SDS and 2% 2-mercaptoethanol and incubated overnight at room temperature. A 15 μ l portion of each sample was then applied to 7.5% polyacrylamide gel for electrophoresis.

Isoelectric focusing

Isoelectric focusing was carried as reported previously [10].

Periodic acid/Schiff staining

After SDS/PAGE of the purified enzyme, the gel was fixed overnight in 50% methanol containing 7% acetic acid, washed with water and transferred to 1% periodic acid at 4 °C for at least 1 h. After being washed with distilled water, the gel was kept overnight in the presence of Schiff's reagent at 4 °C. Destaining was carried out in 10% (v/v) acetic acid at room temperature.

Table 1 Purification of the protease

Enzyme activity towards BAPNA at pH 8.0 was measured as described in the Materials and methods section

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery of activity (%)
KCl-extracted pellet	420.00	6310	15.0	1.00	100.00
Deoxycholate-soluble fraction	221.00	9984	45.2	3.01	158.20
$(\text{NH}_4)_2\text{SO}_4$ precipitation	19.30	5300	274.6	18.30	84.00
Sephadex G-100	9.20	4642	504.6	33.60	73.60
DEAE-cellulose	3.80	3430	902.1	60.10	54.40
Native PAGE	0.16	513	3206.2	213.70	8.13
HPLC	0.07	435.4	6220.0	414.00	6.90

ConA–Sephacryl column chromatography

A ConA–Sephacryl column (0.7 cm × 6 cm) was equilibrated with 20 mM Tris/HCl buffer, pH 6.8, containing CaCl_2 and MnCl_2 (1 mM each). The enzyme (60 μg) loaded on the column was kept for 6 h at 4 °C. The column was washed with the equilibrating buffer and the enzyme was eluted with 50 mM methyl α -D-mannopyranoside.

Kinetic analysis

Kinetic parameters, K_m , V_{max} , and k_{cat} were calculated from Eadie and Hofstee plots, which were constructed by the use of a computer and 'Sigma plot'.

Immunization procedure

Antiserum to the protease was raised by four subcutaneous injections of the purified enzyme in two rabbits at days 0, 7, 15 and 35. The first and second injections contained 300 μg and 200 μg of protein respectively in Freund's complete adjuvant and each subsequent booster injection contained 150 μg of protein in Freund's incomplete adjuvant. On the 10th day after the last injection, blood was drawn from the animals by cardiac puncture under aseptic conditions. Before the first antigen injection, rabbits were bled to obtain non-immune serum. The antiserum and non-immune serum were partially purified by adding solid $(\text{NH}_4)_2\text{SO}_4$ to sera to 45% saturation. The precipitates obtained were dissolved in 50 mM Tris/HCl, pH 7.5, and dialysed overnight against the same buffer.

Analysis of fibrinopeptides released by the enzyme

Release of fibrinopeptides from bovine fibrinogen by the mitochondrial enzyme and bovine thrombin was analysed by HPLC using a reverse-phase μ Bondapak C_{18} column (4 mm × 30 cm). The method, based on the work of Martinelli and Scheraga [14], was essentially as described by Higgins and Schafer [15]. Fibrinogen solution (3 mg/ml) was prepared in 0.15 M NH_4HCO_3 , pH 7.8. The fibrinogen solution (3.0 ml) was incubated with mitochondrial enzyme (5.0 μg) or thrombin (3 NIH units) for 4 h at room temperature. The clots that appeared were removed by centrifugation at 11 000 g (12 000 rev./min) for 15 min; the supernatants were lyophilized and then redissolved in 0.3 ml of 0.083 M ammonium phosphate, pH 3.1. An aliquot (150 μl) of the sample was applied to an HPLC column equilibrated with 14% acetonitrile/86% 0.083 M ammonium phosphate, pH 3.1. After 5 min of elution with the starting buffer, the elution was performed in a solvent system containing 19% acetonitrile and 81% ammonium phosphate, pH 3.1. The flow rate was 1.0 ml/min and the eluted peaks were monitored by measuring A_{210} .

Protein was determined by the Bradford method [16].

RESULTS

During purification of mitochondrial membrane protease from rat submaxillary gland, the enzyme fraction after ion-exchange chromatography was resolved into an SBTI-sensitive protease (fraction A) and an SBTI-insensitive protease (fraction B) on non-denaturing PAGE [10]. The SBTI-insensitive protease in fraction B was further purified to homogeneity by gel-filtration HPLC (Figure 1). Five protein peaks appeared, and the enzyme activity was found in the major one. Table 1 records the quantitative aspects of the enzyme purification. The enzyme was 414-fold purified over the KCl-extracted pellet. The purified

Figure 2 SDS/PAGE of the protease

Lane A, 10 μg of purified protease; lane B, marker proteins: BSA, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; SBTI, 20.1 kDa; β -lactalbumin, 14.2 kDa. Electrophoresis was carried out at a constant current of 15 mA for 3 h at 20 °C.

Figure 3 pH profile of the protease

The buffers used were: ○, 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). Protease activity was measured in buffer (90 mM) at different pH values with BAPNA as substrate; 0.55 μg of enzyme was used in each assay.

enzyme preparation showed a single protein band on SDS/PAGE under reducing conditions (Figure 2). Furthermore, when rechromatographed quickly on the same gel-filtration HPLC column, the enzyme was eluted as a single protein peak, also suggesting homogeneity.

Characterization of the enzyme

The enzyme was found to have a molecular mass of 45 kDa, as determined from its electrophoretic mobility on SDS/PAGE as well as from its elution position on a Sephadex G-100 column

Table 2 Effect of various inhibitors on the protease activity

Enzyme was preincubated with different inhibitors for 10 min at 37 °C. Inhibition was calculated as a percentage of the activity in the absence of any inhibitor. The amount of enzyme used in each assay was 0.6 µg. Enzyme activity was measured by studying BAPNA hydrolysis in 90 mM Tris/HCl buffer, pH 8.0. Other details are given in the Materials and methods section.

Inhibitor	Inhibition (%)
Dithiothreitol (1.0 mM)	8.0
Iodoacetamide (1.0 mM)	7.0
<i>N</i> -Ethylmaleimide (1.0 mM)	6.4
EDTA (1.0 mM)	15.0
1,10-phenanthroline monohydrate (1.0 mM)	2.0
Puromycin (50 µg)	10.4
Pepstatin A (50 µg)	1.0
SBTI (10 µg)	4.0
(50 µg)	2.0
(200 µg)	2.0
LBTI (50 µg)	0.0
(100 µg)	2.0
Aprotinin (5 kallikrein-inhibitory units)	90.0
(10 kallikrein-inhibitory units)	95.0
Benzamidine (50 µg)	32.0
(100 µg)	47.0
DFP (0.5 mM)	71.0
(1.0 mM)	84.0
Antipain (50 µg)	61.0
Chymostatin (50 µg)	10.0
TosPheCH ₂ Cl (1.0 mM)	0.0
TosLysCH ₂ Cl (1.0 mM)	0.0

(1.6 cm × 80 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.0, containing 100 mM NaCl. A similar molecular mass was found for the enzyme (44 kDa) when determined by gel-filtration HPLC (0.75 cm × 30 cm column equilibrated with 250 mM Tris/HCl buffer, pH 6.7). The molecular mass was estimated by using a calibration curve obtained under identical conditions with proteins of known molecular mass. Marker proteins were monitored spectrophotometrically at 280 nm and the protease by assaying its BAPNA-hydrolysing activity. The isoelectric point of the enzyme was estimated by isoelectric focusing to be 3.25. The enzyme was found to be a glycoprotein as evidenced by positive staining with periodic acid/Schiff. We observed that the protease could bind to a ConA-Sepharose column and the bound enzyme was released by methyl α-D-mannopyranoside, which provides further evidence that the enzyme is a glycoprotein. Significant activity was observed in the pH range 8.0–11.5, the maximum being at pH 10.5 (Figure 3). However, considering

Figure 4 Plasma coagulation by the protease

Clotting time was measured in the presence of various amounts of the enzyme. Data represent means ± S.E.M. ($n = 4$). The procedure for measuring plasma coagulation is given in the Materials and methods section.

that many of the reagents to be tested for their effects on the activity might be affected at high alkaline pH, we carried out most studies with the enzyme at pH 8.0. Table 2 shows the effect of several inhibitors on the activity. Di-isopropyl fluorophosphate (DFP), aprotinin and antipain produced strong inhibition whereas benzamidine inhibited only moderately. SBTI, lima-bean trypsin inhibitor (LBTI), pepstatin A, EDTA, TosLysCH₂Cl and TosPheCH₂Cl were without significant effect.

Hydrolysis of synthetic substrates

Peptide specificity was examined by studying the activity towards several synthetic substrates. Substrates (*p*-NA derivatives) with arginine at the P₁ position and a blocked N-terminal residue were effectively hydrolysed (results not shown), but those containing other amino acids at the P₁ position were not cleaved. A study using ester substrates revealed that the enzyme could hydrolyse

Table 3 Kinetic constants for the hydrolysis of different synthetic substrates

Purified protease (0.68 µg) was assayed with each of the indicated substrates, each at a concentration of 5–100 µM. Experimental details and kinetic measurements are as described in the Materials and methods section.

Substrate	$V_{\max}$ (µmol/min per mg)	K_m (mM)	k_{cat} (s ⁻¹)	$10^{-3} \times (k_{\text{cat}}/K_m)$ (s ⁻¹ · M ⁻¹)
<i>N</i> -Bz-Pro-Phe-Arg- <i>p</i> NA	8.78	0.22	6.43	28.90
<i>N</i> -Bz-Phe-Val-Arg- <i>p</i> NA	4.10	0.07	3.01	40.72
<i>N</i> -Bz-Val-Gly-Arg- <i>p</i> NA	7.20	0.29	5.20	18.24
<i>N</i> - <i>t</i> -Boc-O-Bz-Ser-Gly-Arg- <i>p</i> NA	13.20	0.30	9.60	29.89
<i>N</i> - <i>t</i> -Boc-L-Leu-Gly-Arg- <i>p</i> NA	4.02	0.20	2.94	14.72
L-BAPNA	3.95	0.07	2.89	38.13
D,L-BAPNA	3.33	0.18	2.40	13.50

Figure 5 Immunoinhibition study of plasma coagulation by the protease

Various amounts of partially purified immune serum (experimental) or non-immune serum (normal) were used; 3 μg of enzyme was used in each assay. Means $\pm$ S.E.M. ($n = 3$) are presented. In the control experiment, coagulation was initiated by CaCl_2 in the absence of the enzyme but in the presence of different amounts of immune sera.

N-benzoyl-L-arginine ethyl ester, but not *p*-tosyl-L-arginine methyl ester (TAME) or *N*-benzoyl-L-arginine methyl ester.

Kinetic studies

Kinetic studies (Table 3) using several synthetic substrates that were completely hydrolysed by the enzyme show that $k_{\text{cat.}}/K_m$ was highest ($40.72 \times 10^3 \text{ s}^{-1} \cdot \text{M}^{-1}$) for *N*-Bz-Phe-Val-Arg-*p*NA hydrolysis whereas hydrolysis of *N*-*t*-Boc-O-Bz-Ser-Gly-Arg-*p*NA showed the highest $k_{\text{cat.}}$ value (9.6 s^{-1}).

Plasma coagulation

Figure 4 demonstrates that the enzyme coagulated plasma in a dose-dependent manner. Antiserum raised against the purified enzyme inhibited its plasma-coagulating activity (Figure 5), whereas non-immune serum had no effect. Partially purified antiserum and non-immune serum were used in the study. The cross-reactivity of the antiserum with the enzyme was tested by immunodiffusion. It was found that ConA did not alter the coagulating activity of the enzyme.

Fibrinogen coagulation by the enzyme is demonstrated in Figure 6. Clot formation was observed at 100 s with 0.5 μg of enzyme. Clotting time decreased with increasing enzyme content; however, fibrinogen clotting was not observed even up to 720 s in the absence of the enzyme. In two experiments, clots formed in the presence of the enzyme were removed by centrifugation. Measurement of protein content in the reaction mixture before clot formation and after removal of clots showed a marked reduction in protein content. The effect of the enzyme in clotting plasma and fibrinogen in the absence of Ca^{2+} is shown in Table 4. A jelly-like clot appeared at 210 s in the absence of CaCl_2 whereas clot was observed within 100 s in the presence of CaCl_2 . In the fibrinogen-clotting reaction, a scattered fibre-like substance appeared in the absence of CaCl_2 , whereas addition of CaCl_2 in this incomplete polymerization reaction produced clots within 30–50 s. Ca^{2+} accelerates the polymerization of fibrin

Figure 6 Fibrinogen clotting by the protease

Clotting time was measured in the presence of various amounts of the enzyme. Means $\pm$ S.E.M. ($n = 4$) are presented. Purified fibrinogen (600 μg in 0.1 ml) was used instead of plasma, and fibrinogen clotting was measured by the same procedure as that used for plasma coagulation.

Table 4 Plasma and fibrinogen clotting by the protease in the absence of CaCl_2

Clotting of plasma and fibrinogen by the enzyme was studied in the presence and absence of CaCl_2 (8 mM). Experimental details are given in the Materials and methods section. In each assay, 3 μg and 1.5 μg of enzyme were used for plasma and fibrinogen clotting respectively. Data represent the average value of two determinations.

System	Clotting time (s)
Plasma clotting	
+ CaCl_2	98
– CaCl_2	210 (Jelly-like appearance)
Fibrinogen clotting	
+ CaCl_2	50
– CaCl_2	190 (Scattered fibre-like appearance)

monomers produced by thrombin from fibrinogen to form clots [17]. We found that CaCl_2 had no effect on BAPNA hydrolysis by the enzyme.

SDS/PAGE analysis of fibrinogen incubated with either the purified mitochondrial enzyme or thrombin showed very similar patterns for both. The two fibrinogens contained similar subunits. Figure 7 shows HPLC separation of clot supernatants from our enzyme-catalysed and thrombin-catalysed reactions. Mitochondrial enzyme released the same peptide fragments as those released in the reaction with thrombin. We observed that fibrinogen clotting by the enzyme was blocked in the presence of the protease inhibitor aprotinin (5 kallikrein-inhibitory units), suggesting that clot formation occurred through proteolytic processing of fibrinogen. Furthermore, the enzyme did not

Figure 7 HPLC profile of peptides released from bovine fibrinogen by the action of (b) mitochondrial protease and (a) thrombin

Procedures are described in the Materials and methods section.

produce clots at least up to 720 s in the presence of antiserum to the enzyme, indicating inhibition of the fibrinogen clotting.

DISCUSSION

Conversion of fibrinogen into fibrin clots, the last step in mammalian blood coagulation, is catalysed by thrombin. Many snake venoms are also known to contain thrombin-like enzymes that can catalyse this reaction [18]. The present study demonstrates that a mitochondrial membrane protease from rat submaxillary gland can convert fibrinogen into fibrin, a fact not previously reported. Inhibition by serine protease inhibitors such as DFP, aprotinin, antipain and benzamidine suggests it to be a serine protease. SBTI and LBTI which inhibit many submaxillary proteases [5,6,10,19] had no significant effect. Neither TosLysCH₂Cl, an inhibitor of trypsin-like enzymes, nor chymotrypsin inhibitors such as TosPheCH₂Cl and chymostatin inhibited the enzyme. Experiments with several synthetic substrates reveal that the protease reported here is an endopeptidase as evidenced by its affinity towards substrates containing arginine at P₁ with the N-terminal residue blocked and a reporter group at the C-terminus.

Kinetic analysis (Table 3) using several synthetic substrates that were completely hydrolysed by the enzyme gave the highest $k_{cat.}/K_m$ with *N*-Bz-Phe-Val-Arg-*p*NA. Incidentally, *N*-Bz-Phe-Val-Arg-*p*NA is a specific substrate for thrombin [20,21]. Thrombin is known to coagulate plasma in the mammalian system and catalyses the formation of fibrin clots from fibrinogen. Our study (Figure 4) demonstrates the plasma-coagulating activity of the enzyme. Although the study was carried out with rabbit plasma, the enzyme can also clot human plasma. Furthermore, the enzyme can catalyse clot formation from purified fibrinogen (Figure 6), which demonstrates it to be a thrombin-like enzyme.

We observed that hirudin, an inhibitor of thrombin [22,23] at a concentration of 1.5 µg/ml, affected neither BAPNA-hydrolysing activity nor fibrinogen-clotting activity of the enzyme, which excludes the possibility of thrombin contamination. Several properties of thrombin such as the molecular mass, sensitivity towards *p*-tosyl-L-arginine methyl ester hydrolysis and inhibition by TosLysCH₂Cl suggest differences between our enzyme and

Table 5 Comparison of SBTI-insensitive protease with SBTI-sensitive protease from rat submaxillary mitochondrial membrane

Property	SBTI-insensitive protease	SBTI-sensitive protease [10]
Molecular mass (kDa)	45	22 or 24
Isoelectric point	3.25	4–4.25
Optimum pH	10.5	8.5
DFP	Inhibition	Inhibition
SBTI	No inhibition	Strong inhibition
LBTI	No inhibition	Slight inhibition
Aprotinin	Inhibition	Inhibition
Hydrolysis of TAME	Insensitive	Insensitive
Glycoprotein	Yes	No
Plasma-clotting activity	Yes	Yes
Fibrinogen-clotting activity	Yes	No

thrombin. Also, antiserum against our enzyme did not cross-react with thrombin.

From Lundblad et al. [24], 1.0 NIH unit of thrombin causes a clot after about 10–12 s. However, the mitochondrial enzyme described here clots fibrinogen in 42–55 s (1.5–1.0 µg of protein; 300 µl of assay mixture), indicating that it is much less active than thrombin. Also, thrombin has higher affinity towards the synthetic substrate, *N*-Bz-Phe-Val-Arg-*p*NA, than our enzyme. The $k_{cat.}/K_m$ ($40.72 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) of our enzyme is nearly 50 times less than that reported for bovine thrombin [21]; other kinetic constants such as K_m and $k_{cat.}$ are also higher for thrombin [21].

Thrombin releases fibrinopeptides A and B from fibrinogen [15]. HPLC analysis (Figure 7) of the supernatant after prolonged incubation of fibrinogen with the mitochondrial enzyme showed the same peptide fragments as those released by thrombin, suggesting that the mitochondrial enzyme can release both fibrinopeptides A and B from fibrinogen.

The presence of different proteases in mitochondria has been reported [25–30]. Several physicochemical properties of our enzyme distinguish it from the known mitochondrial proteases. Also, it differs in several properties from other proteolytic enzymes (see Table V of ref. [10]) from submaxillary gland.

It has been reported that SBTI-sensitive mitochondrial membrane protease shows significant plasma-coagulating activity [10]. Despite the fact that the enzyme reported here is different from the SBTI-sensitive protease in several properties (Table 5) and the antiserum against the enzyme did not cross-react with the previous protease, it also possesses significant blood-coagulating activity. However, its site of action is different. The enzyme reported here is able to initiate fibrinogen clotting whereas the SBTI-sensitive enzyme that can activate prothrombin does not have fibrinogen-clotting activity [10]. However, it is interesting to note that both membrane proteases from submaxillary-gland mitochondria have significant blood-coagulating activity. The physiological role of these enzymes in the extramitochondrial blood coagulation process is not at present known. The leakage of mitochondrial enzymes in different pathogenic and abnormal physiological states [31,32] may indicate the possibility that in some abnormal physiological conditions these enzymes leach out of the mitochondria into the blood and participate in blood coagulation.

Furthermore, the conversion of fibrinogen into fibrin occurs through specific cleavage of Arg-Gly peptide bonds. The ability of this enzyme to catalyse the reaction establishes that it has a processing function. We suggest that the enzyme may participate

as a processing enzyme in the mitochondrial function of the submaxillary gland.

We acknowledge the Council of Scientific and Industrial Research (CSIR) for awarding a Senior Research Fellowship to Mrs. M. Bharadwaj and Mr. D. Bharadwaj. Technical assistance from Mr. B. Tewari is gratefully acknowledged. Also we thank H. J. Datta and S. Sahoo for art work.

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