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Articles

Purification and Characterization of a Serine Protease (Esterase B) from Rat Submandibular Glands†

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ABSTRACT: A new protease has been purified to homogeneity from rat submandibular gland homogenate by using DEAE-Sephadex chromatography, chromatofocusing, aprotinin–Sepharose affinity chromatography, and high-performance liquid chromatography. The enzyme has been named esterase B, since it represents the second major esterolytic peak on DEAE-Sephadex chromatography of submandibular gland homogenate. It is an acidic protein (p*I* = 4.45) with an apparent molecular weight of 27 000. It is heat-stable and has an optimum pH of 9.5. Esterase B hydrolyzed the synthetic substrates tosyl-L-arginine methyl ester and Val-Leu-Arg-*p*-nitroanilide (S2266). It also cleaved dog plasma kininogen to produce a kinin, identified as bradykinin on reverse-phase high-performance liquid chromatography. Esterase B, however, is only a weak kininogenase, since it had only 5% of the kininogenase activity of equimolar concentrations of glandular kallikrein and had no effect on rat mean blood pressure or on the isolated rat uterus. Esterase B activated plasminogen and had caseinolytic activity. It was inhibited by aprotinin, soybean trypsin inhibitor, lima bean trypsin inhibitor, phenylmethanesulfonyl fluoride, antipain, leupeptin, and *p*-tosyl-L-lysine chloromethyl ketone. On double immunodiffusion, when reacted with kallikrein and tonin antisera, esterase B showed partial identity with kallikrein but not with tonin. On immunoelectrophoresis against kallikrein antisera, esterase B formed a precipitin arc at a position different from that of kallikrein. Esterase B appears to be a trypsin-like serine protease having some homology with glandular kallikrein.

Esterolytic activity in mammalian salivary glands was first demonstrated by Hüfner in 1873, as cited in Willslatter (1924). While this activity varies from species to species, rats have remarkably high esterolytic activity in their salivary glands (Junqueira & Fapa de Moraes, 1965). A number of proteases with esterolytic activity have been identified in rat salivary glands (Riekkinen & Hopsu-Havu, 1965; Minato et al., 1967). Riekkinen and co-workers partially purified and characterized two of these esterases, namely, salivain and glandulain (Riekkinen et al., 1966, 1967). However, Brandtzaeg and co-workers reported that salivain was a mixture of kallikrein and an unidentified protease (Brandtzaeg et al., 1976). Although rat salivary gland kallikrein (Brandtzaeg et al., 1976) and tonin (Boucher et al., 1974) have been well characterized, there is very little information available regarding other salivary gland proteases. Here, we report the purification and characterization of a protease from rat salivary gland homogenate. This enzyme is a weak kininogenase that belongs to the serine protease family and has properties distinctly different from those of glandular kallikrein, glandulain, salivain, and tonin.

Materials and Methods

The following reagents were obtained from commercial sources: DEAE-Sephadex A-50, Polybuffer anion exchanger (PBE 94), Polybuffer 74, low molecular weight protein markers (LMWK), and isoelectric pH markers (Pharmacia Fine Chemicals); tosyl-L-arginine [^{3}H]methyl ester ([^{3}H]-TAME)[1] (Amersham Corp.); acrylamide, *N*,*N*′-methylene-

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bis(acrylamide), TEMED, and Coomassie brilliant blue R-250 (Eastman Kodak); sodium dodecyl sulfate (SDS) (Pierce); bovine fibrinogen containing plasminogen (Miles); agar nobel (Difco); Val-Leu-Arg-*p*-nitroanilide (S2266) (Kabi); human angiotensin I (Bachem); soybean trypsin inhibitor (SBTI), phenylmethanesulfonyl fluoride (PMSF), Schiff's reagent, and urokinase (Sigma Chemical Co.). All other reagents used were of analytical grade. Aprotinin was a kind gift from Dr. G. L. Haberland, A. G. Bayer, Wuppertal, West Germany.

Preparation of Submandibular Gland Homogenate and Purification Procedures. Fifty adult male Sprague-Dawley rats (400–500 g) were sacrificed by decapitation, and their salivary glands were removed, placed in cold 0.9% saline, and rinsed free of blood. After separation of the sublingual glands, the submandibular glands (SMG) were finely minced and homogenized in 50 mL of 0.25 M sucrose (pH 7.0) containing 1 mM EDTA by using a Polytron homogenizer. The homogenate was centrifuged at 480*g* for 10 min to eliminate debris. The supernatant was centrifuged first at 17000*g* for 10 min and later at 105000*g* for 90 min in a Beckman L-5 ultracentrifuge. The 105000*g* supernatant was concentrated with Aquacide and dialyzed against 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA. This SMG extract was used for further purification procedures, which were carried out at 4 °C.

The SMG extract was applied to a DEAE-Sephadex A-50 column (50 × 700 mm), preequilibrated with 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA (buffer A). After washing, the column was eluted with a linear salt gradient (0–0.15 M NaCl dissolved in buffer A) at a flow rate of 40 mL/h. The fractions with the second major esterolytic activity were pooled, concentrated on an Amicon ultrafiltration system using a PM10 membrane, and pressure dialyzed against 0.025 M imidazole buffer (pH 7.4). This fraction was called esterase B and was loaded (10 mL) on a PBE 94 column (1 × 48 cm). It was eluted with Polybuffer 74 (pH 4.0) until the OD_{280} was less than 0.005. The fractions with esterolytic activity eluting between pH 5 and pH 4 (esterase B) were pooled and dialyzed against 0.1 M sodium phosphate buffer (pH 8.0) containing 0.5 M NaCl. The pooled esterase B fraction (25.2 mL, 1 mg of protein/mL) was mixed with aprotinin–CH-Sepharose gel at room temperature for 2 h, and then at 4 °C for 18 h. The gel (30 mL) was packed on a column (2.5 × 10 cm) and washed once with 0.1 M sodium acetate buffer containing 0.5 M NaCl (pH 6.4) and thereafter with 0.1 M sodium acetate buffer (pH 6.4) until the OD_{280} of eluate was less than 0.005. Total volume of washings was 300 mL. Esterase B was eluted with 0.1 M acetate buffer containing 1 M NaCl (pH 4.0) at a flow rate of 60 mL/h. Fractions of 2 mL each were collected. To avoid prolonged exposure to acidic pH, a solution of 2 M Tris-HCl, pH 8.5, was simultaneously added to the tubes at the same flow rate as the eluting buffer. Fractions with esterolytic activity were pooled, concentrated to 3 mL, and dialyzed at 4 °C against distilled water. Esterase B was further purified by size exclusion in high-performance liquid chromatography (HPLC), using a Waters automated gradient system. The column (Bio-Sil TSK-250, 300 × 7.5 mm) was preequilibrated with 0.1 M sodium phosphate buffer (pH 6.8) and eluted isocratically with the same buffer at a flow rate of 1 mL/min. Between 0.1 and 0.2 mg of protein was loaded in each run.

Protein determination was done by the method of Bradford (1976) using bovine serum albumin as the standard. Protein concentration in column effluents was measured at 280 nm (OD_{280}).

Enzyme Assays. Esterase activity was determined by using [^{3}H]TAME (specific activity, 213 mCi/mmol; Amersham) as a substrate (Beaven et al., 1971) during the purification. A standard preparation of hog pancreatic kallikrein of known activity, as determined by colorimetric assay (Roberts, 1958), was used as the standard. For kinetic studies, the hydrolysis rate of TAME was determined at pH 8.0 and 22 °C by titration with standarized 0.005 M NaOH in 0.1 M NaCl using an Autotitrator 11 with Autoburette ABU 12 (0.25-mL buret) from Radiometer. The reaction volume was 5 mL, and the vessel was constantly flushed with nitrogen. The reaction was started by adding the enzyme as the last component, after a blank value for spontaneous hydrolysis of the ester had been obtained. For the evaluation of kinetic constants, initial velocities were determined in triplicate at each substrate concentration, which ranged from 0.01 to 0.2 mM. A nonlinear least-squares program was used to fit the data to the Michaelis–Menten equation. Tonin activity was measured fluorometrically with angiotensin I as substrate (Boucher et al., 1976). Amidolytic activity was measured with the synthetic substrate Val-Leu-Arg-*p*-nitroanilide (S2266) (Amundsen et al., 1978). Kininogenase activity was determined by using a method previously discussed in detail (Carretero et al., 1976). Shortly, a 0.05-mL aliquot of a solution of esterase B (20 μg/mL) or purified rat urinary kallikrein (800 ng/mL) in 0.1 M phosphate buffer, pH 8.5, was incubated at 37 °C for 15 min with semipurified dog kininogen having 2000 ng of kinin releasing capability, in the presence of 30 mM EDTA and 3 mM 1,10-phenanthroline. The reaction was stopped by an addition of 5 volumes of redistilled ethanol. The precipitate was separated by centrifugation and the supernatant containing the released kinins evaporated under a nitrogen stream. After reconstitution in 0.1 M Tris-HCl buffer, pH 7.4, kinins were measured by radioimmunoassay (RIA). The concentration of the enzymes used released less than 5% of the substrate's total kinin releasing capability.

In addition, identification of kinins liberated during kininogenase assay was carried out by isolating the kinins by reverse-phase HPLC. Kinin solution was injected into the column (μBondapak C_{18}), and the column was eluted with a linear gradient of 10–20% acetonitrile in 0.05 M triethylammonium formate (TEAF), pH 4.4, at a flow rate of 1.0 mL/min. Kinins eluted from HPLC were measured by RIA (Carretero et al., 1976). For caseinolytic activity estimation, 1% casein was incubated with enzyme solution or bovine trypsin (0.09 mM) in 1.0 mL of 0.1 M phosphate buffer (pH 7.4). Triplicate samples were removed at 0-, 5-, and 20-min periods after incubation at 37 °C. Samples were precipitated with 15% TCA, kept at 4 °C for 1 h, and centrifuged, and the OD_{280} was determined. Under these conditions, trypsin released 0.38 A_{280} units in 20 min. This value was used to calculate the relative caseinolytic activity of esterase B.

Plasminogen activating capability was determined by fibrin-plate assay (Schumacher & Schill, 1972). Bovine fibrinogen containing plasminogen was used as substrate. Ten

[1] Abbreviations: TAME, tosyl-L-arginine methyl ester; [^{3}H]TAME, tosyl-L-arginine [^{3}H]methyl ester; S2266, valylleucylarginine-*p*-nitroanilide; TCA, trichloroacetic acid; BSA, bovine serum albumin; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TLCK, *p*-tosyl-L-lysine chloromethyl ketone; SMG, submandibular gland homogenate; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; LBTI, lima bean trypsin inhibitor; DFP, diisopropyl fluorophosphate.

Table I: Purification of Rat Submandibular Gland Esterase B

purification step	total protein (mg)	sp act. (TAME units)[a]	act. (TAME units/mg)	recovery (%)	purification factor
(1) DEAE-Sephadex	93.0	11300	121	(100)	1
(2) chromatofocusing, gel pH range 7–4	25.2	6000	238	53	2
(3) aprotinin–CH-Sepharose	3.5	2900	818	25	6.7
(4) HPLC on TSK-250	3.3	2800	840	25	6.9

[a] One TAME/unit is defined as that amount of enzyme that hydrolyzes 1 μmol of TAME/min at pH 8.0 and at 30 °C.

microliters of standard urokinase (20 Plough units) or purified enzyme (4 μg) was placed into each well with 1% agar gel containing 4% fibrinogen. The diameter of the lytic zone was measured after 24-h incubations at room temperature.

Electrophoretic Characterization. Discontinuous polyacrylamide gel electrophoresis (disc PAGE) on 16% vertical gel slabs was carried out as described by Studier (1972) using the buffer system of Laemmeli (1970). The gels were fixed overnight in methanol/acetic acid/water (40:10:50) and stained with silver nitrate with Bio-Rad silver stain (Oakley et al., 1980). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by using Laemmeli's modified discontinuous buffer system on 12.5% separating gels containing 0.2% SDS. Pharmacia's low molecular weight kit was used as the standard. The gels were fixed overnight in methanol/acetic acid/water (40:10:50) and silver stained as described above. The molecular weight of esterase B was determined as previously described (Weber & Osborn, 1969). Gels were also stained for glycoproteins by using periodic acid and Schiff's reagent.

High-pressure liquid chromatography (HPLC) was conducted in a Waters automated gradient system. The column (Bio-Sil TSK-250, 300 × 7.5 mm) was preequilibrated with 0.1 M sodium phosphate buffer (pH 6.8) and eluted isocratically at a flow rate of 1 mL/min.

Protein determination was done by the method of Bradford (1976) using bovine serum albumin as the standard. Protein concentration in column effluents was measured at 280 nm (OD_{280}).

Isoelectric focusing on polyacrylamide slab gels was carried out on an LKB 2117 multiphore electrophoresis system with pH gradients 3–10 and 3.5–5.5, using ampholine polyacrylamide gel plates (1 mm thick). The gels were prefocused at 800 V for 1 h. The protein sample or isoelectric point (p*I*) markers were applied directly on the gel and focused at 1000 V until complete. The gel slabs were immediately fixed in 10% TCA solution for 1 h, stained overnight in Allen's stain (0.2% Coomassie brilliant blue R-250; 135 mL of absolute ethanol, 30 mL of glacial acetic acid), and destained in ethanol/acetic acid/water (500:200:1300) until the background was colorless. The isoelectric pH (or p*I*) of esterase B was calculated by comparison with the electrophoretic mobility of the p*I* markers.

Amino Acid Analysis. The amino acid composition of heavy and light chains of reduced esterase B was determined by reverse-phase HPLC of acid-hydrolyzed protein (Koop et al., 1982). Cysteine and methionine residues were analyzed as cystic acid in a duplicate run on intact protein.

Carbohydrate analysis was carried out by the method of Perini and Peters (1982).

Contractile activity was determined in the isolated rat uterus preparation as previously described by Perry (1970). Blood pressure responses were tested in male Sprague-Dawley rats (200 g) anesthetized with pentobarbital (50 mg/kg ip). Purified esterase B (10 μg) or glandular kallikrein (10 μg) dissolved in 0.9% saline was injected into the ascending aorta through a catheter (PE50) inserted via the carotid artery. Blood pressure in the femoral artery was monitored with a micron blood pressure transducer.

Immunological Characterization. Cross-reactivity of esterase B against tonin or kallikrein antiserum was tested by immunodiffusion, immunoelectrophoresis, and RIA. Immunodiffusion was carried out as described by Sharpless and LoGrippo (1965). Twenty microliters of sample/antiserum per well was allowed to diffuse for 40 h at room temperature. For immunoelectrophoresis, 2-μg samples were electrophoresed on 1% agar in 0.05 M barbital buffer (pH 8.6) at 300 V for 1.5 h, followed by diffusion against antiserum for 24 h at room temperature.

Radioimmunoassay for glandular kallikrein and tonin was performed as previously described (Rabito et al., 1982; Schiller et al., 1976). Cross-reactivity was calculated as the concentration of pure enzyme giving 50% displacement on the standard curve.

Results

The purification of esterase B from rat SMG is summarized in Table I. The TAME esterolytic activity after the anionic exchange chromatography step was taken as 100% instead of the activity in the original homogenate because the esterolytic activity in the latter is due not only to esterase B but also to kallikrein, tonin, and other as yet unidentified proteases. Ion-exchange chromatography of SMG on DEAE-Sephadex A-50 (Figure 1a) with a linear salt gradient (0–0.15 M NaCl) eluted two major peaks with [^{3}H]TAME esterase activity. The first esterolytic peak coincided with tonin activity and represented the majority of protein eluted from the column. The second [^{3}H]TAME esterolytic peak (esterase B) was clearly distinct from tonin and had the strongest esterolytic activity. Ion-exchange chromatography was considered as the first step in purification, and the enzyme recovered from it was taken as 100%.

The second step of purification (Figure 1b), chromatofocusing, resulted in further separation of tonin from esterase B and a 2-fold increase in the specific activity. Esterase B eluted between pH 4 and pH 5.5 (Figure 1b). Further purification of esterase B on aprotinin–Sepharose resulted in a complete separation of esterase B from tonin, and a 3.35-fold increase in specific activity was achieved (Table I).

High-performance liquid chromatography of purified esterase B (Figure 1c) gave a single sharp esterolytic peak without any significant increase in specific activity (Table I), indicating that the fraction after aprotinin–Sepharose was already highly purified. All further characterization was performed with pooled material eluted from HPLC. The amino acid composition of esterase B is given in Table II. No carbohydrates were found upon carbohydrate analysis. Disc gel electrophoresis of esterase B (8 μg) on 16% gel slabs showed a single band (Figure 2A) with silver nitrate staining, indicating homogeneity of the preparation. It also gave a single band on a zymogram using the chromogenic substrate *N*-acetyl-L-methionine α-naphthyl ester (data not shown). SDS–PAGE gave a single band with nonreduced esterase B (apparent

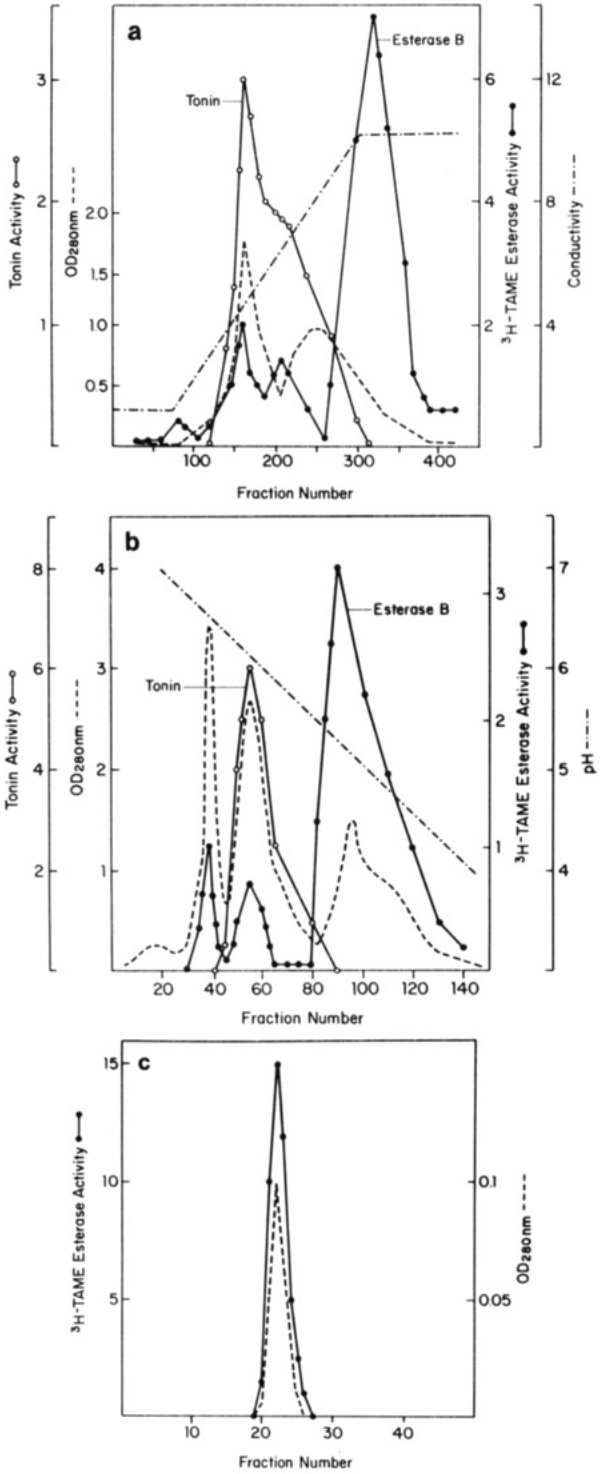

FIGURE 1: (a) DEAE-Sephadex A-50 chromatography of rat submandibular gland homogenate. Five hundred milligrams of protein was loaded onto a column (50 × 700 mm) and preequilibrated with 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA. The column was eluted with a linear salt gradient (0–0.15 M NaCl) in 0.02 M Tris-HCl (pH 8.0). Tonin activity is in nmol of His-Leu mL^{-1} min^{-1}. [^{3}H]TAME esterase activity is in cpm/mL × 10^{-8}. Conductivity is in millimhos. Flow rate = 40 mL/h; volume per fraction = 4.0 mL. (b) Chromatofocusing of esterase B on PBE 94. Ninety-three milligrams of protein was loaded onto a column (48 × 1 cm) preequilibrated with 0.025 M imidazole buffer, pH 7.4, and eluted with Polybuffer 74 (1:8 dilution), pH 4.0. Flow rate = 30 mL/h; volume per fraction = 4 mL. Tonin activity is in nmol of His-Leu mL^{-1} min^{-1}. [^{3}H]TAME esterase activity is in cpm/mL × 10^{-9}. (c) High-pressure liquid chromatography of esterase B on a TSK-250 column. One hundred micrograms of esterase B (1 mL) was injected onto a column, preequilibrated with 0.1 M phosphate buffer (pH 6.8), and eluted isocratically with the same buffer. Flow rate = 1 mL/min; volume per fraction = 0.5 mL. [^{3}H]TAME esterase activity is in cpm/mL × 10^{-8}.

Table II: Amino Acid Composition of Esterase B[a]

residue	no./mol heavy chain	no./mol light chain	residue	no./mol heavy chain	no./mol light chain
Asp	19	5	Met[c]		
Thr	9	3	Ile	6	1
Ser	20	7	Leu	12	3
Glu	25	17	Tyr	3	0
Pro	7	nd[d]	Phe	5	1
Gly	10	6	Lys	10	3
Ala	10	7	His	5	1
Cys[b]	nd[d]	nd[d]	Arg	8	2
Val	7	4			
			total[e]	156	60

[a] Estimated M_r 26 000. [b] Cys = 9, as determined in the intact molecule. [c] Met = 5. [d] nd = not detected. [e] Total number of amino acid residues.

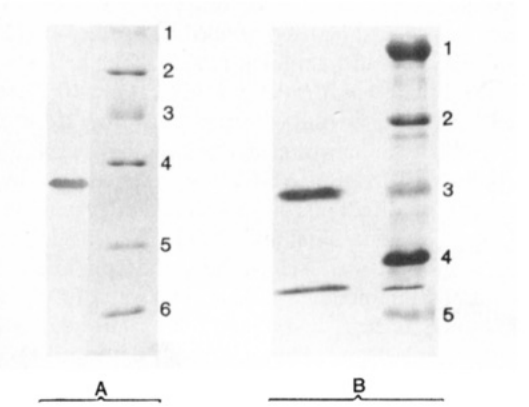

FIGURE 2: SDS–polyacrylamide gel electrophoresis of esterase B. (A) Nonreduced gel: left lane, esterase B (10 μg) stained with silver nitrate; right lane, molecular weight standards stained with Coomassie brilliant blue G-250 (1, phosphorylase B, M_r 94 000; 2, bovine serum albumin, M_r 67 000; 3, ovalbumin, M_r 43 000; 4, carbonic anhydrase, M_r 31 000; 5, soybean trypsin inhibitor, M_r 21 500; 6, lysozyme, M_r 14 400). (B) Reduced gel: left lane, esterase B (8 μg); right lane, molecular weight standards (1, ovalbumin; 2, carbonic anhydrase; 3, soybean trypsin inhibitor; 4, lysozyme; 5, insulin, $M_r \simeq 3200$). Gel was stained with silver nitrate; due to the high number of spurious protein bands revealed with this stain in the molecular weight standards, major components were first identified by staining with Coomassie brilliant blue G-250.

molecular mass, 27 000 daltons) and two bands with reduced esterase B (treated with mercaptoethanol) (Figure 2B) having molecular masses of 21 000 and 6500 daltons. Analytic electrofocusing of esterase B showed a single protein band at pH 4.45.

Esterase B hydrolyzed TAME and S2266, which are frequently used as synthetic kallikrein substrates. It had a pH optimum of 9.5 and was stable when heated at 56 °C for 30 min. Diluting the sample with either buffer or distilled water up to 1000-fold did not result in any significant decrease in enzymatic activity, nor did lyophilization alter the activity. The kinetic properties of esterase B and rat glandular kallikrein are shown in Table III. Purified esterase B, when incubated with dog kininogen, released 54 μg of immunoreactive kinins (mg of protein)$^{-1}$ min^{-1}; under identical conditions, rat glandular kallikrein generated 1000 μg of immunoreactive kinins (mg of protein)$^{-1}$ min^{-1}. The kinins generated contracted the rat uterus. There was good agreement between concentrations obtained by bioassay and by RIA. When kinins generated by esterase B were purified on HPLC, the immunoreactive kinin eluted in the same position as synthetic bradykinin. Esterase B (1–15 μg) had no direct oxytocic effect on the isolated rat uterus while kallikrein (100 ng) strongly contracted the rat uterus preparation. When injected into the ascending aorta, esterase B (10 μg) did not alter mean blood pressure, but 10 μg of glandular kallikrein caused a transient decrease in blood

Table III: Kinetic Parameters of Esterase B and Glandular Kallikrein

substrate	esterase B			glandular kallikrein		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
TAME[a]	0.096	29	600	0.103	3	2544
S2266	0.084	45	532	0.045	47	1025

[a] TAME esterolytic activity was measured by pH-stat titration method at 22 °C and at pH 8.0.

Table IV: Effects of Protease Inhibitors on Rat Esterase B, Rat Kallikrein, and Bovine Trypsin[a]

inhibitor (concn)	% inhibition		
	esterase B	kallikrein	trypsin
aprotinin (10^{-5} M)	60	83	86
soybean trypsin inhibitor (10^{-5} M)	49	0	92
phenylmethanesulfonyl fluoride (10^{-6} M)	76	61	66
antipain (10^{-4} M)	91	100	–[b]
p-tosyl-L-lysine chloromethyl ketone (10^{-4} M)	49	4	65
leupeptin (10^{-5} M)	74	58	100
Cu^{2+} (10^{-3} M)	0	0	–
Ca^{2+} (10^{-6} M)	0	0	–

[a] Ten microliters each of easterase B, rat urinary kallikrein, and bovine trypsin (=20 milliunits/mL, amidolytic activity) was preincubated with 10 μL of inhibitor at room temperature for 30 min, and amidolytic activity was measured as described by Amundsen et al. (1978). The results are expressed as percent inhibition of amidolytic activity as compared to control enzymatic activity in the absence of any inhibitor. [b] A dash (–) indicates that the value was not determined.

pressure (≃30 mmHg). Esterase B had 10% of the caseinolytic activity of an equimolar concentration of bovine trypsin. When tested for plasminogen activation, esterase B (4 μg) formed a clear radial halo (approximately 50% of the diameter given by 20 Plough units of urokinase) in plasminogen containing fibrinogen agar plates. When the sensitivity to several serine protease inhibitors of esterase B, kallikrein, and trypsin was compared, qualitative similarities between esterase B and trypsin were observed. In contrast, esterase B and kallikrein differ in their sensitivity to SBTI and TLCK since these compounds inhibited esterase B but did not affect kallikrein. Those data are shown in Table IV.

Immunological Characterization. Double immunodiffusion of esterase B against tonin and kallikrein antibodies showed that it had partial identity with kallikrein antibodies (Figure 3a) but none with tonin antibodies. On immunoelectrophoresis against kallikrein antibodies, esterase B formed a precipitin arc that corresponded to a second arc of SMG and was at a different position compared to kallikrein (Figure 3b). On RIA for glandular kallikrein and tonin, esterase B showed <0.01% cross-reactivity.

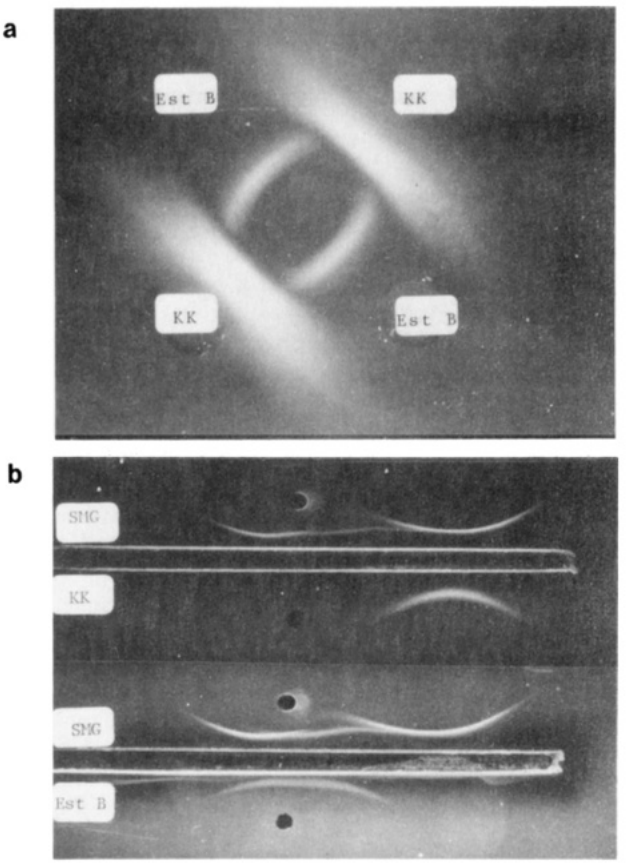

FIGURE 3: (a) Double immunodiffusion of esterase B (Est B) and glandular kallikrein (KK) against kallikrein antiserum (central well). (b) Immunoelectrophoresis of esterase B (Est B), glandular kallikrein (KK), and submandibular gland homogenate (SMG) against kallikrein antiserum (trough).

Discussion

In this study, we report on the purification and characterization of a trypsin-like protease from rat SMG. This enzyme represents the second major TAME esterolytic peak on DEAE-Sephadex ion-exchange chromatography of SMG homogenate and is arbitrarily named esterase B. A homogeneous preparation of esterase B was achieved following different chromatographic procedures as shown by a single protein band (stained with the highly sensitive silver nitrate staining technique) on 16% polyacrylamide gel, a single band on electrofocusing, and a single peak on gel filtration on HPLC. The submandibular gland homogenate contains not only esterase B but also kallikrein, tonin, and other proteases, all capable of hydrolyzing TAME (Branztaeg et al., 1976; Riekkinen et al., 1966; Minato et al., 1967). This is reflected in the high esterolytic activity of the crude homogenate, which ranged in several preparations from 120 to 248 TAME units/mg of protein. Thus, no accurate purification coefficients can be reported since the various enzyme components with TAME esterolytic activities present in SMG could not be differentiated until after the first step of purification. Under the conditions used, kallikrein and at least one other potent TAME esterase arbitrarily named esterase E remained bound to the DEAE-Sephadex. Considering DEAE-Sephadex chromatography as the first step, a 7-fold increase in specific activity was achieved. The relatively low purification factor suggests that this enzyme may be present in high concentrations in the rat submandibular glands. Preliminary data obtained by using RIA for esterase B suggested that ≃5% of the total protein concentration in the original submandibular gland homogenate was esterase B. For comparison, the concentration of kallikrein in the same homogenate is around 10% of the total protein (Johansen et al., 1983). Chromatofocusing was very useful in separating esterase B from tonin, another protease present in high concentrations in the submandibular gland (Schiller et al., 1976). The elution from the chromatofocusing column occurred at a pH different from the p*I*. This phenomenon has been discussed before (Fagerstäm et al., 1983). The difference in elution pH and isoelectric pH is a charac-

Table V: Comparison of Esterase B, Tonin, Glandulain, and Salivain

property	esterase B	tonin[a]	salivain[b]		glandulain[c]
M_r	25 000–27 000	28 000	30 000		23 000
isoelectric point	4.45	6.0–6.2	6.0	8.0–9.0	
optimum pH	9.5	6.8	9.2	8.0–8.2	
DFP, PMSF	inhibited	unaltered	inhibited		unaltered
SBTI	inhibited	partial inhibition	unaltered		–
LBTI	inhibited	–[d]	unaltered		inhibited
aprotinin	inhibited	unaltered	inhibited		inhibited
Cu^{2+} ions	unaltered	inhibited	–	inhibited	

[a] Schiller et al., 1976. [b] Riekkinen et al., 1966. [c] Riekkinen et al., 1967. [d] A dash (–) indicates that the property was not determined.

teristic constant for each type of protein and is related to the charge vs. pH relationships around its isoelectric point. This effect may be explained by disproportioning of the buffer molecules, giving a slightly different pH inside the matrix pores than in the soluble phase. Further, as shown by Wakefield et al. (1984), the elution range of a particular protein on chromatofocusing could vary depending on the sharpness of the gradient, presence of salt, or degree of column equilibration. The elution pH on chromatofocusing can be made to be near the p*I* if a suitable protocol is first established (Fagerstäm et al., 1983). Obviously this is not a requisite for separating two particular proteins as shown by the good separation of esterase B from tonin obtained here.

Esterase B is an acidic protein with an isoelectric point of 4.45. The data obtained on SDS–PAGE indicate that esterase B is composed of a heavy chain (M_r 21 000) and a light chain (M_r 6500) linked together through a disulfide bridge.

Esterase B hydrolyzed synthetic and natural protein substrates. It hydrolyzed both TAME and Val-Leu-Arg-*p*-nitroanilide (S2266), which have been used as kallikrein substrates. Although the K_m for TAME was comparable, esterase B had a higher catalytic rate toward this substrate as shown by a 10-fold difference in the k_{cat}, but kallikrein was more specific toward both TAME and S2266 as shown by its higher k_{cat}/K_m. Esterase B generated bradykinin when incubated with kininogen, but it was found to be a weak kininogenase, having only $^1/_{20}$th of the kinin releasing activity of glandular kallikrein under similar conditions. These results were obtained with canine kininogen, and it may be that a different relationship is found with homologous substrate, but this is very unlikely. In contradistinction to kallikrein, esterase B had no direct oxytocic effect on isolated rat uterus and did not induce hypotension when injected into the anesthetized rat. It is probable that these results are due to the low kinin releasing activity of esterase B, although rapid inactivation cannot be discarded. The amino acid composition of esterase B was quite similar to that of rat glandular kallikrein and tonin, but esterase B is not a glycoprotein. Another difference is that esterase B has weak caseinolytic and plasminogen activating activity while kallikrein is inactive toward these substrates.

Esterase B was not inhibited by EDTA, thereby indicating that it does not require metal ions. Nonspecific serine protease inhibitors such as leupeptin, SBTI, and aprotinin inhibited esterase B although not as effectively as they inhibit trypsin. The presence of serine and histidine residues in the catalytic center of esterase B is suggested by inhibition by PMSF, a serine reagent, and TLCK, a histidine reagent. Thus, esterase B appears to differ from kallikrein in its substrate specificity. However, on double immunodiffusion, esterase B had partial identity with glandular kallikrein. Immunoelectrophoresis showed an immunoprecipitin arc against kallikrein antisera, but at a different position than that of kallikrein. These results suggest that esterase B shares some antigenic determinants with kallikrein. In the radioimmunoassay for glandular kallikrein, esterase B had less than 0.01% immunoreactivity. Thus, when the antisera are highly diluted (as in RIA), the small proportion of the antibody against an epitope in common with esterase B is so diluted that only negligible cross-reactivity is observed. Taken together, those data indicate that esterase B is a serine protease that is immunologically related to kallikrein but has broader specificity. Tonin is also immunologically related to kallikrein, which suggests that the submandibular gland of the rat contains a family of mRNAs encoding for proteases that are characterized by different specificity but have high homology in their amino acid sequence. A similar family of mRNAs has been described in the submandibular gland of the mouse (Mason et al., 1983).

A comparison of the properties of esterase B with those reported for other known rat submandibular enzymes such as tonin, glandulain, and salivain indicates that esterase B is a previously unrecognized enzyme (Table V). The present enzyme may correspond to one of the non-kallikrein esterases reported by Brandztaeg et al. (1976).

The apparently high concentration of esterase B in submandibular gland suggests to us that this enzyme could have an important physiological function. Immunologically, esterase B belongs to the family of kallikrein-like enzymes. These enzymes have been identified as subunits of nerve growth factor and epidermal growth factor in the mouse (Bothwell et al., 1979). It has been suggested that enzymes of the kallikrein family such as glandular kallikrein and tonin are processing enzymes that cleave larger precursor polypeptides into shorter biologically active peptides (Mason et al., 1983). Esterase B may also be a processing enzyme. Its capacity to generate bradykinin and convert plasminogen to plasmin, as well as its strong esterolytic activity toward TAME and Val-Leu-Arg-*p*-nitroanilide (S2266), suggests that esterase B cleaves peptides containing basic amino acids. Peptide bonds containing basic amino acids are commonly split during the processing of polypeptides (Douglass et al., 1984). In preliminary experiments, we have observed esterase B immunoreactivity in saliva that is increased under sympathetic stimulation as compared to parasympathetic stimulation (unpublished results), thereby indicating secretion into the saliva.

Although kallikrein-like enzymes have been proposed to have a genetic role in the bioactive processing of growth factors in the mouse, such growth factors are apparently absent in the rat submandibular glands. Thus the precise biological substrates of esterase B, tonin, and kallikrein in submandibular glands or saliva are not clear. Further identification of esterase B in other tissues known to produce biologically active polypeptides may help in understanding the precise role of esterase B as well as other related proteases.

In summary, we have purified a protease from the rat submandibular gland that is related to trypsin in its broad substrate specificity and inhibitor profile and to kallikrein in its immunological properties. Its precise physiological role is unknown, although it could be one of the processing enzymes

known to belong to the kallikrein family identified in the mouse.

Registry No. TAME, 901-47-3; S2266, 62354-41-0; esterase B, 100513-59-5; trypsin, 9002-07-7; kallikrein, 9001-01-8; kininogen, 92307-50-1; bradykinin, 58-82-2; plasminogen, 9001-91-6.

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