Vol. 166, No. 1, 1990
January 15, 1990

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 231-237

OBSERVATION OF TISSUE PROKALLIKREIN ACTIVATION BY SOME SERINE PROTEASES, ARGININE ESTERASES IN RAT SUBMANDIBULAR GLAND

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Received November 22, 1989

SUMMARY: Two serine proteases, arginine esterases (esterases I and II) which showed the activity of tissue prokallikrein activation were identified in rat submandibular gland. These enzymes were separated from the homogenate of rat submandibular gland by two successive DEAE-cellulose chromatographies and were further purified and characterized. Esterases I and II were found to be identical with tonin and esterase B-like enzyme, respectively. Both enzymes activated rat urinary prokallikrein at near neutral pH. Esterase B-like enzyme activated rat urinary prokallikrein better than tonin.

Prokallikrein, the precursor of tissue kallikrein (EC. 3. 4. 21. 35), has been purified and characterized from porcine pancreas (1), human urine (2-3) and rat urine (4). Trypsin is a well known model activator of prokallikrein *in vitro* and thermolysin has also been reported to be a model activator *in vitro* (2). However, these proteases do not seem to be *in vivo* activators of prokallikrein in submandibular gland and kidney, which are rich source of tissue kallikrein. Screening analyses for prokallikrein-activating enzymes in tissues have shown that rat submandibular gland contains unknown prokallikrein activator(s). Also, prokallikrein-activating activity was observed in one of the fractions having arginine esterase activity, obtained after DEAE-cellulose chromatography of a rat submandibular gland extract. This paper reports partial characterization of these enzymes, and activation profiles of prokallikrein by the enzymes, using rat urinary prokallikrein from the same animal species.

MATERIALS AND METHODS

Rat urinary prokallikrein: Rat urinary prokallikrein (RUPK, 58 EUeq/A_{280}) was purified according to the method of Takaoka et al. (4) with some

Abbreviations: RUPK, rat urinary prokallikrein; Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; SBTI, soybean trypsin inhibitor; LBTI, limabean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride; TLCK, tosyl-L-lysine chloromethyl ketone; kDa, kilo daltons; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/90 $1.50

modifications. The amount of RUPK was determined after activation by trypsin according to the method described previously (5). The amount of RUPK was expressed as EU equivalent (EUeq).

Measurement of esterase activity: Esterase activities of kallikrein, trypsin and rat submandibular serine proteases (arginine esterases) were determined photometrically by the method previously described (5), using Bz-Arg-OEt as substrate. One esterase unit (EU) was defined as the amount of enzyme that could hydrolyze 1 μmol of substrate per min at 25°C, pH 8.0.

Active site titrations: The active enzyme concentrations of kallikrein, trypsin and serine proteases (arginine esterases) in rat submandibular gland were determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate according to the method of Fiedler et al. (6).

Analysis of N-Terminal Amino Acid Sequence: Automated amino acid sequence analysis of the N-terminal region of a protein was performed with a gas phase protein sequencer (Model 477A Protein Sequencer, connected to a 120A PTH Analyzer, Applied Biosystems, Foster City, Ca., U.S.A.).

Measurement of prokallikrein activation: (A) Two hundred μl of RUPK solution (2.9 EUeq (= 0.4 nmol)/ml in 0.15 M 1,3-bis[Tris(hydroxymethyl)methylamino]-propane-HCl buffer, pH 7.4 (buffer I)) and 100 μl enzyme solution was mixed and incubated for 19 h (in case of detecting prokallikrein activating enzyme) at 25°C. The reaction was stopped by adding 100 μl of SBTI solution (3 mg/ml in purified water), and the esterase activity of the mixture was measured (value *A*). In a control the following two mixtures, (B) 200 μl of RUPK solution and 100 μl of purified water and (C) 200 μl of buffer I and 100 μl of enzyme solution were separately incubated for 19h at 25°C. After adding 100 μl of SBTI solution to each of them, the esterolytic activities of the mixtures were measured (value *B*, *C*). The esterase activity of active kallikrein generated during the incubation (value *D*) was calculated from the equation: $D = A - B - C$. The amount of prokallikrein activated was calculated as the per cent generated kallikrein activity (EU) from the amount of prokallikrein used (EUeq) and expressed as prokallikrein activation (%). In Fig. 4, the incubation was carried out at 37°C, and 0.15 M 1,3-bis[Tris-(hydroxymethyl)methylamino]propane-HCl buffer, containing 10 mM $CaCl_2$ (pH 8.0), was used instead of buffer I.

Purification procedures: All steps were carried out at 4°C.

(1)*Fractionation of arginine esterases of a supernatant fraction of rat submandibular gland homogenate*---Ninety-two submandibular glands (34g) from 46 Wister male rats (8-14 weeks old, weighing 250-400 g, Nippon Bio Supp. Center Co., Tokyo, Japan) were chopped into small pieces with scissors. Then, 340 ml of 3.7 mS ammonium acetate solution (pH 5.8) was added. The mixture was homogenized with a Polytron PCU-2 (Kinematica, Switzerland) for 2 min, and the homogenate was centrifuged for 25 min at 8000x*g*. After centrifugation, the supernatant fraction was applied to a DEAE-cellulose column (3.6 x 40 cm), equilibrated with 3.7 mS ammonium acetate solution (pH 5.8). After non-adsorbed materials were washed out from the column with 1200 ml of the equilibration solution, adsorbed materials were eluted with a linear gradient of 3.7-20 mS ammonium acetate solution, pH 5.8 (800 ml each), followed by 800 ml of 40 mS ammonium acetate solution, pH 5.8. Five bracketed fractions which showed Bz-Arg-OEt esterase activity were separated (Fig. 1).

(2)*Second DEAE-cellulose chromatography of fraction A*---Fraction A (700 ml) in Fig. 1 was applied to another DEAE-cellulose column (2.8 x 27 cm) after dialysis against 0.02 M Tris-HCl buffer, pH 8.0. Adsorbed materials were eluted with a linear gradient of 0-0.3 M NaCl in 0.02 M Tris-HCl buffer, pH 8.0 (460 ml each). Two peaks of Bz-Arg-OEt esterase activity were eluted from the column, and two bracketed fractions (I and II) were pooled (Fig. 2).

(3)*Further purification of esterase I*---Fraction I in Fig. 2 (125 ml) was applied to a DEAE-Sepharose Fast Flow column (2.2 x 40 cm, Pharmacia-LKB Biotechnology, Uppsala, Sweden) after dialysis against 0.02 M Tris-HCl buffer, pH 8.0. The adsorbed materials were eluted with a linear gradient of 0-0.4 M NaCl in 0.02 M Tris-HCl buffer, pH 8.0. A single peak of Bz-Arg-OEt esterase activity was eluted from the column at 0.1 M NaCl. After esterase I containing fractions were pooled (40 ml) and concentrated to 3 ml, the sample

was dialyzed against Polybuffer 74® (Pharmacia-LKB Biotechnology), diluted 1:10, pH 4.0 (buffer II). The dialyzate was applied to a PBE™ column (1.2 x 15 cm, Pharmacia-LKB Biotechnology) and chromatofocusing was carried out. Esterase I was eluted with a single peak at pH 6.2. Esterase I containing fractions were pooled in a final preparation (specific activity of 16EU/A_{280}, total 80 EU (143 nmol)).

(4)*Further purification of esterase II*---After fraction II in Fig. 2 (101 ml) was concentrated to 3 ml, the sample was dialyzed against buffer II. The dialyzate was applied to the PBE™ column (1.2 x 15 cm) and chromatofocusing was carried out. Esterase II was eluted from the column as a single peak at pH 5.6. Esterase II-containing fractions were pooled (21 ml) and dialyzed against 0.1 M sodium phosphate buffer containing 0.5 M NaCl, pH 8.0. The dialyzate was applied to a guanidinated aprotinin-Sepharose 4B column (3.1 x 6.8 cm). After washing the column with 400 ml of 0.1 M sodium phosphate buffer containing 0.5 M NaCl, pH 6.4, esterase II was eluted with 0.1 M sodium acetate buffer containing 1 M NaCl, pH 4.0 and eluted fractions were immediately neutralized. Esterase II-containing fractions were pooled (227 ml) as the final preparation and concentrated to 6 ml after dialysis against purified water (specific activity of 98 EU/A_{280}, total 340 EU (60.7 nmol)).

RESULTS AND DISCUSSION

Figure 1 shows the first DEAE-cellulose chromatography of the supernatant of rat submandibular gland homogenate. Bz-Arg-OEt esterase activities were apparently separated into five fractions (A-E). Activation of prokallikrein by each eluted fraction was examined by the method described in "MATERIALS and METHODS" with using 3 EU/ml of enzyme solution. Prokallikrein activating activity was detected only in fraction A (non-adsorbed fraction). The second DEAE-cellulose chromatography was carried out with fraction A (Fig. 2), giving two peaks of Bz-Arg-OEt esterase activities (Fractions I and II);

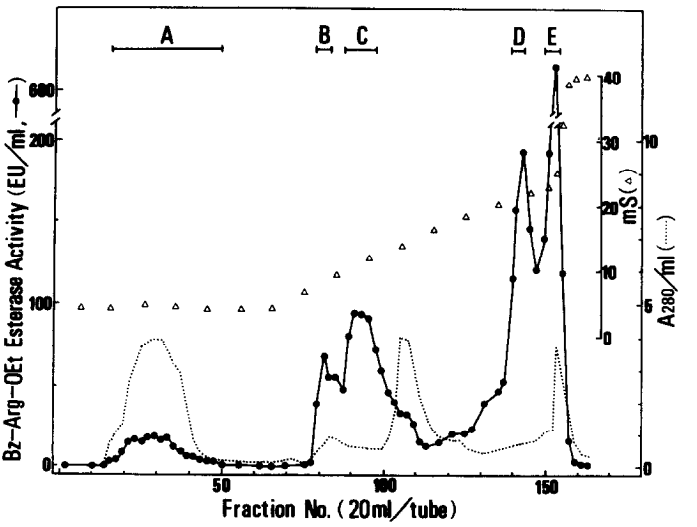

Fig. 1. First DEAE-cellulose chromatography of the supernatant fraction of rat submandibular gland. Flow rate, 36 ml/h. Five bracketed fractions which showed Bz-Arg-OEt esterase activity (A:fraction 18-49, B:fraction 78-83, C:fraction 87-97, D:fraction 139-143, E:fraction 149-154) were separately pooled.

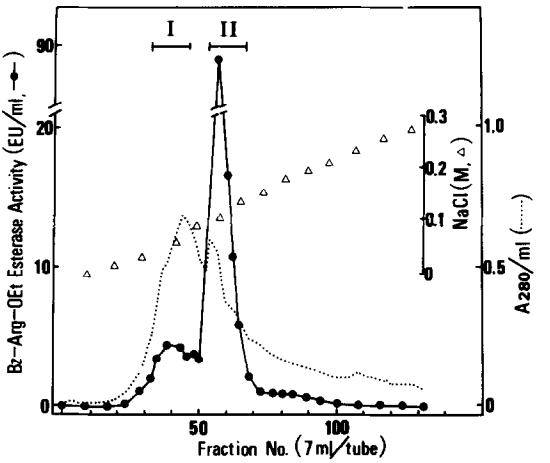

Fig. 2. Second DEAE-cellulose chromatography of the fraction A. Flow rate was 30 ml/h. Two bracketed fractions (I:fraction 33-47, II:fraction 54-68) were separately pooled.

arginine esterase in fraction I was designated as esterase I and that in fraction II as esterase II. On examining prokallikrein activation by esterases I and II (3 EU/ml of enzyme solution was used for assays), 24 % and 74 % of added prokallikrein was activated, respectively.

Esterase I was further purified by DEAE-Sepharose Fast Flow chromatography, followed by chromatofocusing. Purified esterase I was apparently homogeneous on the basis of SDS-PAGE (Fig. 3-A). Molecular mass of esterase I was estimated to be 26 kDa. Reduced esterase I showed a single band at higher

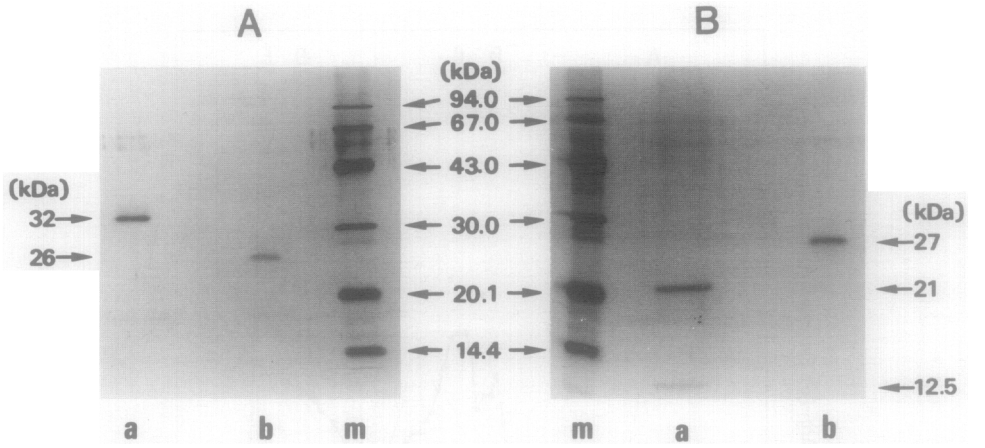

Fig. 3. SDS-PAGE analysis of esterases I and II. This was performed by the method of Laemmli (gel concentration: 12.5 %)(7). After electrophoresis, the gels were stained by silver using Ag-stain DAIICHI kit (Daiich Pure Chemical Co., Tokyo, Japan). (A) lane (a): molecular mass markers (phosphorylase b, 94.0 kDa; bovine albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; α-lactalbumin, 14.4 kDa); lane (b): esterase I reduced with β-mercaptoethanol, 0.01 nmol; lane c: esterase I, 0.01 nmol. (B) lane (a): molecular mass markers; lane (b): esterase II reduced with β-mercaptoethanol, 0.01 nmol; lane (c) esterase II, 0.01 nmol.

Table 1. Effect of enzyme inhibitors on the Bz-Arg-OEt esterase activity of esterases I and II

esterase I		esterase II	
Inhibitor	IC_{50}	Inhibitor	IC_{50}
SBTI	0.33 mg/ml	SBTI	0.75 mg/ml
LBTI	5.5 mg/ml	LBTI	n.d.
aprotinin	6300 KIU/ml	PMSF	0.62 nmol/ml
EDTA	n.d.	benzamidine	n.d.
		TLCK	n.d.
		antipain	2.5 μmol/ml
		leupeptin	9 nmol/ml

IC_{50} indicates final concentration of inhibitors which inhibit 50 % of Bz-Arg-OEt esterase activity of esterases (final concentrations of esterases I and II used were 2.8 nmol/ml and 0.17 nmol/ml, respectively). n.d., inhibitory activity was not detected.

molecular mass (32kDa). Similar phenomena are generally observed with cystine-containing proteins before and after reduction in SDS-PAGE as described by Griffith (8). Therefore, esterase I is believed to have a single polypeptide chain structure. The isoelectric point was pI 6.2. The Bz-Arg-OEt esterase activity of esterase I was inhibited by SBTI, LBTI and aprotinin, but not by EDTA (Table 1). Esterase I also had angiotensin I-converting activity (data not shown). N-terminal amino acid sequence of native esterase I was H-Ile-Val-Gly-Gly-Tyr-Lys-X-Glu-Lys-Asn-. In addition to the elution profile of esterase I on the first and second DEAE-cellulose chromatography, these observations with esterase I indicated that esterase I should be tonin (9-12) itself.

Esterase II was further purified by chromatofocusing followed by guanidinated aprotinin-Sepharose 4B affinity chromatography. The purified esterase II was apparently homogeneous on the basis of SDS-PAGE (Fig. 3-B). Molecular mass of esterase II was estimated to be 27 kDa, and esterase II consisted of two polypeptide chains (light chain of 12.5 kDa linked to a heavy chain of 21 kDa by disulfide bond). The isoelectric point was pI 5.6. The Bz-Arg-OEt esterase activity of esterase II was inhibited by SBTI, PMSF, antipain and leupeptin, but not by LBTI, benzamidine or TLCK (Table 1). These observed properties of esterase II suggest that esterase II should be esterase B-like arginine esterase (9,13).

The activation profiles of RUPK by esterases I and II were investigated. Figure 4-A shows the effect of enzyme concentration on RUPK activation by both enzymes. The activation of RUPK by esterases I and II were both dependent on enzyme concentration. RUPK was 100 % activated by esterase II at final concentration of 3 nmol/ml, but only 13 % of RUPK was activated by esterase I at 4 nmol/ml. Figure 4-B shows the effect of incubation time on RUPK activation by both enzymes and trypsin (each at a final concentration of 3 nmol/ml). Trypsin showed the fastest and most complete activation of RUPK

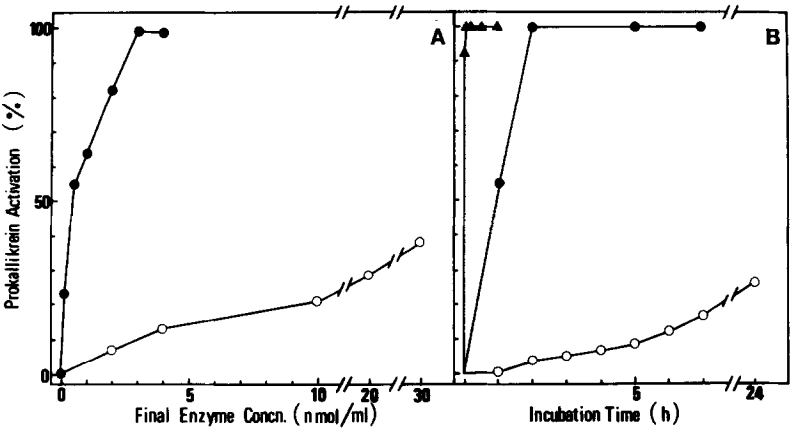

Fig. 4. Activation profiles of rat urinary prokallikrein by esterases I and II. (A) Effect of enzyme concentration on activation of RUPK. Each 100 μl of enzyme solution (esterase I, 6-90 nmol/ml, 5 points, —○— ; esterase II, 0.3-12 nmol/ml, 6 points, —●—) was mixed with prokallikrein solution and incubated for 6 h. (B) Effect of incubation time on activation of RUPK by esterases I and II and trypsin. Each 100 μl of enzyme solution (esterases I and II: 9 nmol/ml in purified water, porcine trypsin: 9 nmol/ml in 0.01 N HCl) was mixed with prokallikrein solution and incubated for the indicated time. (—○—): esterase I, (—●—): esterase II, (—▲—): trypsin. Prokallikrein activation % was determined as described in "MATERIALS and METHODS".

(within 1 min). Esterase II activated 100 % of RUPK within 2 h. But esterase I could activate only 26 % of prokallikrein, even after 24 h incubation. In a control study, the activation of RUPK was inhibited when SBTI was added to the enzyme solution before mixing with RUPK. These results indicate that esterase I and II, i.e. tonin and esterase B-like arginine esterase, had tissue prokallikrein activating activity *in vitro*. In these experiments, large amounts of the enzymes (ten times more amount of prokallikrein) were necessary to activate prokallikrein in our *in vitro* system, so we cannot conclude that these enzymes are prokallikrein activating enzymes *in vivo*. Recently, Nishi et al. (14) reported that thiol proteases from kidney cortex activated prokallikrein. In their case, it also seemed that a much larger amount of the enzyme was necessary for prokallikrein activation. Even in the case of trypsin activation of prokallikrein, a larger amount of trypsin (more than one tenth the amount of substrate) was necessary for complete activation of tissue prokallikrein (2,5) compared to that for proteolytic digestion as, for example, caseinolysis (usually one fiftieth to one hundredth amount of substrate). From these observations, there may be somewhat complicated problems in determining the quantitative relationship between prokallikrein and its activating enzymes in a tissue prokallikrein activation system. An important aspect is to assure whether prokallikrein can be activated or not by even larger amount of the enzyme.

Tonin has been shown to convert angiotensin I to angiotensin II and to generate angiotensin II from renin substrates (10,11), and esterase B has been

shown to have weak kininogenase activity as well as activity in plasminogen activation (13). However, the actual physiological functions of tonin and esterase B are still obscure. In conclusion, we would like to propose that these enzymes have the new activity of prokallikrein activation. If these enzymes indeed react multi-functionally in the body, there may be related factors which reinforce each of the activities.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Science and Culture, of Japan and Sankyo Foundation of Life Science, Japan. We thank Professor Charles C. Sweeley, Department of Biochemistry, Michigan State University, for useful suggestions in preparation of the manuscript.

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