A novel serine protease with vasoconstrictor activity coded by the Kallikrein gene S3*

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A fraction separated from rat submandibular gland homogenates was found to contain a potent vasoconstrictor when tested on isolated rabbit aortic rings. The vasoconstrictor was purified by a series of chromatographic steps. The purified compound (2.77 ± 10^{-6} m) induced 40% of the maximum contractile response to 80 mM KCl. Constriction was slow in onset, long-lasting, rinse-resistant, and unchanged by de-endothelialization; in addition, it was dose-related and inhibited by both EGTA and verapamil, but it was not affected by DUP753, an angiotensin II receptor antagonist. The compound was found to be a protein having a pI of 7.36 and a molecular weight of ~29,000 and exhibiting partial immunologic identity to rat glandular kallikrein and rat tonin. After 2-mercaptoethanol treatment, it migrated on SDS-gel electrophoresis and HPLC to ~19,300 and lacked (~10,700) chains having amino-terminal sequences of Ay(X)HNLMLL and VVGGYN(X)ETNSQ, respectively. We found that they correspond to the amino-terminal and internal sequence of a previously unidentified kallikrein-like serine protease whose mRNA, named S3, has been found in the rat submandibular gland and prostate. The vasoconstrictor is able to hydrolyze t-butoxycarbonyl-valine-proline-arginine-methylcoumarin amide (a thrombin substrate), although its $K_{cat}/K_m$ was only 0.03% that of rat thrombin. Both vasoconstrictor and enzymatic activity on t-butoxycarbonyl-valine-proline-arginine-methylcoumarin amide were completely suppressed by amindinophenylmethylsulfonyl fluoride and soybean trypsin inhibitor; however, they were unaffected by hirudin, a thrombin inhibitor. At pH 6.5, it released angiotensin II when incubated with sheep angiotensinogen, although it had approximately one-tenth the activity of tonin. The submandibular enzymatic vasoconstrictor is a kallikrein-like enzyme, having some properties of both tonin and thrombin. It directly contracts vascular smooth muscle, acting via a mechanism that requires intact enzymatic activity.

We recently found a potent proteinic vasoconstrictor in a fraction separated from a rat submandibular gland homogenate. Contractile activity was blocked by serine protease inhibitors, indicating that it is an enzyme (1). We have named it submandibular enzymatic vasoconstrictor (SEV). The rat submandibular gland is rich in serine proteases; most prominent are the enzymes of the kallikrein family (2-5), some of which may be involved in regulation of vascular tone via generation of vasoactive peptides from protein precursors (5-7). However, SEV contracted isolated vascular tissue directly and did not require preincubation with plasma (1), suggesting that it acts directly upon vascular tissue. Some proteases, including tonin and thrombin (8-11), have recently been reported to increase vascular tone in the absence of plasma trypsin components. The vasoconstrictor we found in the rat submandibular gland could be separated from tonin by column chromatography and is not inhibited by hirudin, a thrombin inhibitor (1), indicating that it is a different protein. This report describes the purification, characterization, and identification of SEV. We have determined that it is a novel enzyme encoded by S3, a gene of the kallikrein family.

EXPERIMENTAL PROCEDURES2

Materials—Rat thrombin and hirudin (a specific thrombin inhibitor (12)) were purchased from Sigma, amindinophenylmethylsulfonyl fluoride (pAPMSF, a general serine protease inhibitor (13)) from Calbiochem, soybean trypsin inhibitor (SBTI) from Worthington, human fibrinogen (grade L) from Kabai Vitrum, sodium pentobarbital (Nembutal) from Abbott, and heparin from Elkins-Sinn. Fluorogenic substrates (peptide-4-methylcoumarin amides, peptide-MCA), in-cluding Boc-Val-Pro-Arg-MCA, Boc-Ile-Gln-Gly-Arg-MCA, Pro-Phe-Glu-Arg-MCA, Glu-Gly-Arg-MCA, Boc-Gln-Lys-Lys-MCA, Suc-Ala-Pro-Ala-MCA, and Suc-Leu-Leu-Val-Tyr-MCA, were obtained from Peninsula, and DEAE-Sephadex A-50, Mono Q HR5/5, and Superose 12 columns and molecular weight calibration kits from Pharmacia LKB Biotechnology Inc. All other chemicals were analytical grade. Purification of rat urinary kallikrein and tonin and preparation of antisera against each enzyme have been described previously (14-16). Angiotensinogen (314 ng of angiotensin I generating capability/mg of protein as determined with excess hog renin) was prepared from sheep plasma as described previously (17).

Purification of SEV.—The method used to purify SEV is described in the supplemental material.

Biological Assay Using Thoracic Aortic Rings.—Vasoconstrictor activity was measured using isolated rabbit thoracic aortic rings. New Zealand White rabbits (2.0-2.5 kg) were killed by exsanguination.

1 The abbreviations used are: SEV, submandibular enzymatic vasoconstrictor; SBTI, soybean trypsin inhibitor; MCA, methylcoumarin amide; Boc, t-butoxycarbonyl; Glu, glutaryl; Suc, succinyl; CU, contractile units; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; pAPMSF, amindinophenylmethylsulfonyl fluoride; HEPE5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenediamine oxycyclohexenyltritol)tetraacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

2 Portions of this paper (including part of "Experimental Procedures," part of "Results," Figs. S1-S4, and Table S1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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from the carotid artery under Nembutal anesthesia (125 mg/animal). The thoracic aorta was isolated and rinsed with Krebs-Henseleit solution (KHB: 118 mM NaCl, 4.6 mM KCl, 27.2 mM NaHCO₃, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 1.75 mM CaCl₂, 30 μM Na₂EDTA, and 11.1 mM d-glucose). The isolated aorta was cut into 4-mm rings, two of which were placed in a 4-ml organ chamber filled with KHB aerated with 95% O₂, 5% CO₂ at 37°C. After equilibration under 1 g of tension for 1 h, the vasoconstrictor response to 60 mM KCl was determined. Changes from the initial tension in response to various agents were measured isometrically. In most cases, contractile activity was expressed in contractile units (CU); 1 CU was defined as the contractile potency obtained with 60 mM KCl in the same aortic ring. To determine the effects of protease inhibitors, two tests were done per ring in random order, one with vasoconstrictor alone (control) and the other with the inhibitor added.

**Enzymatic Assays with Fluorogenic Substrates**—Protease activity was measured fluorometrically using peptide-MCA as substrate. Each substrate was dissolved in 50 mM Tris/HCl buffer (pH 8.0) containing 0.1 M NaCl. The reaction was begun by adding 10–25 μl of solution to 1 ml of the substrate. The mixture was incubated at 25°C and the increase in fluorescent intensity was recorded continuously using a Perkin-Elmer 3000 fluorescence spectrometer with excitation at 380 nm and emission at 460 nm. The amount of enzyme-generated 7-amino-4-methylcoumarin was calculated from the standard fluorescent intensity of authentic 7-amino-4-methylcoumarin.

For kinetic analysis, the activity of 0.31 μg of purified enzyme was measured at various substrate concentrations (0.02, 0.095, 0.26, and 0.4 mM). Kinetic parameters were calculated from Lineweaver-Burk plots.

**Tomin-like Activity (Generation of Angiotensin II)**—Angiotensin II was generated directly by incubating semipurified sheep angiotensinogen with either SEV or tonin. The reaction was initiated by adding the enzyme (0.5 μg of purified enzyme or 0.2 μg of tonin/25 μl) to angiotensinogen (3.25 mg in 475 μl of 74 mM sodium phosphate, 14.7 mM EDTA buffer, pH 6.8). After 30–120-min incubations at 25°C, 50-μl aliquots of the reaction mixture were obtained. The reaction was terminated by adding 50 μl of 0.15 M NaCl and boiling the mixture for 10 min. The precipitate was removed by centrifugation at 10,000 rpm for 12 min in an Eppendorf 5415C centrifuge, and the supernatant was analyzed for angiotensin II by radioimmunoassay (18).

**Protein Determination**—Protein concentration was measured by Bradford's method (19), using bovine serum albumin as a standard.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**—To determine electrophoretic mobility, 15% acrylamide gel (8.3% X 1.5 mm) cast in 0.125 M Tris/HCl, pH 6.8, 0.1% (w/v) SDS was used as a separating gel; 4% gel cast in 0.125 M Tris/HCl, pH 6.8, 0.1% (w/v) SDS was used as a stacking gel. Gels were stained with silver nitrate. The isoelectric point of SEV was 7.36, as reconfirmed by analytical isoelectric focusing with precast ampholytes (pH 5.5–8.5, 10 cm X 10 cm X 1.5 cm). The Merck blue Coomassie Brilliant Blue G-250 in 50% methanol was used for 5 min. After the background had been destained with 50% methanol, 10% acetic acid for 10 min, the membrane was thoroughly washed with HPLC-grade water and air-dried. The stained band on the membrane was excised with a scalpel and subjected to amino acid microsequence analysis using an Applied Biosystems model 470 gas-phase sequenator with on-line HPLC (model 120) and a Nelson analytical chromatography data system.

**RESULTS**

**Molecular Weight and Isoelectric Point of SEV**—The final SEV preparation showed a single band on SDS-PAGE, which migrated at the position corresponding to 28,900 ± 2,300 daltons (mean ± S.D.; n = 3) (Fig. 1A). Under reduced conditions, two protein bands were detected. Molecular weight was shown to be 19,900 ± 900 for the heavy chains and 10,700 ± 900 for the light chains (mean ± S.D.; n = 3) (Fig. 1B).

The isoelectric point of SEV was 7.36, as reconfirmed by analytical isoelectric focusing on precast polyacrylamide gel. This was consistent with the values of 7.07–7.45 obtained during purification with the Rotofor cell (Fig. S2, Miniprint).

**Amino-terminal Sequencing**—Fig. 2 illustrates amino-terminal sequencing of the final SEV preparations. When nonreduced protein was sequenced, two amnio acids were detected in each cycle (Fig. 2A). A single amino acid was detected per cycle in both heavy and light chains (Fig. 2, B and C). The sequence determined to be Val-Val-Gly-Gly-Tyr-Asn-(X)-Glu-Thr-Asn-Ser-Gln for the light chain and Ala-Tyr-Asp-His-Asn-Asp-Leu-Met-Leu for the heavy chain. In each cycle, combinations of amino acids found with nonreduced material coincided with those found for heavy and light chains. In the heavy chain, the 11th and 12th amino acids could not be determined because of the low yield; however, they were estimated to be Leu and His by excluding Ser and Gln (11th and 12th amino acids in the light chain) from the results obtained with nonreduced protein (11th, Leu and Ser; 12th, His and Gln).

**Characteristics of Aortic Contraction Induced by SEV**—Purified SEV (0.5 μg; calculated final concentration in 4-ml organ chamber, 2.77 nm) caused 39.5 ± 10.7% of the maximum contraction obtained with 60 mM KCl (mean ± S.D.; n = 14 aortic rings). A typical SEV-induced contraction is repre-
**Submandibular Enzymatic Vasoconstrictor**

**Fig. 2.** Amino-terminal sequencing of purified rat submandibular gland vasoconstrictor. A, results obtained with nonreduced vasoconstrictor protein. B, results obtained with the heavy chain of the vasoconstrictor. C, results obtained with the light chain of the vasoconstrictor.

**Fig. 3.** Contraction of isolated rabbit aortic rings by SEV. A, purified vasoconstrictor (SEV, 0.3 μg/10 μl) was added to a 4-ml organ chamber filled with aerated Krebs-Henseleit solution, and changes in the tension of the aortic ring were recorded. Repeated washing (W) was needed to lower tension to baseline levels. Each wash represents three changes of the bath fluid. B and C, contractile activity was measured in endothelium-intact (B) and de-endothelialized (C) aortic rings. The rings were precontracted with 15 nM norepinephrine (NE). Endothelium-dependent relaxation was monitored with 1.4 μM acetylcholine. (Ach).

Contraction began slowly and took about 15 min to reach a maximum; it was long-lasting and rinseresistant (Fig. 3A). Both intact and de-endothelialized aortas exhibited contraction (Fig. 3, B and C).

Contraction was completely eliminated by omission of Ca2+ from the assay medium, which was accomplished by replacing the Krebs-Henseleit solution with Ca2+-free Krebs-Henseleit solution containing 5 mM EGTA. Furthermore, 0.5 mM verapamil, a calcium channel blocker, blunted SEV-induced contraction from 0.43 ± 0.12 to 0.23 ± 0.16 CU (mean ± S.D.; n = 8) when Ca2+ was present in the assay medium.

Contractile activity was strongly inhibited by pAPMSF or SBTI in a dose-dependent manner (Fig. 4), becoming complete at 5 and 1.8 μM, respectively. Contractile potency of SEV was not significantly changed by treatment with 0.125 unit/ml of hirudin (final concentration in the organ chamber), being 0.23 ± 0.9% CU as opposed to 0.21 ± 0.7% (control value) (n = 6). This concentration of hirudin completely prevented the coagulating activity of 0.4 pg of rat thrombin on human fibrinogen, increasing from 14 min (control value) to more than 480 min.

**Enzymatic Activity of SEV—**Enzymatic activity and substrate specificity of SEV were examined using various peptide-MCA substrates (Table I). SEV hydrolyzed Boc-Val-Pro-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, and Pro-Phe-Arg-MCA. Boc-Val-Pro-Arg-MCA, an α-thrombin substrate, was preferred and exhibited the highest $K_{cat}/K_m$, though it was poor compared with rat thrombin ($K_{cat}/K_m = 6.58 \times 10^6$ s⁻¹/M⁻¹).

Hydrolyzing rates ($V_{max}$) of Boc-Ile-Glu-Gly-Arg and Pro-Phe-Arg-MCA were about 50 and 30 times lower than for Boc-Val-Pro-Arg-MCA, respectively. Other peptides, including Glu-Gly-Arg-MCA, Boc-Glu-Lys-MCA, Boc-Ile-Lys-MCA, Boc-Ile-Lys-MCA, and Suc-Leu-Leu-Val-Tyr-MCA, were not hydrolized when each 0.02 mM substrate was incubated with 0.31 μg of SEV. With Boc-Val-Pro-Arg-MCA, hydrolyzing activity

<table>
<thead>
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<th>Hydrolysis of peptide-MCA substrates by SEV</th>
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<tr>
<td>Substrate</td>
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<tr>
<td>Boc-Val-Pro-Arg-MCA (α-thrombin)</td>
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<tr>
<td>Boc-Ile-Glu-Gly-Arg-MCA (factor Xa)</td>
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<tr>
<td>Pro-Phe-Arg-MCA (glu- dular kallikrein)</td>
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<td>Glu-Gly-Arg-MCA (urokinase)</td>
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<td>Boc-Glu-Lys-MCA (plasmin)</td>
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<tr>
<td>Suc-Ala-Pro-Ala-MCA (elastase)</td>
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<tr>
<td>Suc-Leu-Leu-Val-Tyr-MCA (chymotrypsin)</td>
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</table>

Each substrate is preferentially cleaved by the enzyme listed in parentheses.
were not affected by DUP753. The results (mean percent of maximum contractions induced with 60 mM KCl) were 31.4 ± 9.4 for angiotensin II before DUP753, 0 (n = 8 rings) after DUP753, 42.1 ± 10 for SEV before DUP753, and 44.9 ± 11 (n = 8 rings) after DUP753.

Immunochemical Characterization—Immunochemical cross-reactivity of SEV to rat glandular kallikrein and tonin was studied using the double immunodiffusion method. SEV formed a precipitation band with anti-kallikrein or anti-tonin antiserum. In both cases, the band did not completely fuse with that formed between the kallikrein or tonin standard and the corresponding antiserum, so that a spur was observed (Fig. 5). Thus, SEV exhibits partial immunologic identity with kallikrein and tonin. Under the same conditions, rat thrombin did not form a precipitation band with anti-kallikrein or anti-tonin antiserum.

Comparison of the Amino Acid Sequence of SEV with Sequences of Related Enzymes—Fig. 6 compares the partial amino acid sequence of SEV with the known sequences of selected kallikrein-like enzymes and thrombins, as well as with the predicted sequences derived from submandibular gland kallikrein cDNA. The amino acid sequence of SEV coincided with that predicted from the nucleotide sequence of kallikrein gene S3 cDNA.

**FIG. 5.** Immunochemical characterization of rat SEV with anti-kallikrein and anti-tonin antisera. Double immunodiffusion analysis was performed on a 1% agarose gel plate for 12–18 h at 25°C. A, A-K, anti-rat urinary kallikrein rabbit antiserum (10 μl); RUK, rat urinary kallikrein (0.25 μg/5 μl); SEV (0.3 μg/10 μl); TH, rat thrombin (0.8 μg/10 μl); B, A-T, anti-tonin rabbit antiserum (10 μl); TONIN, submandibular gland tonin (0.75 μg/5 μl); SEV (0.3 μg/10 μl); TH, rat thrombin (0.8 μg/10 μl). After immunodiffusion, the gel plate was hydrated and the precipitated bands stained with 1% (w/v) Amido Black, 5% (v/v) acetic acid solution.

**FIG. 6.** Partial amino acid sequence for rat SEV and comparison with sequences of various related serine proteases. Amino acid sequences were 31.4 ± 9.4 for angiotensin II before DUP753, 0 (n = 8 rings) after DUP753, 42.1 ± 10 for SEV before DUP753, and 44.9 ± 11 (n = 8 rings) after DUP753.

**DISCUSSION**

We have determined that SEV is a novel enzyme. The submandibular gland contains high concentrations of many members of the kallikrein family of the kallikrein family of serine proteases, among them glandular (the best known), tonin, esterase B and others which were well characterized (6, 7, 22–27). In the rat, it is estimated that the kallikrein family consists of up to 20 genes (28–31), eight of which have already been sequenced (29, 32, 33). Following the previously proposed nomenclature for mRNA or cDNA genes, PS encodes glandular kallikrein and S2 encodes tonin. There are other similar but distinct genes. S1, K1, and P1 are probably similar to kallikrein in terms of substrate specificity, since amino acids Tyr-99 and Trp-205 (thought to determine kallikrein-like specificity) are retained in the polypeptide encoded by them (29, 32, 33). Also, there is high overall sequence conservation between PS, S1, K1, and P1. The kallikrein-related S3 mRNA, which encodes for SEV, is very similar to tonin, since it retains the amino acid residues Asp-183, Ala-217, His-93, and Gly-205, which determine the kallikrein-like en-

was completely inhibited by 0.28 mM PAPMSF and 4 μM SBTI. Upon incubation with sheep kallikrein, SEV and tonin generated 0.94 and 11.7 ng of angiotensin II/mg of protein/min, respectively.

We investigated whether the contractile activity of the enzyme on isolated vascular rings was due to in situ generation of angiotensin II. For this, we studied whether the SEV-induced contractions were affected by DUP753, a nonpeptidic angiotensin II receptor antagonist. At a final organ chamber concentration of 125 ng/ml, DUP753 completely prevented the contractions induced by angiotensin II (final concentration, 25 ng/ml). In contrast, contractions induced by SEV were not affected by DUP753. The results (mean ± S.D.; percent of maximum contractions induced with 60 mM KCl)
its heavy chain between amino acids 55 and 101 is exactly the same as that found in kallikrein and that predicted from the cDNA sequence of other known kallikrein genes (32, 33). Also, amino acids 1-6 of the light chain of SEV and the amino-terminal end of kallikrein are exactly the same.

Comparison of the amino acid sequence of the light chain and the nucleotide sequence reported for S3 demonstrated that the first 12 amino acids of the light chain and those encoded by S3 mRNA coincided exactly, whereas S2 differed in the amino acid sequence of its amino-terminal region. Furthermore, the sequence of amino acids we found in the heavy chain coincided with the internal sequence of S3 at Ala-88 to Leu-97 (Fig. 6). Thus, SEV is very likely the protease encoded by the S3 gene. It is possible that during purification, SEV undergoes partial proteolysis, since only a single chain was predicted for the amino acid sequence indicated by S3 mRNA. We found that Ala was the amino terminus of the heavy chain, so that proteolytic cleavage should have occurred between Arg-87 and Ala-88. The theoretical molecular weight of SEV is 25,761 (calculated from the sequence of S3 protein reported by Ashley and MacDonald (32) as a possible mature active protease), which is smaller than that obtained with SDS-PAGE (~29,000). Upon testing to see whether SEV was a glycoprotein, using a glycan detection kit, we observed a faint band corresponding to SEV (not shown). Thus, the fact that the molecular weight was higher than that predicted from the amino acid sequence could be due to the additional carbohydrate chain.

The most notable property of SEV is its capacity to contract vascular tissue in vitro. Tonin is also known to induce direct vasoconstriction of the rabbit aorta (8). In addition, SEV released angiotensin II from angiotensinogen, also a property of tonin (35). These findings confirm that SEV is the enzyme coded by the kallikrein gene S3, predicted to be similar to tonin (29, 32). In addition, S3 mRNA is also found in the rat prostate, where it appears to be androgen-regulated (36-38). In preliminary work, we found that an extract of the adult rat prostate contains an SBTI-sensitive and hirudin-resistant enzymatic vasoconstrictor having the same physicochemical properties as SEV. Although we do not know its amino acid sequence, it is reasonable to conclude that it is SEV. (We excluded tonin because its gene, S2, is not expressed in the rat prostate [37, 38].)

Although we concluded that SEV is the protein encoded by the S3 kallikrein gene, there are other genes, as yet unsequenced, which also encode kallikrein-like enzymes (29-31). As unlikely as it seems, we cannot completely exclude the possibility that SEV is encoded by a still unidentified kallikrein gene that may have extensive homology to S3.

The contractions induced by SEV and tonin are very similar to thrombin and its smaller derivatives, being slow in onset, prolonged, independent of the endothelium, and resistant to antithrombin (9-11). In addition, when we stained the substrate specificity of SEV using small peptide analogues, we found that the best was Boc-Val-Pro-Arg-MCA, a classic thrombin substrate. Since the same property has been reported for tonin (39), SEV and tonin may share substrate specificity with thrombin. However, SEV and rat thrombin are easily distinguished. The two agents differ in their sensitivity to various protease inhibitors: SEV was inhibited by SBTI and resistant to hirudin, whereas the reverse was true for thrombin. Both SEV and thrombin require an intact catalytic center to contract vascular smooth muscle (10, 11). In the case of thrombin, contractility can be dissociated from coagulant activity, since γ-thrombin and mesothrombin contract vascular tissue but are practically devoid of clotting activity (40). At this time, we have no information about the nature of the substrate cleaved by SEV that leads to the contractile response. It is probably present on the surface of vascular smooth muscle cells, since contractions are not affected by elimination of the endothelium, and it is unlikely that a protein the size of SEV would be rapidly internalized. Contractions induced by SEV were not affected by a competitive receptor antagonist and angiotensin II, indicating that they are not due to in situ generation of angiotensin II from vascular angiotensinogen. Using rabbit aortic rings deprived of endothelium, we observed only weak contractions with rat thrombin, whereas SEV was a more potent vasoconstrictor. We speculate that SEV and thrombin interact with a common substrate to induce contraction of vascular tissue, SEV being the more potent enzyme. As mentioned before, the cDNA of the kallikrein gene S3 has been found in the submandibular gland and prostate. However, kallikrein-like enzymes are present in several other tissues, among them arteries and veins (30, 31, 41). Thus, whether or not SEV is present in tissues other than the submandibular gland and prostate remains to be explored.

At physiological pH, kallikrein acts on protein precursors to release kinins, which are potent vasodilator peptides; tonin and SEV release angiotensin II, a potent vasoconstrictor (5, 7). In contrast to SEV and tonin, we found that purified preparations of kallikrein (up to 10 µg/bath) do not alter vascular tone. In contrast, SEV and tonin directly contract vascular tissue, although SEV is more potent (1). It may be inadequate to include kallikrein, SEV, and tonin under the unifying classification of "kallikrein-like enzymes" (29, 30), since they are functionally dissimilar. SEV and tonin seem to represent a peculiar type of serine protease and should be classified as a discrete subtype separate from kallikrein-like enzymes.

In summary, we have found a vasoconstrictor protease in the rat submandibular gland. It directly contracts isolated vascular tissue by a mechanism that requires an intact catalytic center. This protease, named SEV, is the protease encoded by the kallikrein gene S3 and has properties similar to tonin. SEV also appears to have some of the characteristics of thrombin.

REFERENCES

Table 1. Supramolecular Structures of Cathepsin D

<table>
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<tr>
<th>Fraction</th>
<th>Clumping</th>
<th>Presence of Actin</th>
<th>Presence of Myosin</th>
<th>Presence of Calmodulin</th>
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</table>

The fractions were analyzed for clumping, presence of actin, myosin, and calmodulin using immunofluorescence microscopy. Clumping was defined as the presence of large, irregularly shaped aggregates. Presence of actin and myosin was determined by staining with specific antibodies and visualizing with a fluorescent microscope. Calmodulin was detected using a commercially available kit.

Results:

Table 1 shows the supramolecular structures observed in each fraction of the purified cathepsin D. Fraction 3 and 4 showed the highest degree of clumping and the presence of actin, myosin, and calmodulin, indicating the formation of a stable complex.

Conclusions:

The results suggest that cathepsin D associates with actin, myosin, and calmodulin to form a supramolecular complex, which may play a role in the regulation of cellular processes.

Reference:

**Submandibular Enzymatic Vasoconstrictor**

5017

![Graph](image1)

Fig. 1. Vasoconstrictor fraction obtained by DEAE-Sepharose A-50 chromatography (Fraction 4). The concentrate (10 mg protein/ml) was subjected to isoelectric focusing with a Sepharose cell. Fractions exhibiting contractile activity (Fractions 8 to 11) were pooled and used for the next purification step.

![Graph](image2)

Fig. 2. Gel Filtration on Superose 12. The contractile fraction obtained by isoelectric focusing was concentrated with a Stelof (30 ml) concentrator. The concentrate (10 mg protein/ml) was loaded onto a Superose 12 column equilibrated with 0.1 M potassium phosphate buffer (pH 6.8, containing 0.25 M NaCl) and gel filtration carried out at a rate of 0.5 ml/min. Samples containing were collected at a rate of 0.5 ml/30 sec/tube. The fraction having the highest contractile activity (Fraction 25) was stored. Those obtained from six runs were combined and used for the next step.

![Graph](image3)

Fig. 3. Gel Filtration on Superose 12. The contractile fraction obtained by isoelectric focusing was concentrated with a Stelof (30 ml) concentrator. The concentrate (10 mg protein/ml) was loaded onto a Superose 12 column equilibrated with 0.1 M potassium phosphate buffer (pH 6.8, containing 0.25 M NaCl) and gel filtration carried out at a rate of 0.5 ml/min. Samples containing were collected at a rate of 0.5 ml/30 sec/tube. The fraction having the highest contractile activity (Fraction 25) was stored. Those obtained from six runs were combined and used for the next step.

![Graph](image4)

Fig. 4. Sphingosine and Enzyme Chromatography. The contractile fraction obtained by gel filtration (1.95 mg protein/ml) was diluted with 1% gelatin (100 ml) buffer (pH 10). Buffer B, 1% gelatin (100 ml) buffer (pH 10) containing 0.5 M NaCl, was used as a buffer. The mixture was then divided into three, the enzyme concentration was 1 mg/ml each. The enzyme fraction (Fraction 8 to 11) was collected at a rate of 0.5 ml/30 sec/tube. Fraction(s) being contractile activity were pooled as a final preparation.

**Table 1: Purification of Rat Submandibular Gland Enzymatic Vasoconstrictor (RVF) from 1 g Perfused Tissue**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Specific Activity (CU/mg protein)</th>
<th>Protein (mg)</th>
<th>Specific Activity (CU/mg)</th>
<th>Purification Factor</th>
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<td>(total)</td>
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<tr>
<td>Most RVF-enriched Fraction (No. 5)</td>
<td>36.6</td>
<td>362 (33.1)</td>
<td>26.6</td>
<td>9.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Isoelectric Focusing</td>
<td>8.0</td>
<td>181 (23.4)</td>
<td>8.5</td>
<td>9.1</td>
<td>9.3</td>
</tr>
<tr>
<td>Superose 12</td>
<td>3.0</td>
<td>172 (22.2)</td>
<td>1.1</td>
<td>156.6</td>
<td>24.9</td>
</tr>
</tbody>
</table>

Notes: 1. RVF (contractile unit) was defined as the amount of the vasoconstrictor that induces the same contractile activity in the assay with the same number of rat submandibular gland vesicles. 2. Fractions 1 to 12 were subjected to gel filtration using 50 ml sodium phosphate buffer (pH 7) at a rate of 0.5 ml/min. 3. Fractions were collected, and samples having the highest contractile activity (Fraction 8 to 11) were pooled for further purification.