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Purification and Molecular Cloning of Calobin, a Thrombin-Like Enzyme from *Agkistrodon caliginosus* (Korean Viper)[1]

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A thrombin-like enzyme, calobin, has been purified to homogeneity from the venom of *Agkistrodon caliginosus* by a procedure involving Bio-Gel P-100, Mono S, and Pro-RPC. The enzyme was identified as a monomer with a molecular weight of 34,000 on SDS-PAGE, and its isoelectric point was 6.2. Calobin acted on fibrinogen to form fibrin with a specific activity of 226 NIH equivalent units, and also exhibited arginine esterase activity. The enzyme predominantly cleaved the α-chain of fibrinogen with little degradation of the β-chain. It contained abundant asparagine/aspartic acid residues, but very few tyrosine or methionine residues. The proteolytic activity of the enzyme with TAME as a substrate was higher than that of thrombin. However, it showed neither lysine esterase nor caseinolytic activity. The enzyme activity was strongly inhibited by PMSF, and moderately by benzamidine and soybean trypsin inhibitor, indicating it is a serine protease. On the other hand, the enzyme activity was not inhibited by hirudin or aprotinin. cDNA (1.6 kb) for calobin has been cloned from an *A. caliginosus* cDNA library. The cDNA sequence indicates that calobin is synthesized as a pre-zymogen of 262 amino acids, including a putative secretory signal peptide of 18 amino acids and a proposed zymogen peptide of 6 amino acid residues. The cDNA sequence encodes a 238-amino acid residue molecule exhibiting strong amino acid sequence homology to those of ancrod, batroxobin, and flavoxobin isolated from other snake venoms. Calobin contains 12 cysteine residues. As judged on alignment of the amino acid sequences of other thrombin-like enzymes (batroxobin, ancrod, and flavoxobin), calobin constitute the formation of six disulfide bridges. Amino acid residues, His^{43}, Asp^{88}, and Ser^{182}, which are thought to be the catalytic active site are highly conserved. As calobin is a glycoprotein, its possible glycosylation site, Asn-X-Thr, is located at amino acid residues 81-83.

Key words: ***Agkistrodon caliginosus***, cDNA sequence, purification, thrombin-like enzyme.

Snake venom is known to contain proteases involved in blood coagulation and fibrinolysis. They are usually divided into serine proteases and metalloproteases, depending on their properties (*1-3*). Among the serine proteases of snake venoms, thrombin-like enzymes are widely distributed among the venoms of the *Viperidae* and *Crotalidae* families. They have been studied widely because of their therapeutic potential in myocardial infarction and thrombotic diseases (*4, 5*). The thrombin-like enzymes act enzymatically on a fibrinogen molecule to form a product that cannot be stabilized by factor XIII. Consequently, the thus formed clots are not cross-linked and are susceptible to degradation by plasmin. As they lower serum fibrinogen, they appear to be an appropriate alternative to heparin and may be preferable to it in hemorrhagic complications.

Recently, we studied two kinds of metalloproteases from the eastern cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), and found that the two enzymes exhibited different extents of proteolytic activity toward the α- and β-chains of the fibrinogen molecule (*6*).

Suzuki and Takahashi (*7*) reported the purification of two kinds of thrombin-like proteins from the venom of the Korean viper (*A. caliginosus*). However, neither their amino acid sequences nor their biochemical characteristics were described. In this study, a low molecular weight thrombin-like enzyme has been purified from the Korean viper and its cDNA cloned from a venom-gland cDNA library. Its nucleotide sequence was determined and the total amino acid sequence was deduced based on the nucleotide sequence.

MATERIALS AND METHODS

Venom was pooled from the glands of *A. caligionosus* at the

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Abbreviations: BAA, $N\alpha$-benzoyl-L-argininamide; BAEE, $N\alpha$-benzoyl-L-arginine ethyl ester; NA, *p*-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; TAME, $N\alpha$-*p*-tosyl-L-arginine methyl ester; TLEE, $N\alpha$-*p*-tosyl-L-lysine ethyl ester.

Snake Institute of Seoul, and immediately lyophilized. Mono S™ HR 5/5 and Pro-RPC™ HR 5/5 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Human thrombin, bovine fibrinogen, chromogenic substrates, aprotinin, hirudin, and other reagents were from Sigma Chemicals (St. Louis, USA). All restriction endonucleases, DNA ligase, T4 polynucleotide kinase, and M13mp18/19 were purchased from New England Biolabs (Beverly, USA).

Purification—Calobin was purified sequentially by gel filtration, ion-exchange chromatography, and reverse phase chromatography. The venom (1.3 g) was dissolved in 4 ml of 50 mM Tris-HCl (pH 7.5), and the insoluble material was removed by centrifugation (1,000×*g*) for 15 min at 4°C. The supernatant was applied on a Bio-Gel P-100 column (1.5×110 cm) equilibrated with 50 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.5), and the column was eluted with the same buffer at the flow rate of 5 ml/h. The fractions containing arginine esterase or fibrinogen clotting activity were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5, overnight, and then concentrated with Diaflo UM 10 from Amicon (Beverly, USA). The active fractions were loaded on a Mono S cation-exchange chromatography column equilibrated with 50 mM Tris-HCl (pH 7.5). The bound enzyme was eluted with a linear salt gradient (0–0.6 M) in 50 mM Tris-HCl (pH 7.5) at the flow rate of 1.0 ml/min. The fractions containing arginine esterase activity were pooled, dialyzed against distilled water, and then concentrated by ultrafiltration. The enzyme was subsequently purified by Pro-RPC, with elution with a linear acetonitrile (0–100%) gradient for 60 min at 0.3 ml/min. The elution profile was monitored at 280 nm.

Enzyme Activity Assays—Arginine esterase activity was followed by the spectrophotometric procedure, with TAME as a substrate (*8*). In brief, a reaction mixture containing 1 ml of 1 mM substrate in 10 mM Tris-HCl (pH 7.5) was preincubated at 37°C for 10 min. The enzyme (10 μl) was added to the mixture and then the absorbance was monitored at 247 nm for 10 min. One unit of TAME activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of TAME per min. The absorbance change accompanying the hydrolysis of 1 μmol of TAME per ml was 0.409 $cm^{-1} \cdot mM^{-1}$ (*8*). The other substrates used for esterase activity measurement were BAEE, TLEE, and BAA, with measurement at 256 nm. One unit of arginine esterase activity was defined as the amount of enzyme necessary to hydrolyze 1.0 μmol of substrate per min.

Amidolytic activity was assayed at 25°C in 10 mM Tris-HCl, pH 7.4, by monitoring the hydrolysis of chromogenic substrates containing *p*-nitroanilide groups next to an arginine residue (V-L-K-NA, D-Val-Leu-Lys *p*-nitroanilide; Tos-G-P-R-NA, *Nα*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide; Bz-F-V-R-NA, *N*-benzoyl-Phe-Val-Arg *p*-nitroanilide; and Bz-P-F-R-NA, *Nα*-benzoyl-Pro-Phe-Arg *p*-nitroanilide). The enzyme, which had been preincubated with the buffer for 3 min, was added to each substrate and then the absorbance change was measured at 405 nm. One unit of amidolytic activity was defined as the amount of enzyme necessary to hydrolyze 1.0 μmol of substrate per min.

Assaying of Clotting Activity—The fibrinogen clotting time was measured as described by Hofmann *et al.* (*9*). Briefly, the clotting time of 0.5 ml of bovine fibrinogen (0.7%) in 10 mM Tris-HCl buffer (pH 7.5) was measured after the addition of 2 μg of enzyme. The time to the first appearance of fibrin strands was measured, with magnetic stirring at 37°C. A standard curve was obtained by varying amounts of NIH standard thrombin. Fibrinogen clotting activity was expressed in NIH equivalent units of thrombin.

Fibrinogenolytic Activity—Fibrinogenolytic activity was examined using a 1% bovine fibrinogen solution in saline. The enzyme (10 μg) was added to the solution, followed by incubation at 37°C. Aliquots of the reaction mixture were withdrawn at various time intervals and denatured by boiling for 5 min, and then the clots that formed were removed by centrifugation at 8,000×*g* for 10 min. The supernatant containing fibrinopeptides was analyzed by HPLC. The solution (100 μl) was loaded on a C_{18} column (0.4×22 cm; Applied Biosystems, USA), and then analyzed essentially as described by Shimokawa and Takahashi (*10*). Linear gradient elution (0–30%) between solvent A (0.025 M ammonium acetate, pH 6.0) and solvent B (50% acetonitrile in 0.05 M ammonium acetate, pH 6.0) was performed for 40 min at the flow rate of 1 ml/min, the absorbance at 214 nm being monitored.

Fibrinolytic Activity—Fibrinolytic activity was evaluated by applying 2 μl of a test sample on a plate of fibrin produced by thrombin-mediated polymerization, with further incubation for 17 h at 37°C (*11*). The activity was determined from the area of lysis on the plate.

PAGE and Isoelectric Focusing on Polyacrylamide Gels—SDS-PAGE was performed according to Laemmli (*12*) using 10% polyacrylamide gels. The protein standards used were α-lactalbumin (14,200), soybean trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000). Isoelectric focusing was performed with a Bio-Rad Model 111 Mini Isoelectric Focusing Cell (Richmond, USA) according to the manufacturer's procedure. Proteins were stained with Coomassie Brilliant Blue R-250 or a silver stain kit from Bio-Rad.

Amino Acid Composition—Amino acid composition analysis was carried out by HPLC involving precolumn derivatization of amino acids with phenylisothiocyanate (PITC), followed by reverse phase chromatography (*13*) for the quantitative determination of amino acids, using a Pierce amino acid standard solution (Rockford, USA).

Amino Acid Sequence—The partial amino acid sequence of the purified enzyme was determined by automatic sequencing with a Milligen 6600B amino acid sequencer, and performed at the Korea Basic Science Center in Seoul.

Proteolytic Activity Assay—Proteolytic activity was measured with azocasein according to a previously described method (*6, 14*).

Effects of Divalent Metal Cations and Inhibitors—The enzyme was preincubated with 5 mM of divalent metal cations (Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}) and inhibitors (10 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 5 mM cysteine, 2 mM PMSF, 10 mM β-mercaptoethanol, 50 μM soybean trypsin inhibitor, 1 mM aprotinin, and 10 U hirudin) at 37°C for 2 h. Then the substrate (Tos-G-P-R-NA) was added and the reaction mixture was incubated at 37°C for 2 min. The reaction was stopped by adding 100 μl of 50% acetic acid and then the absorbance was read at 405 nm.

Effect of pH—The enzyme (2 μg) was dissolved in acetate buffer (50 mM, pH 3-6), Tris-HCl buffer (50 mM, pH 7-9), or phosphate buffer (50 mM, pH 10-11), and then incubated at room temperature for 2 h. The effect of pH on proteolytic activity was determined using a chromogenic substrate, Tos-G-P-R-NA, as described above.

Effect of Temperature—The enzyme (2 μg) in 50 mM Tris-HCl buffer (pH 7.5) was incubated for 15 min at 10, 20, 30, 40, 50, 60, 70, and 80°C. The reaction mixtures were then cooled on ice and the effect of temperature on proteolytic activity was determined using a chromogenic substrate, Tos-G-P-R-NA, as described above.

Effect on Platelet Aggregation—This was essentially followed by the method of Willis and Tu (*15*). In brief, rat blood was mixed with 3.8% sodium citrate (9 : 1 v/v) in a plastic tube. Platelet-rich plasma (PRP) was collected after centrifuging the citrated blood at 200×*g* for 10 min. Platelet-poor plasma was obtained by centrifuging the blood at 500×*g* for 15 min. The platelet count was adjusted to 400-450×10⁶/ml by adding PRP and PPP. The effect of calobin was studied according to a turbidimetric method by monitoring the change in light transmission using an aggregometer from Chrono-Log (Havertown, USA). PRP (450 μl) was preincubated with 1 μg of enzyme and 25 μl of 10 mM $CaCl_2$ at 37°C for 2.5 min. The activator (5 μl of 10 μM ADP) was then added, and platelet aggregation was recorded over 5 min. The maximum aggregation response obtained on the addition of ADP in the absence of the enzyme was taken as 100%.

Protein Concentration Determination—Protein concentrations were determined by reading the absorbance at 280 nm or by the method of Bradford (*16*) using bovine serum albumin as a standard.

Construction of a cDNA Library—Total RNA was extracted from the venom glands of one freshly sacrificed adult snake, *A. caliginosus*, by the phenol procedure (*17*). The poly(A)⁺ fraction was purified by oligo (dT)-cellulose chromatography from US Biochemicals (Cleveland, USA). A cDNA library was constructed in λ ZAP II from venom gland poly(A)⁺ RNA using a cDNA kit from Stratagene (La Jolla, USA) according to the manufacturer's instructions, using standard procedures (*18*). Double-stranded cDNA was synthesized from 5 μg of poly(A)⁺ RNA template, ligated to *Eco*RI-*Xho*I digested λ ZAP II DNA, and then packaged. The packaged library was plated on *Escherichia coli* XL1-Blue MRF′ and amplified as a plate lysate on agar plates (*18*). It contained over 10^6 independent phages (96% recombinant).

Design of Oligonucleotide Probes and Screening—A 20-meric oligonucleotide probe (5′-GTC/A ATT GGA GGT GAT GAA TG-3′) was deduced and synthesized according to the N-terminal sequence of calobin and the nucleotide sequence of batroxobin (*19, 20*). The probe was ^{32}P-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (*21*). The membrane replicas were pre-hybridized at 52°C for 3 h in pre-hybridization buffer [0.9 M sodium chloride, 0.09 M sodium citrate (pH 7.0), 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 0.2% (w/v) BSA, 0.5% SDS, and 100 μg/ml sheared, denatured salmon sperm DNA]. The membrane replicas were then hybridized at 52°C for 15 h in 30 ml of hybridization buffer [0.9 M sodium chloride, 0.09 M sodium citrate (pH 7.0), 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 0.2% (w/v) BSA, 0.5% SDS, 10 mM EDTA, and 100 μg/ml sheared, denatured salmon sperm DNA] containing 10 pmol of labeled oligonucleotide probe. Subsequent washes of the hybridized membrane filters were carried out in 6×SSC (1×SSC=0.15 M NaCl/0.015 M sodium citrate) and 0.1% (w/v) SDS as follows: three times at 25°C for 5 min each and once 50°C for 2 min. The filters were then exposed to X-ray films at −70°C using intensifying screens. The positive plaques were detected by autoradiography and further purified by isolating single plaques.

Sequencing of cDNA Clones—Phage inserts were excised from the λ ZAP II vector and recircularized in the presence of the ExAssist interference-resistant helper phage to form the pBlueScript SK-phagemid according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy chain-termination method (*22*) on excised insert DNA, and cloned DNA digested with appropriate restriction enzymes and subcloned into M13 mp18/19.

RESULTS

Purification—The thrombin-like enzyme was isolated by a combination of gel filtration, Mono S ion exchange, and Pro-RPC chromatographies (Fig. 1s), and it was named calobin. Most fibrinogen clotting activity as well as arginine esterase activity was seen in the arrow region. The homogeneity of the final fraction is shown in Fig. 2s. Table Is shows that the specific activity of the purified calobin, which was obtained in a purification yield of 2.1%, was 226 U/mg. The purification was 25-fold compared to that of the crude venom of *A. caliginosus*.

Chemical Properties—The molecular weight was estimated to be 34,000 by SDS-PAGE under reducing conditions (Fig. 2sA). The isoelectric point of calobin, as determined by isoelectric focusing, was 6.2 (Fig. 2sB). Treatment of calobin with endoglycosidase F changed the electrophoretic mobility of the protein, suggesting that it is a glycoprotein containing Asn-linked oligosaccharides (data not shown). The optimum pH for the enzyme activity was 8.0 when examined with Tos-G-P-R-NA as the substrate. The enzyme was stable in the range of pH 6 to 10. Its proteolytic activity decreased outside the range of pH 4 to 10. Its optimum temperature was 40°C and it was stable at 10 to 50°C, but the activity drastically decreased at above 60°C. The substrate specificity of calobin was investigated using various kinds of substrates (Table I). The enzyme hydrolyzed arginine esters, such as TAME and BAEE, but did not cleave TLEE or BAA. Calobin did not show any caseinolytic activity.

TABLE I. **Arginine esterase activity and chromogenic substrate assay.**

Substrate	ΔAbsorbance/min/mg	Activity (units/mg)
TAME	130.0	318
BAEE	115.0	281
TLEE	6.6	16
BAA	ng[a]	
D-Val-Leu-Lys-NA (plasmin)		211
N-*p*-Tosyl-Gly-Pro-Arg-NA (thrombin)		9,884
N-Benzoyl-Phe-Val-Arg-NA (trypsin)		6,650
N-Benzoyl-Pro-Phe-Arg-NA (kallikrein)		244

[a]ng means negligible.

Aprotinin, benzamidine, and soybean trypsin inhibitor showed partial inhibition, but PMSF had a significant inhibitory effect on the enzyme activity, suggesting that calobin is a serine-type protease (Table II). Hirudin, a specific thrombin inhibitor from *Hirudino medicinalis*, did not show an inhibitory action on calobin. A chelating agent, EDTA, did not affect the hydrolysis of Tos-G-P-R-NA, indicating calobin is not a metalloenzyme (Table II). The activity of calobin was enhanced by Mg^{2+}, Mn^{2+}, and Ba^{2+}, but inhibited by Zn^{2+} and Cu^{2+} (Table IIIs).

The amino acid composition of calobin after HCl hydrolysis is shown in Table IIs. Asx and Glx are abundant, but there are very few tyrosine and methionine residues.

Sequence Homology—The N-terminal amino acid sequence of calobin was compared with those of several serine proteases. Calobin showed high homology to other thrombin-like enzymes including ancrod and batroxobin with some deletions and insertions, and moderate homology to human α-thrombin and human kallikrein (Fig. 1). The N-terminal amino acid was found to be valine, as it is in most other thrombin-like enzymes.

Thrombin-Like Activity—Thrombin-like enzyme activity was found by three independent methods. First, when chromogenic substrates were treated with calobin, Tos-G-P-R-NA and Bz-F-V-R-NA, which are specific for thrombin, were easily hydrolyzed (Table I). Second, a fibrin clot was clearly visualized on mixing calobin with fibrinogen. The clotting activity was equivalent to 226 NIH thrombin units. Third, the HPLC profile of incubation mixtures of fibrinogen with calobin or thrombin demonstrated the cleavage of three chains, Aα, Bβ, and γ, fibrinopeptides A and/or B being released. The peaks with retention times of 23 min and 32 min were fibrinopeptides A and B, respectively (Fig. 2A). Under the conditions used, calobin only released fibrinopeptide A in 1 h (Fig. 2B), while thrombin released both. Calobin was an Aα and Bβ type of enzyme hydrolyzing the Aα chain of fibrinogen fast but the Bβ chain slowly. After 6 h incubation with calobin, fibrinopeptides A and B released were almost equivalent (data not shown). In the absence of calobin, fibrinogen cleavage was negligible.

TABLE II **Effects of various inhibitors on the activity of calobin.** Calobin (2 μg) was preincubated at 37°C with 0.1 ml of an inhibitor for 2 h. Then, enzyme activity was determined using a chromogenic substrate for thrombin, as described under "MATERIALS AND METHODS." These values represent % of the control and the means of triplicate experiments.

Inhibitors	Concentration	Relative activity (%)
None		100
Benzamidine	5 mM	75
EDTA	5 mM	103
EGTA	5 mM	83
Cysteine	5 mM	93
PMSF	2 mM	5
β-Mercaptoethanol	10 mM	44
Soybean trypsin inhibitor	50 μM	68
Aprotinin	50 μM	102
	1 mM	94
Hirudin	0.5 U/ml	100
	10 U/ml	91

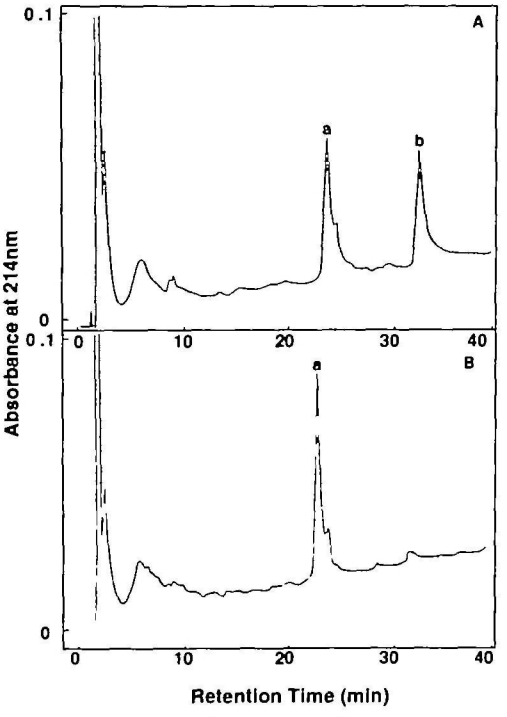

Fig 2. **HPLC analysis of fibrinopeptides released on incubation of fibrinogen for 60 min with thrombin (A) or calobin (B).** The experimental conditions are given under "MATERIALS AND METHODS." As the incubation time for calobin and fibrinogen was increased, fibrinopeptide B appeared, and equivalent amounts of fibrinopeptides A and B were released

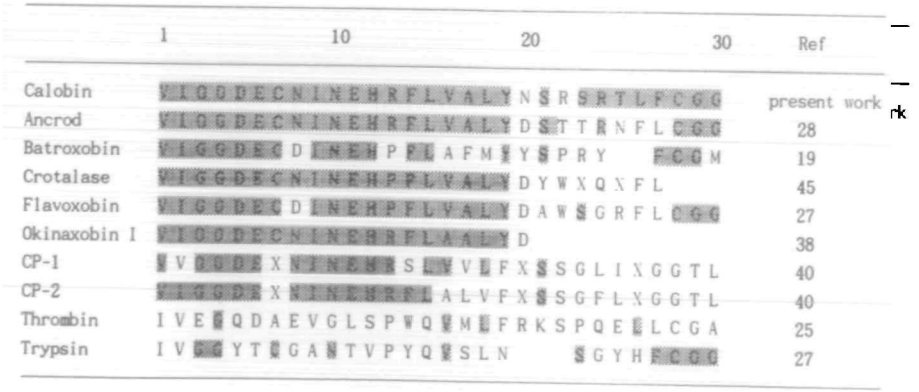

Fig. 1 **Comparison of the amino terminal sequences of calobin and other serine proteases.**

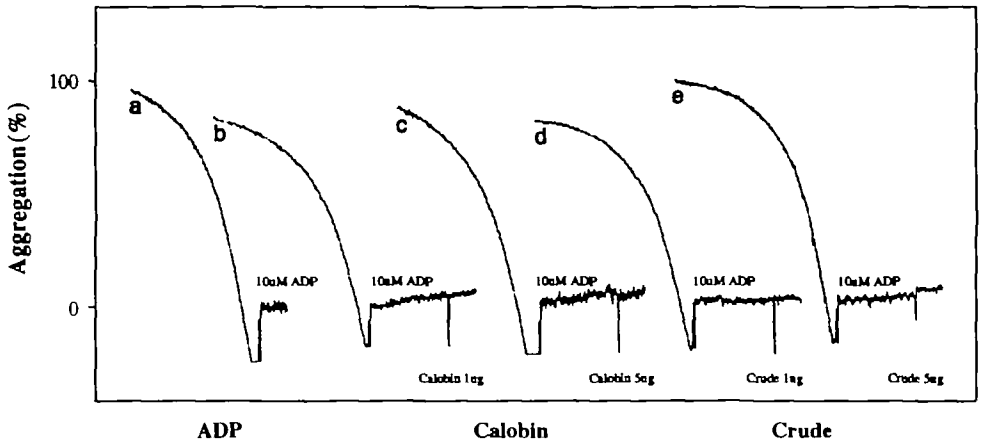

Fig. 3. **Effects of crude venom and calobin on platelet aggregation.** (a) The platelet aggregation induced with 10 μM ADP, without protease, was considered to be 100% aggregation. (b) Platelet aggregation in the presence of 1 μg of calobin with 10 μM ADP added after 2.5 min. (c) Platelet aggregation in the presence of 5 μg of calobin with 10 μM ADP added after 2.5 min. (d) Platelet aggregation in the presence of 1 μg of crude venom with 10 μM ADP added after 2.5 min. (e) Platelet aggregation in the presence of 5 μg of crude venom with 10 μM ADP added after 2.5 min.

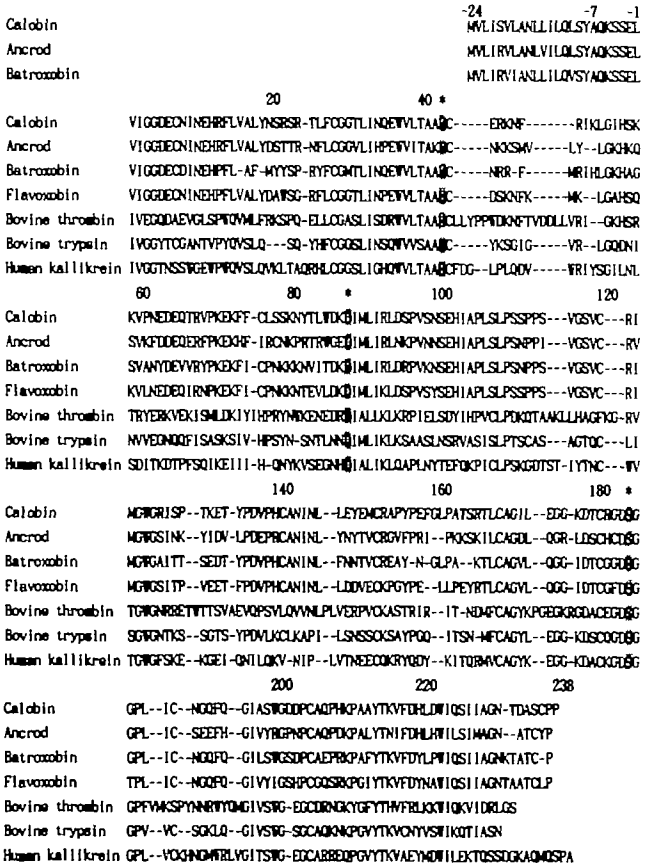

Fig. 5. **Aligned amino acid sequences of the calobin precursor, ancrod precursor, batroxobin precursor, flavoxobin, bovine thrombin, bovine trypsin, and kallikrein.** The numbering starts from the N-terminal residue of calobin. Gaps have been introduced to compare the sequence similarity. Catalytic sites are indicated by stippled boxes.

Effect on Platelet Aggregation—The effects of crude venom and calobin on platelet aggregation were compared as to the aggregation response induced by 10 μM ADP (Fig. 3). Crude venom and calobin did not affect ADP-induced platelet aggregation at 1 and 5 μg, respectively.

Screening of a cDNA Library—Nine positive clones were obtained on the screening of about 6×10^4 phages. They were subcloned into the pBlueScript SK-phagemid. The sizes of the cDNA inserts in each positive clone were determined to be 1.4 to 1.7 kb through digestion with *Eco*RI and *Xho*I. They could be classified into three groups after the inserts had been digested with *Eco*RI and *Bam*HI (data not shown).

Determination of the Nucleotide Sequence of cDNA—The partial restriction map and nucleotide sequence of the clone carrying the longest cDNA insert were determined (Figs. 3s and 4). The translation-initiation site was assigned to the first methionine codon, ATG (nucleotides 178-180), and termination codon TGA was found at nucleotides 964 to 966. The 3′-noncoding region contained a poly(A)$^+$ sequence of 19 nucleotides and the putative polyadenylation signal sequence, AATAAA (nucleotides 1559-1564), which was located at a site 15 nucleotides upstream from the poly(A)$^+$ sequence (Figs. 3s and 4).

Amino Acid Sequence Analysis—A long open reading frame covering 786 nucleotides was found in the nucleotide sequence, which putatively codes for 262 amino acids (Fig. 4). The amino acid sequence deduced from the coding region of the cloned cDNA was compared with those of other thrombin-like enzymes (Fig. 5) (*19, 23-28*). The sequence deduced from the cloned cDNA exhibited notable similarity to those of the thrombin-like enzymes, ancrod (61% sequence identity) and batroxobin (72% sequence identity). As calobin is a glycoprotein, its possible glycosylation site, Asn-X-Thr, is located at amino acid residues 81-83.

DISCUSSION

A number of thrombin-like enzymes have been isolated from the *Viperidae* and *Crotalidae* families of snakes, such as *Agkistrodon rhodostoma* (*29*), *Bothrops asper* (*30*), *Bothrops atrox* (*31*), *Bothrops moojeni* (*31*), *Bitis gabonica* (*32*), *Crotalus adamanteus* (*33*), *Crotalus horridus horridus* (*34*), and *Trimeresurus flavoviridis* (*35*). Of them, ancrod and batroxobin were being clinically tested for the treatment of thrombotic diseases because of their fibrinogenolytic effects (*36*).

The molecular weight of calobin was close to that of enzyme A reported by Suzuki and Takahashi (*7*), but the

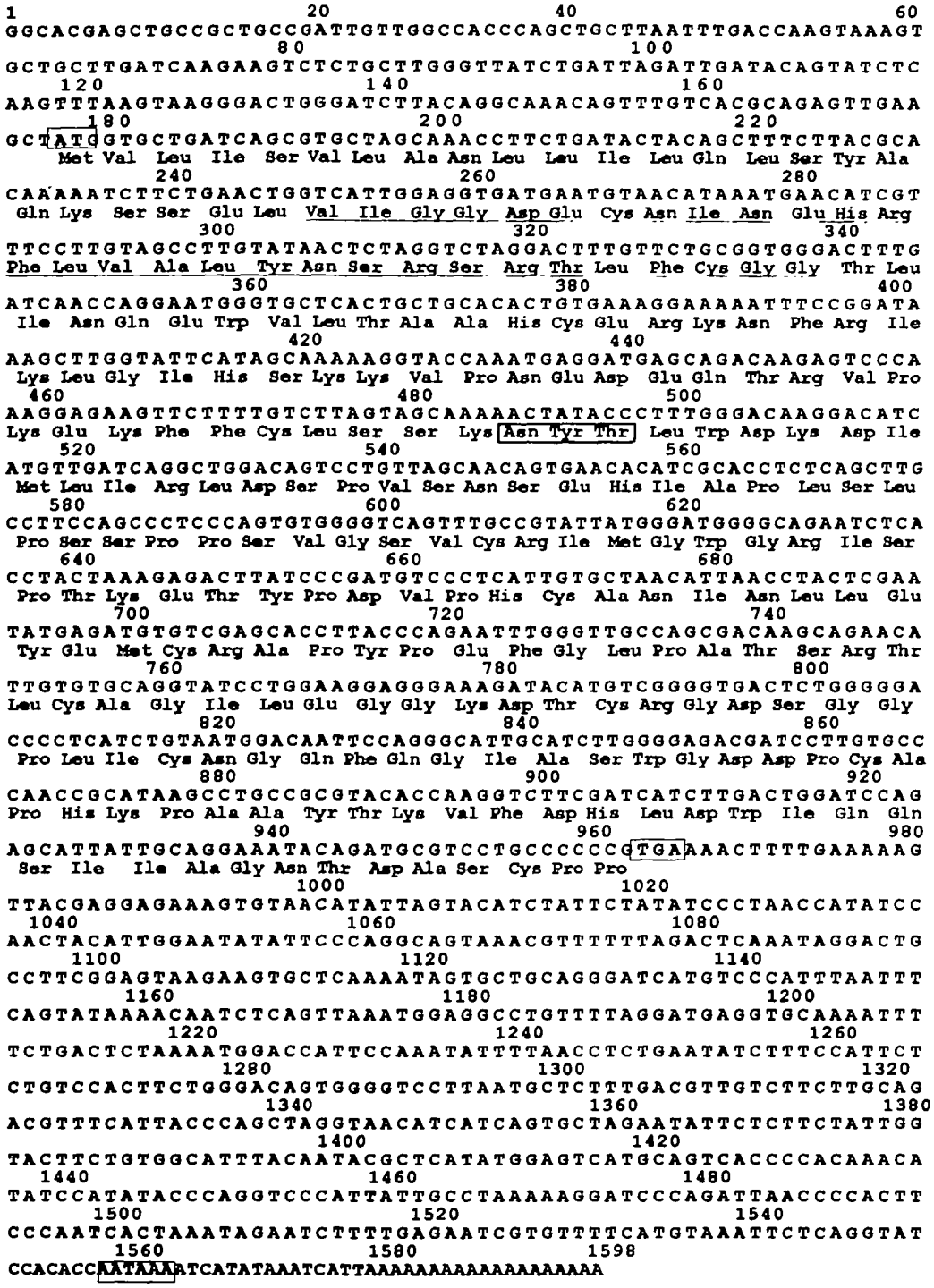

Fig. 4. **The cDNA sequence of calobin and the predicted amino acid sequence of the precursor protein.** The start ATG codon in the open reading frame and the stop TGA codon are boxed. The putative glycosylation site (Asn-Tyr-Thr) and the polyadenylation sequence (AATAAA) are also boxed. The amino acid residues that were confirmed on amino acid sequence analysis of calobin are underlined.

specific activity of calobin was higher. In fact, a second thrombin-like enzyme, which is thought to be enzyme B, was observed in the first gel-filtration step. Calobin did not exhibit microheterogeneity on isoelectric focusing.

Calobin has 238 amino acid residues with a molecular weight of 30,527, based on the DNA sequence from molecular cloning. This is pretty close to the result of SDS-PAGE after deglycosylation with endoglycosidase F (data not shown). PMSF caused a rapid loss of amidolytic activity of calobin, and treatment with EDTA, EGTA, and cysteine did not affect the enzyme activity, indicating that calobin is a serine protease not a metalloprotease found in snake venom. The fibrinogen clotting activity of calobin, as well as that of batroxobin and okinaxobin, was unaffected

by hirudin. Thus, it is speculated that the region of the amino acid sequence of α-thrombin interacting with hirudin is different that of calobin. Calobin was found to act on the Aα chain of fibrinogen fast, but the Bβ chain slowly. Most of thrombin-like enzymes from snake venoms, including ancrod (*29*) and batroxobin (*30*), act on fibrinogen to release fibrinopeptide A preferentially, while the enzymes from *Trimeresurus mucosquamatus* (*37*) and okinaxobin I (*Trimeresurus okinavensis*) readily split off fibrinopeptide B (*38*). Calobin hydrolyzed the ester bonds in TAME and BAEE as well as the amide bonds in Tos-G-P-R-NA and Bz-F-V-R-NA, which are specific substrates for thrombin. Arginine esterase activity was observed for β-fibrinogenases from other snake venoms as well (*39*).

On alignment of the N-terminal amino acid sequences of calobin, ancrod, batroxobin, crotalase, okinaxobin, and flavoxobin, they were found to be highly homologous to one another, although okinaxobin is a β-fibrinogenase. Interestingly, capillary permeability-increasing (CPI)-enzyme-1 and CPI-enzyme-2 from *A. caliginosus* showed remarkable similarities in their amino acid sequences to calobin. These are the same type of arginine esterase, but do not show thrombin-like activity (*40*). When endoglycosidase F was incubated with calobin, clotting activity decreased to 70% of the enzyme activity. This suggests that the carbohydrate moiety may play a role in stabilizing calobin (*41*). Several snake venoms were reported to contain platelet anti-aggregation peptides (*42, 43*), but the effects of crude venom from *A. caliginosus* and calobin on platelets were negligible at two concentrations of crude venom and calobin.

The predicted amino-terminal residue of calobin, valine, is preceded by 24 amino acids of prepeptides (signal peptides) and propeptides (zymogen peptides). Other serine proteases (trypsin and kallikrein) are each synthesized as a pre-zymogen before maturation (*23, 24*). A common feature of the signal peptides is a central core region rich in hydrophobic amino acid residues with large side chains and terminating in an apolar residue having a small side chain (alanine, glycine, and serine) (*44*). Therefore, we could predict that a possible site for cleavage of the prepeptide in the precursor is present after the Ala residue at position -7. The zymogen peptide is characterized by containing a region that is rich in a hydrophilic peptide. The hydrophilic peptide (amino acids -6 to -1) is probably a zymogen peptide. Based on the homology with other mature serine proteases, we could deduce the catalytic amino acid residues to be His^{43}, Asp^{88}, and Ser^{184}. The sequences around them are more highly conserved. This deduction is verified by the fact that calobin is strongly inhibited by PMSF. Calobin contains 12 cysteine residues. On alignment of the amino acid sequences of other thrombin-like enzymes (ancrod, batroxobin, and flavoxobin) (*19, 27, 28*), it may be presumed that the 12 cysteine residues in the sequence are all involved in the formation of six disulfide bridges (Cys^{7}-Cys^{141}, Cys^{28}-Cys^{44}, Cys^{76}-Cys^{236}, Cys^{120}-Cys^{190}, Cys^{152}-Cys^{169}, and Cys^{180}-Cys^{205}).

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Supplemental Materials

TABLE Is. **Specific activity and yield of calobin at each stage of purification.**

Step	Total protein (mg)	Total activity	Specific activity (NIH units)
Crude venom	1,300.0	11,700.0	9.0
Bio-Gel P-100	113.5	7,943.6	70.0
Mono S	36.2	3,623.0	100.0
Pro-RPC	1.1	248.6	226.0

TABLE IIs. **Amino acid content of calobin (residues/mol).**

Amino acid	Calculated value[c]	Amino acid	Calculated value[c]
Asx[a]	35.1 (25)	Met	4.4 (3)
Thr	16.0 (12)	Ile	17.2 (17)
Ser	20.8 (19)	Leu	22.2 (19)
Glx[a]	24.2 (19)	Tyr	6.1 (6)
Pro	20.4 (19)	Phe	13.9 (8)
Gly	27.4 (19)	His	8.4 (7)
Ala	18.8 (14)	Lys	10.1 (12)
Val	18.1 (10)	Arg	17.4 (12)
Cya[b]	14.5 (12)	Trp	14.2 (5)
		Total	309.1 (238)

[a]Asx and Glx mean the sums of asparagine and aspartic acid, and glutamine and glutamic acid, respectively. [b]Cya means the sum of cysteic acid and oxidized cystine. [c]Values in parentheses are the numbers of amino acid residues predicted from the cDNA sequence.

TABLE IIIs. **Effects of divalent metal cations on the activity of calobin.** Calobin (2 μg) dissolved in 50 mM Tris-HCl (pH 7.5) was preincubated with each divalent cation at 37°C for 2 h. Activity was determined using a chromogenic substrate for thrombin, as described under "MATERIALS AND METHODS." These values represent % of the control and the means of triplicate experiments.

Divalent cations	Concentration (mM)	Relative activity (%)
None		100
Mg^{2+}	5	118
Mn^{2+}	5	104
Zn^{2+}	5	21
Ba^{2+}	5	108
Cu^{2+}	5	5

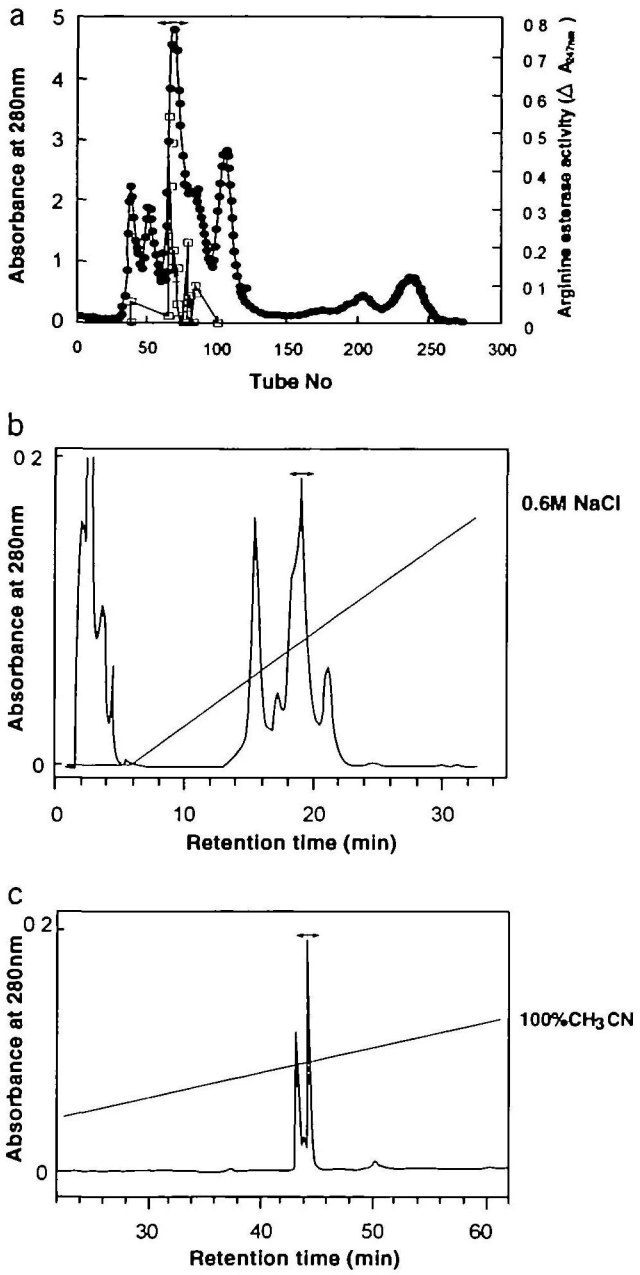

Fig. 1s. **Isolation of calobin from the venom of *A. caliginosus*.** The procedure is described under "MATERIALS AND METHODS." (a) Chromatography on Bio-Gel P-100. The eluant was monitored by spectrophotometry at 280 nm (●) Arginine esterase activity (□) was measured and the active fractions were pooled (–). (b) Mono S chromatography, (c) Pro-RPC chromatography.

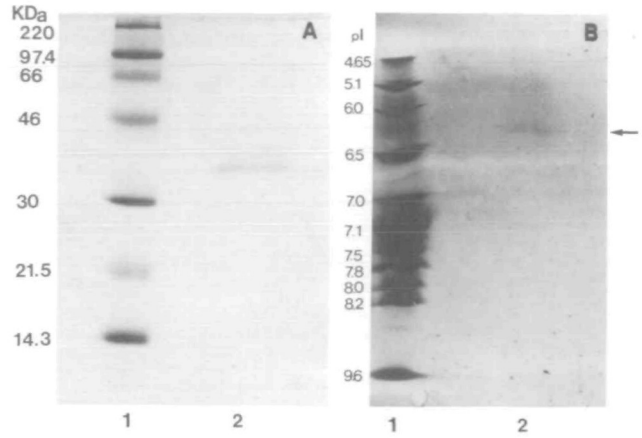

Fig. 2s **Homogeneity of the purified calobin from a Pro-RPC column.** Polyacrylamide gel electrophoresis and isoelectric focusing of calobin The conditions are given under "MATERIALS AND METHODS." (A) SDS-PAGE under reducing conditions with SDS, (B) IEF on a 10% acrylamide gel containing 2% ampholyte (pH 3-10) Lane 1, pI markers; and lane 2, calobin

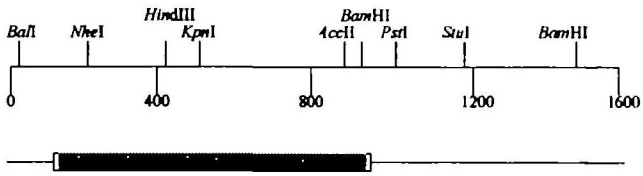

Fig. 3s. **Partial restriction map of the longest clone sequenced.** The open reading frame is shown in a stippled box