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Purification and Amino Acid Sequence of Brevilysin L6, a Non-Hemorrhagic Metalloprotease from *Agkistrodon halys brevicaudus* Venom

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A non-hemorrhagic proteinase, brevilysin L6 (L6), has been purified to homogeneity from *Agkistrodon halys brevicaudus* venom by gel filtration and DEAE-Toyopearl 650M chromatography. It is an acidic protein with an isoelectric point of 4.8, and its molecular mass was estimated to be 21.5 kDa by SDS-PAGE. The optimum pH of L6 was about 9. EDTA and *o*-phenanthroline inhibited the proteolytic activity, suggesting that L6 is a metalloprotease. Cysteine also inhibited the activity of L6, but glutathione did not. The protein was stable in the pH range of 5-8.5 and below 40°C. Calcium ions had no effect on the proteolytic activity of L6 or on its thermal stability. The enzyme preferentially cleaved X-Leu, X-Phe, X-Val, and X-His bonds. L6 showed weak α-fibrinogenase activity. The complete amino acid sequence of L6 was also determined by manual Edman degradation. L6 is a non-glycosylated single-chain polypeptide consisting of 203 residues with an N-terminal pyroglutamic acid and a calculated molecular weight of 22,713 Da. Its entire sequence is highly homologous to those of other metalloproteases from various snake venoms. A zinc-binding motif, HEXXHXXGXXH, is located at residues 143-153 in the sequence of L6.

Key words: *Agkistrodon halys brevicaudus*, amino acid sequence, fibrinogenolytic, metalloprotease, snake venom.

Snake venom contains various biologically active proteins related to blood coagulation and fibrinolytic systems (*1*). Hemorrhaging is one of the most striking manifestations of Crotalid and Viperid poisoning (*2*). Many hemorrhagic factors (hemorrhagins) have been purified and their amino acid sequences have also been elucidated (*3*). Such factors are divided into two classes according to their molecular sizes: high-molecular mass (more than 60 kDa) hemorrhagins such as HR1B (*4*), and low-molecular mass (22-26 kDa) ones such as HR2a (*5*). They all belong to the zinc metalloprotease subfamily because of their high homologies to venomous metalloproteases (*6*), and are capable of hydrolyzing basement membrane components including laminin, nidogen, fibronectin, and type IV collagen (*2, 7*). Some of them also show fibrinolytic activity (*8*). Sequence analysis of a high-molecular mass hemorrhagin, HR1B, from *Trimeresurus flavoviridis* revealed that the protein is composed of three domains, *i.e.* metalloprotease, disintegrin-like and Cys-rich domains (*4*). The metalloprotease domain is equivalent to the entire sequence of low-molecular mass proteases.

Chinese viper (*Agkistrodon halys brevicaudus*) venom contains several metalloproteases. In order to correlate the substrate specificity and hemorrhagic activity with the primary structure, we intended to characterize these enzymes. In previous studies, we have already isolated two metalloproteases designated as protease H6 (brevilysin H6) (*9*) and protease L4 (brevilysin L4) (*10*) from this venom. Brevilysin H6 is a 60 kDa protein with weak hemorrhagic activity, while brevilysin L4 is a low molecular mass (22 kDa) protein and has no hemorrhagic activity. Here, we report the purification and amino acid sequence of another low-molecular-mass metalloprotease, brevilysin L6, from the same venom. On comparison with brevilysins L4 and H6, L6 showed a much wider substrate specificity.

MATERIALS AND METHODS

Materials—A low-M_r protein fraction was prepared from crude *A. halys brevicaudus* venom as described previously (*10*). The sources of the materials used were as follows; 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate from Dojindo Lab. (Kumamoto); arginylendopeptidase (Arg-C), *Achromobacter* protease I, *Staphylococcus aureus* V8 protease, endoproteinase Asp-N, pyroglutamate aminopeptidase, and bovine insulin from Takara (Kyoto); bovine and human fibrinogens from Nacalai Tesque (Kyoto); carboxypeptidase Y from Wako Pure Chem. (Osaka); and Z-Gly-Pro-Leu-Gly-Pro from Peptide Inc. (Osaka). Other oligopeptides were synthesized in our laboratory. Performic acid-oxidized insulin was prepared according to Sanger (*11*). FTC-casein was prepared according to Twining (*12*).

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Abbreviations: Arg-C, arginylendopeptidase; CM, *S*-carboxymethylated; FTC-casein, fluorescein thiocarbamoyl casein; L6, brevilysin L6 from *A. halys brevicaudus* venom; LH-RH, luteinizing hormone-releasing hormone; PE, *S*-pyridylethylated; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

Chromatography—All the purification steps were carried out at 4°C. A sample solution was put on a Sephacryl S-200HR column (2.6×80 cm, Pharmacia) equilibrated with 0.5 mM $CaCl_2$-0.1 M NaCl-50 mM Tris-HCl (pH 7.4). Elution was carried out with the same buffer, 3.5 ml fractions being collected. Anion-exchange chromatography was performed on a DEAE-Toyopearl 650M column (1.6×17 cm, Tosoh). Proteins were eluted with a linear gradient of 0-0.3 M NaCl in 0.5 mM $CaCl_2$-50 mM Tris-HCl (pH 7.4) (total volume, 500 ml), 4 ml fractions being collected. Protease activity was measured using casein in 0.2 M Tris-HCl (pH 8.5) at 37°C, as described by Satake *et al.* (*13*).

Electrophoresis—SDS-PAGE was carried out according to Laemmli (*14*). Protein bands were detected by staining gels with 0.1% Coomassie Brilliant Blue R-250. Isoelectric focusing was performed as described by Wrigley (*15*) using a Model 111 mini-IEF apparatus (Bio-Rad) and 7.5% polyacrylamide gels containing 2% ampholine (pH 3-10). The gels were stained with 0.04% Coomassie Brilliant Blue G-250 in 3.5% perchloric acid.

Thermal and pH Stability—The stability of the protease was examined in 50 mM veronal-HCl (pH 3.4-9.0) or 50 mM Gly-NaOH (pH 8-10.9). After 50 μl of the enzyme solutions (1 mg/ml) had been kept at 37°C for 1 h, 200 μl of 1% FTC-casein in 0.2 M TES-NaOH (pH 9.0) was added and then the increase in fluorescence was measured with a FP-550A spectrofluorometer (Jasco) at 525 nm with excitation at 490 nm to determine the remaining activity. The thermal stability was examined in 100 mM TES-NaOH (pH 7.0). The enzyme solution (1 mg/ml) was heated at 40-70°C for 30 min in a water bath in the presence or absence of 1 mM $CaCl_2$ and then cooled in ice. The residual activity was measured as described above.

Action of Brevilysin L6 on Peptide and Protein Substrates—A substrate (100 μg each of LH-RH and oxytocin, 230 μg each of synthetic pentapeptides, or 600 μg of performic acid-oxidized insulin) was incubated with L6 (25 μg) at 37°C for 1 h in 0.1 M TES-NaOH (pH 9.0). The resultant peptide fragments were separated by reverse-phase HPLC on a Biofine RPC SC-18 column (0.46×25 cm, Jasco) with an appropriate gradient of acetonitrile in 0.1% TFA. The positions of cleavage sites were determined by the amino acid composition of each fragment.

Fibrinogenolytic activity was analyzed by SDS-PAGE. Human fibrinogen (400 μg) was treated with 50 μg of L6 in 100 μl of 2 mM $CaCl_2$-50 mM imidazole-HCl buffer (pH 7.4) at 37°C. The reaction was stopped by the addition of 10 mM EDTA (25 μl). The mixture was lyophilized, and then the residue was dissolved in 5 M urea-2% SDS-2% 2-mercaptoethanol-10 mM phosphate buffer (pH 7.2). Electrophoresis was performed under reducing conditions. The degradation of fibrin clots was determined as described previously (*10*).

Sequence Analysis of L6—*S*-Pyridylethylation and *S*-carboxymethylation of L6 were carried out according to Friedman *et al.* (*16*) and Hirs (*17*), respectively. PE-L6 was digested at 37°C with arginylendopeptidase (E/S=1:50) for 6 h in 0.25 M phosphate buffer (pH 8.0), *S. aureus* V8 protease (E/S=1:100) for 6 h in 0.1 M NH_4HCO_3, or CNBr (100 equivalent) for 24 h in 70% HCOOH. CM-L6 was digested with *Achromobacter* protease I (E/S=1:100) at 37°C for 6 h in 0.1 M NH_4HCO_3. Some N-terminal peptides were digested with pyroglutamate aminopeptidase (0.02 U/mg peptide) at 37°C for 20 h in 50 mM dithiothreitol-0.1 M phosphate buffer (pH 8.0). The digests were lyophilized, dissolved in 0.1% TFA, and then fractionated by reverse-phase HPLC on a TSK-gel ODS-120T column (0.46×25 cm, Tosoh) in 0.1% TFA with an appropriate gradient of acetonitrile. The amino acid sequences of the peptides were determined by manual Edman degradation using the 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate double coupling method (*18*). Amino acid-derivatives were identified by an HPLC method (*19*).

RESULTS

Purification of Brevilysin L6—We began to purify L6

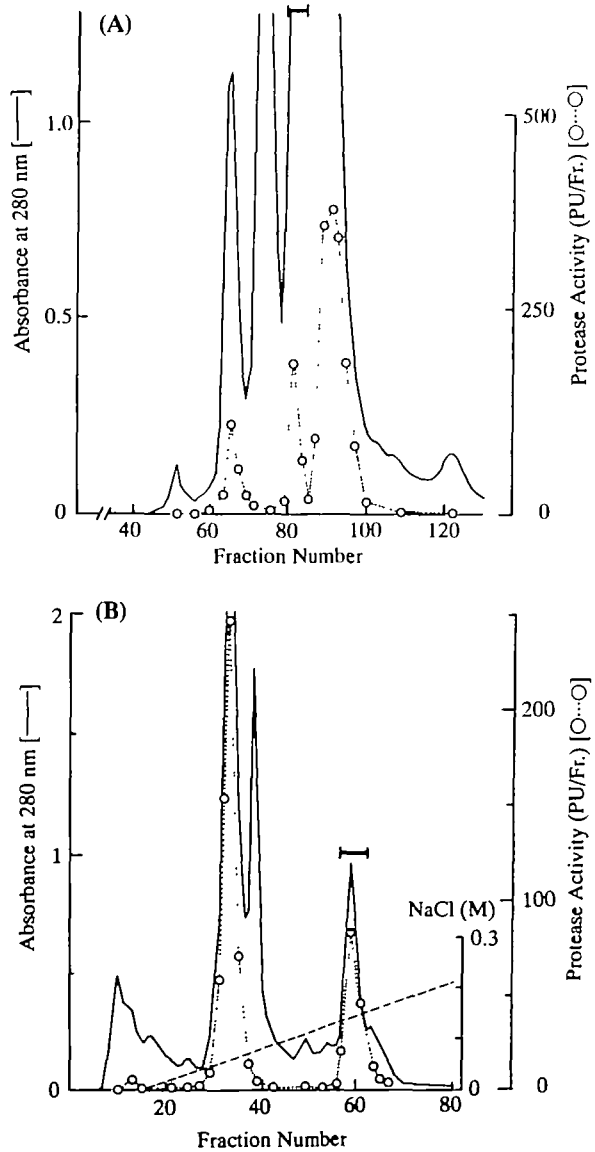

Fig. 1. **Purification of brevilysin L6.** (A) Gel filtration of low M_r proteins from *A. halys brevicaudus* venom (*12*) on a Sephacryl S-200HR column. (B) Anion-exchange chromatography on a DEAE-Toyopearl 650M column. The fractions indicated by a bar were collected.

using low-M_r proteins (*10*) from venom. Gel filtration on Sephacryl S-200HR gave three protease peaks (Fig. 1A). The fractions indicated by a bar in Fig. 1A were collected and applied to a DEAE-Toyopearl 650M column, which yielded two proteolytically active peaks, as shown in Fig. 1B. SDS-PAGE analysis showed that L6 was present in the last peak. The first peak contained phospholipase A_2 and a small amount of brevilysin L4. Approximately 6 mg of pure L6 was obtained from 141 mg of the low-M_r proteins. The purified enzyme gave single band on SDS-PAGE, its molecular weight being estimated to be 21,500 (Fig. 2A). The isoelectric point of L6 was about 4.8 (Fig. 2B). L6 exhibited no hemorrhagic activity up to 100 μg of protein per mouse nor fibrinogen clotting activity.

Properties of Brevilysin L6—The protease activity of the enzyme was measured in buffers of various pHs using FTC-casein as the substrate. The optimum pH of L6 was about 9.

Metalloproteases are influenced by some metal ions and inactivated by metal ion chelators that can remove a zinc ion from the active site of an enzyme. The effects of several metal ions, metal chelating agents and proteinase inhibitors

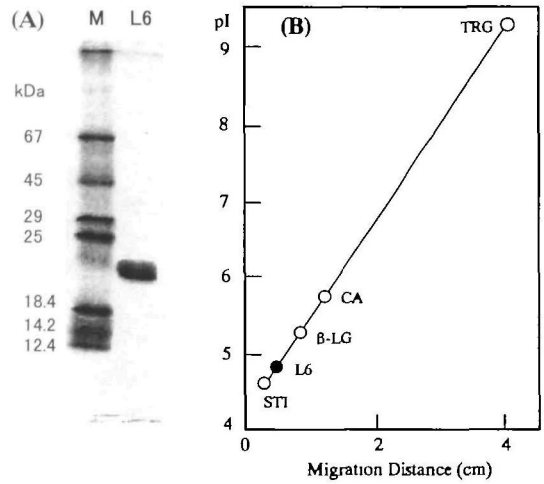

Fig. 2. **Homogeneity and isoelectric point of the purified brevilysin L6.** (A) SDS-PAGE analysis using a 12% gel. M, marker proteins: bovine serum albumin, ovalbumin, carbonic anhydrase, chymotrypsinogen, β-lactoglobulin, α-lactalbumin, and cytochrome *c* (from top to bottom). (B) IEF analysis on a 7.5% gel containing 2% Ampholine (pH 3-10). STI, soybean trypsin inhibitor; β-LG, β-lactoglobulin A; CA, bovine carbonic anhydrase II; TRG, bovine trypsinogen.

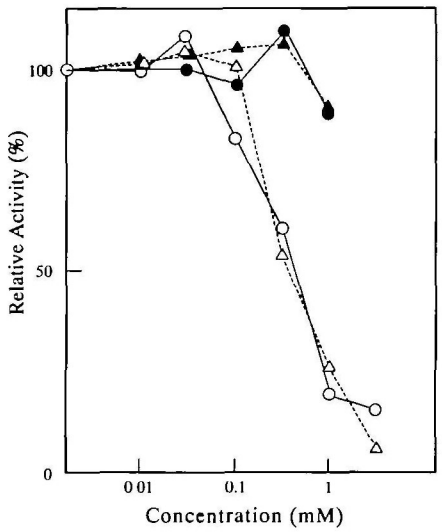

Fig. 3. **Effects of cysteine and the reduced form of glutathione on the caseinolytic activities of brevilysins L6 and L4.** Protease activity was assayed in 50 mM Tris-HCl (pH 8.5) at 37°C using FTC-casein as the substrate. Solid lines, L6; broken lines, L4. Open symbols, cysteine; closed symbols, glutathione.

TABLE I. **Effects of metal ions, metal-chelating agents, and protease inhibitors on brevilysin L6 from *Agkistrodon halys brevicaudus* venom.**

Additive	Conc. (mM)	Relative activity (%)[a]
None	–	100
$MgCl_2$	1	95
$CaCl_2$	1	93
$SrCl_2$	1	101
$BaCl_2$	1	106
$CoCl_2$	1	32
$ZnCl_2$	1	79
$MnCl_2$	1	57
$CuCl_2$	1	10
$HgCl_2$	1	42
$FeCl_3$	1	71
$CrCl_3$	1	85
o-Phenanthroline	1	24
EDTA	1	11
Glutathione, reduced	1	88
L-Cysteine	1	20
α_2-Macroglobulin	1[b]	106
α_1-Antitrypsin	3[b]	100
Ovoinhibitor	4[b]	116
Aprotinin	4[b]	103
Phosphoramidon	200[b]	100

[a]Protease activity was measured in 0.2 M Tris-HCl (pH 8.5) at 37°C using FTC-casein as the substrate. [b]Mole ratio of inhibitor to L6.

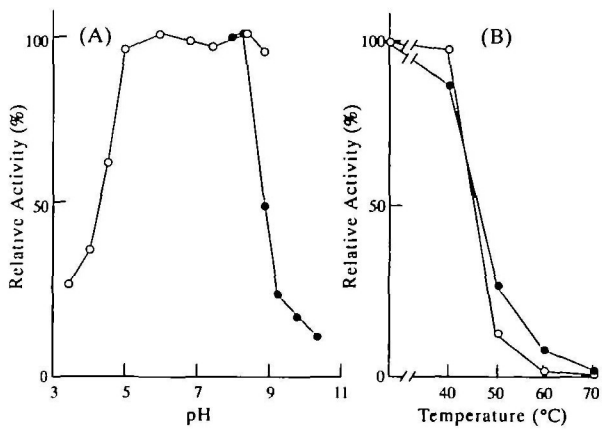

Fig. 4. **Stability of brevilysin L6.** (A) Thermal stability of L6. The protease solution was left for 30 min at 0-70°C in TES-NaOH (pH 7.0) in the presence or absence of 1 mM $CaCl_2$, and then the residual activity was measured in TES-NaOH (pH 9.0) at 37°C. (B) Stability at various pHs. Protease activity was assayed after pre-incubation in 50 mM veronal-HCl (pH 3.4-9.0) or 50 mM Gly-NaOH (pH 8-10.9) for 1 h at 37°C. FTC-casein was used as the substrate.

on the caseinolytic activity of L6 were examined. The enzyme was inhibited by EDTA and *o*-phenanthroline (Table I), indicating that L6 is a metalloprotease. The activity was decreased profoundly by Co^{2+} and Cu^{2+} ions, and weakly by Hg^{2+}. In contrast to brevilysins H6 and L4 (*9, 10*), Ca^{2+} ions had no enhancing effect on L6. Phosphoramidon and several proteinase inhibitors were also ineffective. Cysteine dose-dependently inhibited the caseinolytic activity of L6 and L4 (Fig. 3). A thiol compound, glutathione, had little effect on any of the brevilysins (Fig. 3).

The stability of L6 was examined after treatment in buffers of various pHs at 37°C for 1 h. The enzyme was stable in the pH range of 5 to 8.5 (Fig. 4A). L6 was heated in 0.1 M TES-NaOH (pH 7.0) at 40-70°C for 30 min in the presence and absence of 1 mM $CaCl_2$, and then the residual activity was quantified. The activity was markedly reduced above 50°C (Fig. 4B). The addition of Ca^{2+} ions did not affect the thermal stability.

Substrate Specificity and Fibrinolytic Activity of Brevilysin L6—The substrate specificity of L6 was examined using some natural or synthetic peptides and proteins. The enzyme digested the A and B chains of oxidized insulin, as summarized in Fig. 5. It preferentially attacked the amino side of peptide bonds adjacent to hydrophobic residues such as Leu, Phe, and Val as well as X-His bonds. LH-RH was cleaved at the Gly^6-Leu^7 bond. Oxytocin, Z-$(Leu)_4$-Gly, Z-Val-Orn-Leu-Gly-Gly, Z-Gly-Pro-Leu-Gly-Pro, and human placental type IV collagen were not digested.

Degradation of fibrinogen was assayed by SDS-PAGE (Fig. 6). Brevilysin L6 cleaved the Aα chain of fibrinogen without affecting its Bβ and γ chains. The enzyme also digested fibrin clots at the rate of 0.55 mg per hour with 50 μg of the enzyme.

Amino Acid Sequencing of Brevilysin L6—Arg-C digestion of PE-L6 followed by separation of the fragment peptides by HPLC gave ten peptides, R1-R10 (data not shown). On Edman degradation no amino acid was liberated from R1. Because the direct sequencing of PE-L6 was also unsuccessful, the N-terminus of L6 might be blocked, probably by a pyroglutamyl residue. Carboxypeptidase Y digestion of R1 confirmed the sequence of X-Gln-Arg. Since peptide R7 was too large to determine its whole sequence by the manual sequencing method, it was further digested with V8 protease to give five fragments, R7V1-R7V5. PE-L6 was also subjected to V8 protease digestion and chemical cleavage with CNBr, peptides V1-V11 and M1-M5 being yielded, respectively. Peptide V5 was a mixture of two peptides (V5a and V5b), which were further separated by HPLC under different conditions. Since we could not obtain N-terminal fragments through the above digestions,

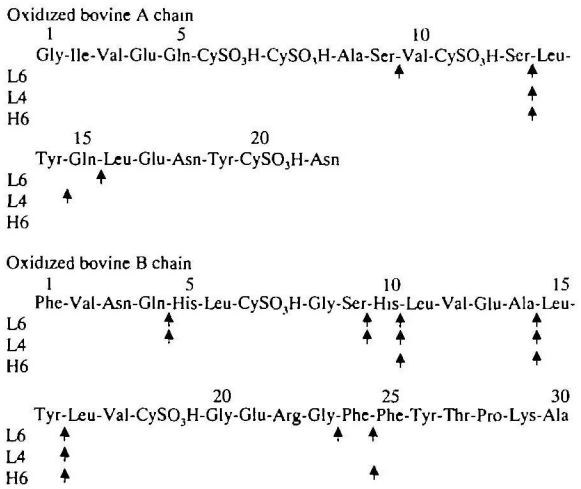

Fig. 5. **Comparison of the substrate specificities of brevilysins.** The cleavage sites of the oxidized bovine insulin A and B chains are indicated by arrows.

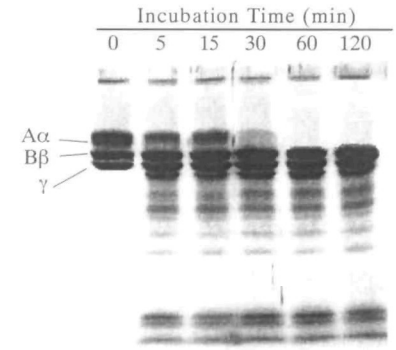

Fig. 6. **SDS-PAGE analysis of fibrinogen digestion by brevilysin L6.**

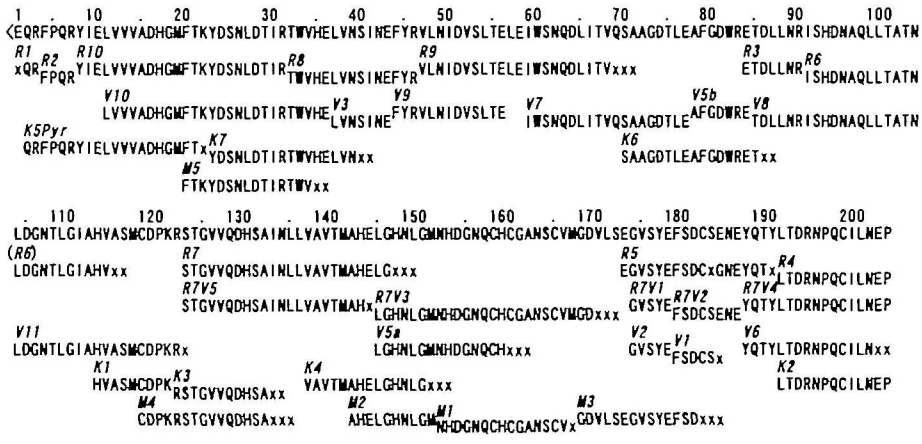

Fig. 7. **Summary of the amino acid sequence analysis of brevilysin L6.** Peptides derived from *S*-alkylated L6 on digestion with Arg-C (R), *S. aureus* V8 protease (V), CNBr (M), and *Achromobacter* protease I (K) are shown above the sequence. Pyr denotes the sub-digestion peptide with pyroglutamate aminopeptidase. Residues not identified are indicated by x.

CM-L6 was digested with *Achromobacter* protease I to yield seven peptides, K1-K7. Peptide K5 was the N-terminal fragment, and was sequenced after the N-terminal pyroglutamyl residue had been removed with pyroglutamate aminopeptidase.

The arrangement of the fragments and the complete amino acid sequence of brevilysin L6 are summarized in Fig. 7. The protein is composed of 203 amino acid residues (22,713 Da). The presence of a conservative zinc chelating sequence, HEXXHXXGXXH (*20*), at residues 143-153 proves that L6 is a typical metalloprotease. A *N*-glycosylation site (Asn-X-Ser/Thr) was not found in its sequence.

DISCUSSION

As reported previously (*10*), gel filtration of crude *A. halys brevicaudus* venom gave two peaks of caseinolytic activity. Brevilysin L6 was present in the second peak, together with brevilysin L4 (*10*), phospholipases A_2, and a thrombin-like enzyme (*21*). We could completely remove these contaminating enzymes by anion-exchange chromatography.

Cysteine inhibited the activity of L6 (Fig. 3). This seems to be common to venom metalloproteases since brevilysins L4 and H6 were inhibited similarly. 2-Mercaptoethanol also inhibited the enzymes but to a lesser extent (data not shown). Glutathione may be released from necrotic tissues after a snake bite and is expected to inhibit venom proteases. However, the reduced form of glutathione had little effect on the enzymes (Fig. 3). These data suggest that the inhibition was not merely to be due to the reducing potency of the agents.

Venomous metalloproteases cleave peptides and proteins on the amino side of peptide bonds adjacent to hydrophobic residues (*1, 22*). Brevilysin L6 digested LH-RH and oxidized insulin at X-Leu, X-Phe, and X-Val bonds as well as the X-His bond. On comparison with other brevilysins, L6 showed a much wider substrate specificity than L4 and H6 (Fig. 5). However, L6 did not cleave the Pro^7-Leu^8 bond in oxytocin that was attacked by brevilysin H6 (Terada *et al.*, unpublished data). No synthetic peptapeptides containing Leu residues acted as substrates for brevilysin L6 either.

Most venomous α-fibrinogenases, which degrade the Aα chain, are zinc metalloproteases (*23*). These enzymes also act on the Bβ chain of fibrinogen, but at a slower rate. Brevilysin H6 degraded the Aα chain, and the Bβ chain slowly (*9*), while brevilysin L4 only attacked the Aα chain (*10*). Brevilysin L6 is also classified as an α-fibrinogenase based on its ability to preferentially attack the Aα chain. However, the velocity of degradation of fibrinogen by L6 was much lower than that by brevilysin L4.

The sequence of L6 was established by manual Edman degradation of overlapping peptides generated with a variety of selective cleavage procedures. We observed two unexpected cleavages on Arg-C digestion of PE-L6 at Ser^{172}-Glu^{173} and Tyr^{190}-Leu^{191} (Fig. 7). Furthermore, the Arg^{194}-Asn^{195} bond was not attacked by Arg-C. Brevilysin L6 has only two lysyl residues (Lys^{22} and Lys^{121}), and *Achromobacter* protease I digestion should give three fragments. However, seven major peaks were obtained owing to four unexpected cleavages at Gln^{69}-Ser^{70}, Ala^{112}-His^{113}, Leu^{136}-Val^{137}, and Tyr^{190}-Leu^{191} (Fig. 7). The reason for the occurrence of these abnormal cleavages is not clear at present.

In Fig. 8, the amino acid sequences of nine venomous proteases including the metalloprotease domain of a disintegrin precursor (*29*) are compared. L6 belongs to the three-disulfide bond proteinase class. The highly conserved cysteine residues allow the prediction of the same pattern of disulfide bonds for L6 (Cys^{118}-Cys^{198}, Cys^{158}-Cys^{165}, and Cys^{160}-Cys^{183}) as determined for fibrolase (*25*). Brevilysin L6 is highly homologous to a hemorrhagic factor, LHF-II (*27*) (72% identity), as well as to a non-hemorrhagic fibrolase (*25*) (69.5% identity). Thus, the similarity between the entire sequences of these proteins did not provide any structural information concerning the characteristics of hemorrhagic proteases. The hemorrhagic activity is correlated with the ability to digest basement membrane components (*30*). Hite *et al.* (*6*) proposed that three residues are involved in the hemorrhagic activity, *i.e.* His-93, Gln-97, and Ile-102 of hemorrhagins (the residue numbers correspond to those of brevilysin L6) because these residues are absent in non-hemorrhagic H_2-proteinase (*24*). However, His-93 and Gln-97 are present in L6. In addition, the fibrolase has all of these residues (*25*). Despite its wide substrate specificity, L6 did not degrade type IV collagen, one of the basement membrane components. This explains the non-hemorrhagic property of L6. The ability of snake venom proteases to attack the basement membrane might be linked rather to the restricted substrate specificity, which would be determined by the subsite architecture in the active site cleft.

Snake venom contains plural metalloproteases as well as related proteins. For example, we could find at least four P-III class enzymes (brevilysins H1, H2, H3, and H6), two P-I class ones (L4 and L6), and two disintegrin precursors

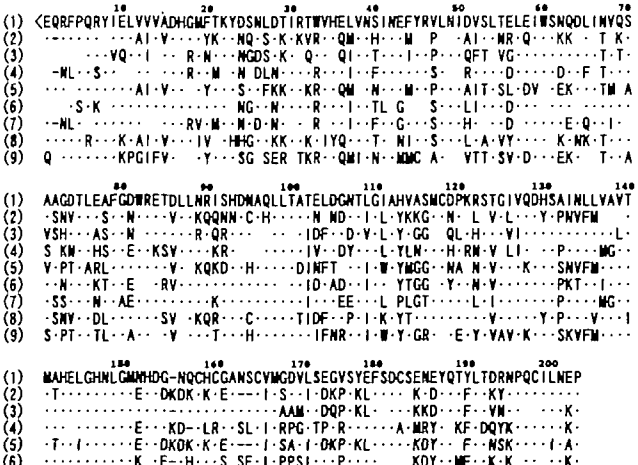

Fig. 8. **Comparison of the amino acid sequence of brevilysin L6 with those of other venomous metalloproteases.** (1) L6, (2) H_2-proteinase (*24*), (3) fibrolase (*A. contortrix contortrix*) (*25*), (4) HT-2 (*C. ruber ruber*) (*26*), (5) HR2a (*5*), (6) LHF-II (*Crotarus atrox*) (*27*), (7) hemorrhagic protease Ht-d (*C. atrox*) (*28*), the metalloprotease domains of (8) HR1B (*4*), and (9) the putative precursor of trigramin (*T. gramineus*) (*29*). H_2-proteinase, HR2a and HR1B were isolated from the venom of *T. flavoviridis*. The residues are numbered according to the sequence of L6. Dots indicate the residues identical to those of L6.

in *A. halys brevicaudus* venom. They may have evolved from a common ancestral protein through repetitive gene duplications, which generated many similar proteases with slightly different substrate specificities as well as different hemorrhagic potencies. This may allow the venom to attack a wide variety of proteins and cause much wider damage in the victims. L6 may have evolved for the extensive degradation of proteins.

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