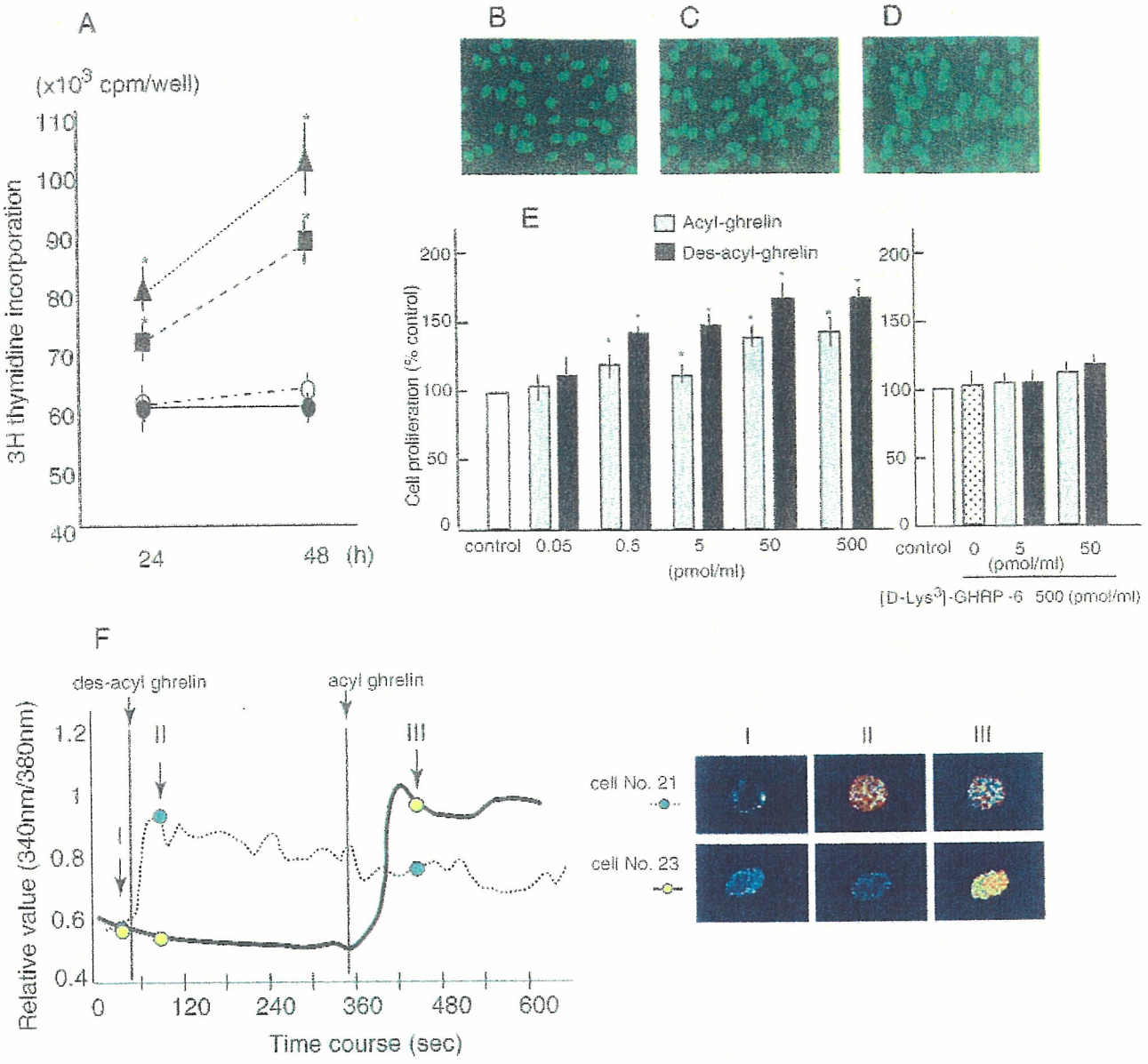

FIG. 5. A, The effect of acyl ghrelin on [^{3}H]thymidine incorporation by cultured fetal (E17) skin cells. One day after initiation of culture, acyl ghrelin (●, 0 pmol; ○, 0.5 pmol; ■, 5 pmol; ▲, 50 pmol/ml) and [^{3}H]thymidine (2 μCi/ml) were added for a 24- or 48-h incubation period. Each *symbol* and *vertical line* represents the mean ± SEM (n = 16 wells). *Asterisks* indicate significant differences ($P < 0.05$ *vs.* ●). B–D, Example of BrdU incorporation (shown in E) into the nucleus of cultured fetal (E17) skin cells incubated for 24 h with 50 pmol/ml acyl ghrelin (C), 50 pmol/ml des-acyl ghrelin (D), or without ghrelin (B). E (*left*), The effect of various doses of acyl and des-acyl ghrelin on BrdU incorporation by cultured fetal skin cells. Fetal (E17) skin cells were cultured for 24 h with or without ghrelin. E (*right*), Effect of [D-Lys3]-GHRP-6, a GHS-R antagonist, on the ghrelin-stimulated proliferation of fetal skin cells. Various doses of acyl or des-acyl ghrelin were added to the culture medium together with 500 pmol [D-Lys3]-GHRP-6. F, Calcium imaging analysis of fetal skin cells. The skin cells were prepared from E17 fetuses. Cell no. 21 responded to des-acyl ghrelin, but not to acyl ghrelin. In contrast, cell no. 23 responded to acyl ghrelin, but not to des-acyl ghrelin. Photographs on the *right* represent the calcium response in cells at points I, II, and III. The *red and yellow color spots* indicate high intracellular Ca^{2+} concentration. The *blue color* shows the basal condition. Des-acyl ghrelin and acyl ghrelin were added to the culture medium at the point indicated by the *arrow*. The *graph on the left* represents the transition of the relative value of Ca^{2+} concentration analyzed by an imaging scanner.

acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation. These results clearly indicate that both acyl ghrelin and des-acyl ghrelin stimulate proliferation of fetal skin cells. Acyl ghrelin induces neurogenesis in the dorsal motor nucleus (46) and stimulates bone formation (47). During pregnancy, maternal ghrelin is likely transferred to the fetal circulation, and then would prompt fetal growth through stimulation of cell proliferation. Calcium-imaging analysis revealed that two types of cells exist in cultured fetal skin cells: one responds only to des-acyl ghrelin, and the other one responds only to acyl ghrelin. These results strongly suggest that fetal skin cells have different type of receptors: one is a classical receptor for acyl ghrelin, GHS-R 1a, and the other is a novel receptor for des-acyl ghrelin that mediates intracellular calcium mobilization.

In this study, we detected high levels of des-acyl ghrelin

in the fetal circulation and amniotic fluid. These findings suggest that amniotic fluid serves, in part, as an incubation medium to provide des-acyl ghrelin to the fetus. In this way, des-acyl ghrelin may act on fetal development by direct stimulation of proliferation. If this is true, however, the lack of an effect of des-acyl ghrelin treatment on neonatal body weight at birth (Fig. 3) remains to be explained. We speculate that, late in pregnancy, high endogenous quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid saturate the GHS-R 1a subtype des-acyl ghrelin receptors, effectively preventing the exogenous des-acyl ghrelin from exerting an effect. It has been reported that ghrelin knockout mice do not exhibit any changes in development (probably including fetal development) (13). We do not know the reason for the discrepancy of neonatal body weights between mothers passive-immunized against acyl ghrelin and ghrelin knockout mice. Further studies are required to elucidate this discrepancy.

In conclusion, the present study has demonstrated that maternal ghrelin is easily transferred to the fetal circulation, and then prompts fetal growth through stimulation of cell proliferation during the late half of pregnancy. Recent reports that ghrelin directly stimulates bone formation (47) also supports this hypothesis. These findings may have implications for the clinical application of ghrelin for pregnant subjects.

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CHARACTERIZATION OF A NOVEL METALLOPROTEINASE IN DUVERNOY'S GLAND OF *RHABDOPHIS TIGRINUS TIGRINUS*

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ABSTRACT — During the characterization of hemorrhagic factor in venom of *Rhabdophis tigrinus tigrinus*, so-called Yamakagashi in Japan, one of the Colubridae family, a novel metalloproteinase with molecular weight of 38 kDa in the Duvernoy's gland of Yamakagashi was identified by gelatin zymography and by monitoring its proteolytic activity using a fluorescence peptide substrate, MOCAc-PLGLA$_2$pr(Dnp)AR-NH$_2$, which was developed for measuring the well-known matrix metalloproteinase (MMP) activity.

After purification by gel filtration HPLC and/or column switch HPLC system consisting of an affinity column, which was immobilized with a synthetic BS-10 peptide (MQKPRCGVPD) originating from propeptide domain of MMP-7 and a reversed-phase column, the N-terminal amino acid sequence of the 38 kDa metalloproteinase was identified as FNTFPGDLK which shared a high homology to *Xenopus* MMP-9.

The 38 kDa metalloproteinase required Zn^{2+} and Ca^{2+} ions for its proteolytic activity. In addition, the proteolytic activity was almost completely inhibited by BS-10, a MMP inhibitor, but not by the serine proteinase inhibitors, cysteine proteinase inhibitors and aspartic proteinase inhibitors.Together these results demonstrated that the 38 kDa proteinase is a novel snake verom metalloproteinase (SVMP) containing HExGHxxGxxH motif which possesses high affinity to the BS-10 peptide, into its molecule, and the enzymatic properties are closed to that of MMPs.

Based on the results obtained in the present study, we concluded that the 38 kDa metalloproteinase is a novel metalloproteinase whose activity may be regulated by the cysteine switch mechanism, and could be classified as one of the matrix metalloproteinases rather than snake venom metalloproteinases.

KEY WORDS: Snake venom metalloproteinase, Matrix metalloproteinase, Yamakagashi, *Rhabdophis tigrinus tigrinus*, Cysteine switch mechanism, Column switch HPLC

INTRODUCTION

Rhabdophis tigrinus tigrinus, so-called Yamakagashi in Japan, is one of the Colubridae that has been recognized as a nonpoisonous snake for a long times. In most cases, continuous bleeding from the gums, old abrasions and needle points, bright-red urine and decrease in fibrinogen contents were observed when bitten by this snake (Mittleman and Goris, 1974; Mori *et al.*, 1983). In a serious case, disseminated intravascular coagulation (DIC), acute renal failure and brain hemorrhage often occurr (Sakai *et al.*, 1990; Akimoto *et al.*,

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1991).

Morita *et al.* have reported that mammalian coagulation factor was activated by 170 kDa protein in the Duvernoy's gland as a venomous coagulant and that activators of coagulation factor appeared to be single-chain enzymes with molecular weights ranging between 50 and 90 kDa. These proteins activated prothrombin, but were different from factor X (Morita *et al.*, 1988).

It is believed that snake venom metalloproteinases (SVMPs) play an impotant role in the bleeding. To date over a hundreds SVMPs have been reported, and classified into the following four classes according to the domain structure/molecular size: P-I, composed of only metalloproteinase domain/20-30 kDa; P-II, containing metalloproteinase and disintegrin domains/30-50 kDa; P-III, consisting of metalloproteinase, disintegrin-like and Cys-rich/50-80 kDa; and P-IV, containing an additional C-type lectin sequence at the C-terminal of P-III/80-100 kDa (Hite *et al.*, 1994; Kini and Evans, 1992). All the metalloproteinases have zinc-binding sites (HExGHxxGxxH), and cysteine residue in prodomain chelates to zinc in the latent form. They are most likely stored as inactive zymogens in order to prevent autodigestion. Moreover, the active form of these metalloproteinases has a shortage of prodomain from precursor protein. This activation mechanism has been suggested to be similar to the cysteine switch mechanism as proposed for matrix metalloproteinases (MMPs) (Grams *et al.*, 1993). Although there are a large number of studies dealing with snake venoms, no report of the metalloproteinase has been found from the Duvernoy's gland of *Rabdophis tigrinus tigrinus*.

During the determination of the hemorrhagic factor, a novel metalloproteinase was found in the Duvernoy's gland of Yamakagashi. In the present study, characterization of this new MMP was undertaken.

MATERIALS AND METHODS

Materials

Gelatin and Triton X-100 were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Molecular weight marker low range for electrophoresis was obtained from BIO-Rad Laboratories (Calfornia, USA). All electrophoresis media were from nacalai tesque, inc. (Kyoto, Japan). Tris was from ICN. Biomedical inc. (OH, USA). Sodium dodecyl sulfate (SDS), calcium chloride and zinc chloride were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) was purchased from Kishida Chemicals (Osaka, Japan). 1,10-phenantholine was from nacalai tesque, inc. (Kyoto, Japan). *O,O'*-Bis(2-aminoethyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA), phosphoramidon, luepeptin, phenylmethylsulfonyl fluoride (PMSF), antipain, E-64, and pepstatin A were purchased from Calbiochem Merck KGaA (Darmstadt, Germany). All other reagents were analytical grade from commercial sources. Fluorescence substrates, 3163-v: (7-methoxycoumarin-4-yl)acetyl-Pro-Leu- Gly-Leu-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-Ala-Arg-NH_2, 3167-v: (7-methoxycoumarin-4-yl) acetyl-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH_2, and 3168-v: (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH_2 were purchased from Peptide Institute Inc. (Osaka Japan).

Venom

The venom was collected from the Duvernoy's gland of *Rhabdophis tigrinus tigrinus* at the Japan Snake Institute (Gunma, Japan). This gland was excised and minced, and the venom was extracted by water. The insoluble material was removed by centrifugation, and the supernatant was then lyophilized. The lyophilized venom was stored at –40°C before use, and dissolved in distilled water in use (Sakai *et al.*, 1983).

Preparation of recombinant MT1-MMP

Recombinant human soluble MT1-MMP was prepared according to the method described previously (Itoh *et al.*, 1996; Oku *et al.*, 2003). In brief, the cDNAs for procatalytic domains of human MT1-MMP were prepared by polymerase chain reactions using sets of primers (5' primer, GGCGGATCCATGCTCGCCTCCCTCGGCTCG, 3' primer, GCCGTCGACGTTCCCGTCACAGATGTTGGG) based on the reported sequences, and the template (poly $(A)^+$ RNA isolated from a human rectal carcinoma cell line pME18S-MTMMP having a 3.5 Kb cDNA fragment of MT1-MMP). The resulting PCR fragments were inserted into the bacterial expression plasmid pTH-72, having a tandem repeat of the T7 promoter and a hexahistidine-Tag encoding sequence. The expression, purification, and refolding of the human recombinant MMPs were performed by the following procedures: Human recombinant proMMPs were produced in *E. coli* strain BL21 (DE3) transfected with the corre-

sponding expression plasmids derived from pTH-72, solubilized in 8 M urea/10 mM Tris-HCl/100 mM Na-phosphate (pH 8.0)/100 mM β-mercaptoethanol, purified with Ni-NTA resin (QIAGEN Inc., U.S.A), and refolded reducing the urea concentration.

Microplate assay

Inhibitory effects of various inhibitors were measured by a microplate reader assay method with fluorescence substrates described previously (Oku *et al.*, 2003). Each 20 μl of inhibitor solution, assay buffer (50 mM Tris, pH 8.0, 5 mM $CaCl_2$, 200 mM NaCl, and 50 μM $ZnCl_2$, 0.1% Brij), distilled water and proteinase solution were mixed and pre-incubated in each well of 96 well microplates at 37°C for 15 min. Then 120 μl of fluorogenic substrate peptide (2.5 μM as final concentration) was added to initiate the proteolytic reaction. The fluorescence intensity transcribed resonance fluorescence unit (RFU) was measured at 320 nm for excitation and 390 nm for emission every 15 min for 2 hr by using an fmax fluorescence microplate reader (Molecular Devices, USA). The inhibitory effects were calculated based on the reaction data at 2hr. For measuring the proteolytic activities in each elution from HPLC, 20 μl of distilled water was added instead of the test sample solution. For the measurement of proteolytic activity of enzymes, 20 μl of distilled water was added instead of inhibitor solution. The concentration of enzymes (100 – 200 RFU at 2 hr) was determined by measuring the proteolytic activity of various dilution enzymes using 3163-v.

Purification of the 38 kDa metalloproteinase by gel-filtration HPLC

A gel-filtration HPLC was carried out with TSK-gel G-3000sw column (Tosoh, Tokyo, Japan; 600 × 7.5 mm i.d.) using 200 mM Tris-HCl (pH 6.7) buffer below 4°C. The flow rate was set at 0.6 ml/min, and chromatogram monitored at UV 220 nm with CD detector CD-1595 (Jasco, Japan).

Preparation of synthetic peptides

BS-10 (MQKPRCGVPD) was synthesized by the Fmoc method with 433A peptide synthesizer (Applied Biosystems, CA., U.S.A.). After deprotection according to the manufacturer's protocol, peptide was purified by a reversed-phase HPLC column (Shiseido co., Ltd.,Tokyo, Japan; Capcell Pak C18, 250 × 10 or 15 mm i.d.) with a linear gradient elution system from 0.1% TFA to 50% CH_3CN containing 0.1% TFA for 30 min. The peptide purity was confirmed by an analytical HPLC system via reversed-phase HPLC using a Capcell Pak C18 (Shiseido co., Ltd.,Tokyo, Japan; Capcell Pak C18, 250 × 4.6 mm i.d.) with a linear gradient elution system from 0.1% TFA to 70% CH_3CN containing 0.1% TFA for 30 min monitoring with a photodiode array detector.

Column switch HPLC system

The column switching HPLC consisted of an affinity column (BS-10 column) for the first separation and a reversed-phase column for the second separation (Fig. 1). For the first separation, an affinity HPLC column was prepared according to the following steps: TSK-GEL Tresyl-5PW packed with $CH_2OSO_2CH_2CF_3$ group conjugating resin (Tosoh; 40 × 6.0 mm i.d.) was washed with water for 10 min, and then equipped with 0.5 M phosphate buffer (pH 8.0) for another 10 min. For immobilization of BS-10, 10 ml of BS-10 solution (1.87 g/ml) in 0.5 M phosphate buffer (pH 8.0) was passed into the column and recycled for 24 hr, and then non-immobilization resin was blocked with 0.2 M Tris-HCL (pH 8.0) for 1 hr. During all steps, the flow rate was set at 0.5 ml/min. The amount of immobilized BS-10 was 8.98 μmole.

In the first separation, the flow rate was set at 1.0 ml/min, and the elution was monitored by UV absorption at 280 nm (Detector A). The BS-10 column (Affinity column) was equipped with 10 mM Tris-HCl (pH 8.0) containing 400 μM $ZnCl_2$ (Binding buffer) for 15 min before injection. Various test samples were loaded into the BS-10 column, and then the column was washed with the binding buffer for 8 min. In this step, the peptide or protein was bound to BS-10 column (Fig. 1b, left). Then the elution solvent changed to 10 mM Tris-HCl (pH 8.0) containing 3 mM EDTA instead of $ZnCl_2$ (Elution buffer) using solvent selection unit by manual mode. At the same time, the column selection unit was switched by manual manner, and the elution from BS-10 column was loaded into a second column with on-line. During another 10 min, the elution of the first column was loaded to the second column (Fig. 1b, center). The second separation was carried out using a reversed-phase HPLC column (Kanto Chemical, Tokyo, Japan; Migthysil RP-18, 75 × 4.6 mm i.d.). After that, the elution of the second column was changed to 0.1% TFA, and kept for another 12 min (Fig. 1b right). In this step, the elution buffer was washed out from the second column. A linear gradient system from 0.1% TFA to 50 % CH_3CN containing 0.1% TFA for 30 min was then started 12 min after switching the column. The flow rate of the second col-

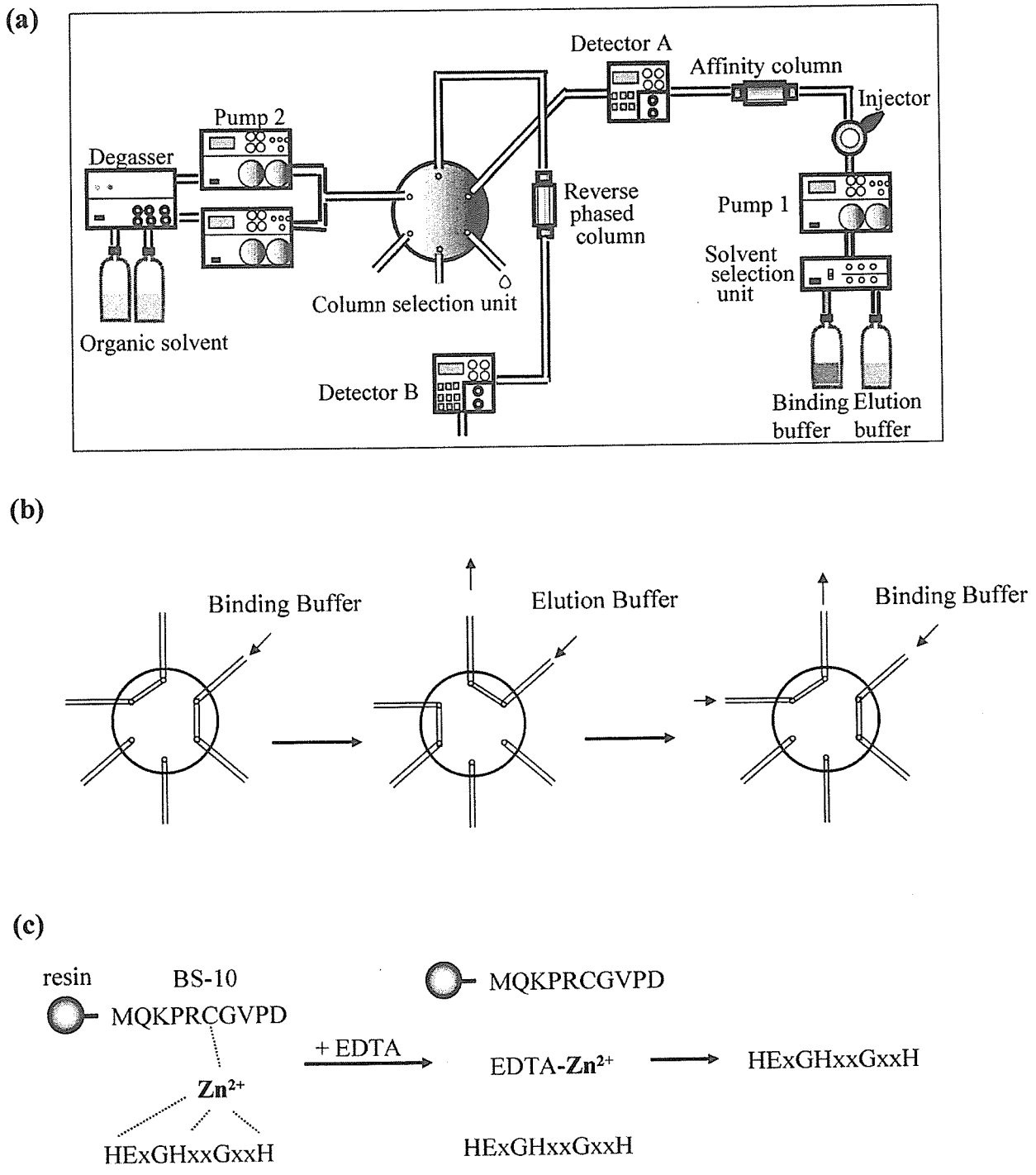

Fig. 1. (a) Apparatus of column switch HPLC system, (b) Connection of column selection unit, (c) Principle of affinity column for purification of 38 kDa metalloproteinase.

umn was set at 1.0 ml/min. The chromatogram was monitored at UV absorption at 220 nm (Detector B).

Gel electrophoresis and Zymography

SDS-polyacrylamide gel electrophoresis (PAGE) was done in a Tris-glycine buffer system as described using a 3% stacking gel and 12.5% separation gel. Zymography was performed as previously described (Miyazaki *et al.*, 1990; Chin *et al.*, 1985). Briefly, samples were mixed with SDS sample buffer in the absence of a reducing agent, incubated for 20 min at 37°C and separated on a 12.5% polyacrylamide gel containing 1 mg/ml of casein or gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 h then digested by incubating the gel in 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, 5 mM $CaCl_2$ and 50 μM $ZnCl_2$ at 37°C for overnight. The gels were stained with 0.1% Coomassie Brilliant Blue G250, and the location of proteolytic activity was detected as clear bands in a background of uniform staining.

Analysis by reversed-phase column

Crude venom (500 μg) was loaded into a reversed-phase column (Waters; Puresil C18 150 × 4.6 mm i.d.) monitoring with photodiode array detector. The flow rate was set at 1.0 ml/min. Each 200 μl of elution was collected into a 96 well microplate twice under linear gradient elution for 30 min from H_2O to 70% CH_3CN containing 0.1% TFA. A 20 μl of each fraction was used for the measurement of the proteolytic activity by microplate reader assay.

N-terminal sequence analysis

For the N-terminal amino acid sequence, 50 μl of crude venom (10 mg/ml) was loaded into a reversed phase column (see **Analysis by reversed-phase column**) twice, and the active fractions were collected into micro tube. The combined fraction obtained was concentrated to approximately 30 μl by Speed vac concentrator (Savant, U.S.A). A 13 μl of concentrated sample was treated with the same volume of SDS, and then 20 μl was applied to SDS-PAGE (see **Gel electrophoresis and Zymography**). After transfer, PVDF membrane was stained by 0.1% Coomassie Brilliant Blue G250. The band of the 38 kDa metalloproteinase was cut off and destained twice by small volume of methanol. The amino-terminal of the purified 38 kDa metalloproteinase on PVDF membrane was determined by Edman degradation with a Procise-cLC sequencer (PE Biosystems, Branchburg, N.J.) operated according to the manufacturer's protocol.

RESULT

Purification of the 38 kDa metalloproteinase

In preliminary experiments, we found the crude venom of *Rhabdophis tigrinus tigrinus* contained many proteins with molecular weights of 30 to 90 kDa. Among them, only 38 kDa protein possessed proteolytic activity in gelatin and casein zymography. In addition, the proteolytic activity was inhibited by EDTA but not PMSF, a serine protease inhibitor, suggesting that a 38 kDa protein may be a metalloproteinase such as SVMP or MMP.

To characterize the 38 kDa protein, the crude venom from Yamakagashi was purified by column switch HPLC system (Fig. 1). The first separation in this system was perfomed based on cysteine switch mechanism using a BS-10 peptide affinity column. BS-10 peptide (MQKPRCGVPD) consisting of cysteine switch with essential zinc atom was chemically synthesized. The molecule containing the zinc-binding motif (HExGHxxGxxH) was retained by the column and eluted by EDTA solution. Our preliminary results indicate that the BS-10 column possesses high affinity to the catalytic domain of MMP or SVMP.

The partially purified enzyme from the snake venom was prepared by gel filtration HPLC (Fig. 2a). As shown in Fig. 2c, fraction A contained several proteins. However, only a single protein band with molecular weight of 38 kDa was identified by gelatin zymography (Fig. 2c). The enzyme in fraction A was further purified by a column switch HPLC. The proteolytic activity was identified in the fraction eluted at 29 min by using 3163-v as a substrate (Fig. 2b). The active fractions at 29 min were combined as fraction α. SDS-PAGE analysis of fraction α revealed that the 38 kDa protein was purified as a single band by a column switch HPLC (Fig. 2d). These result suggest that the 38 kDa metalloproteinase binds to BS-10 through a cysteine switch mechanism and possesse a homolugious amino acid sequence to MMPs and SVMPs

Analysis of N-terminal amino acid sequence

To analyze the N-terminal amino acid sequence of the 38 kDa protein, crude venom was purified by a reversed-phase column chromatography twice. The active fractions were separated by SDS-PAGE, and then transferred onto PVDF membrane. The 38 kDa protein was cut off and analyzed by automatic Edoman sequencer. N-terminal amino acid sequence of the 38 kDa protein was determined as FNTFPGDLK. This sequence possessed a high homology to the active site

N-terminal of *Xenopus* MMP-9 (Table 1). These results suggested that the 38 kDa might be a novel SVMP.

Enzymatic property of the 38 kDa metalloproteinase

Since the N-terminal amino acid of the 38 kDa metalloproteinase shared a high homogogy to *Xenopus* MMP-9, we next examined the substrate specificity of

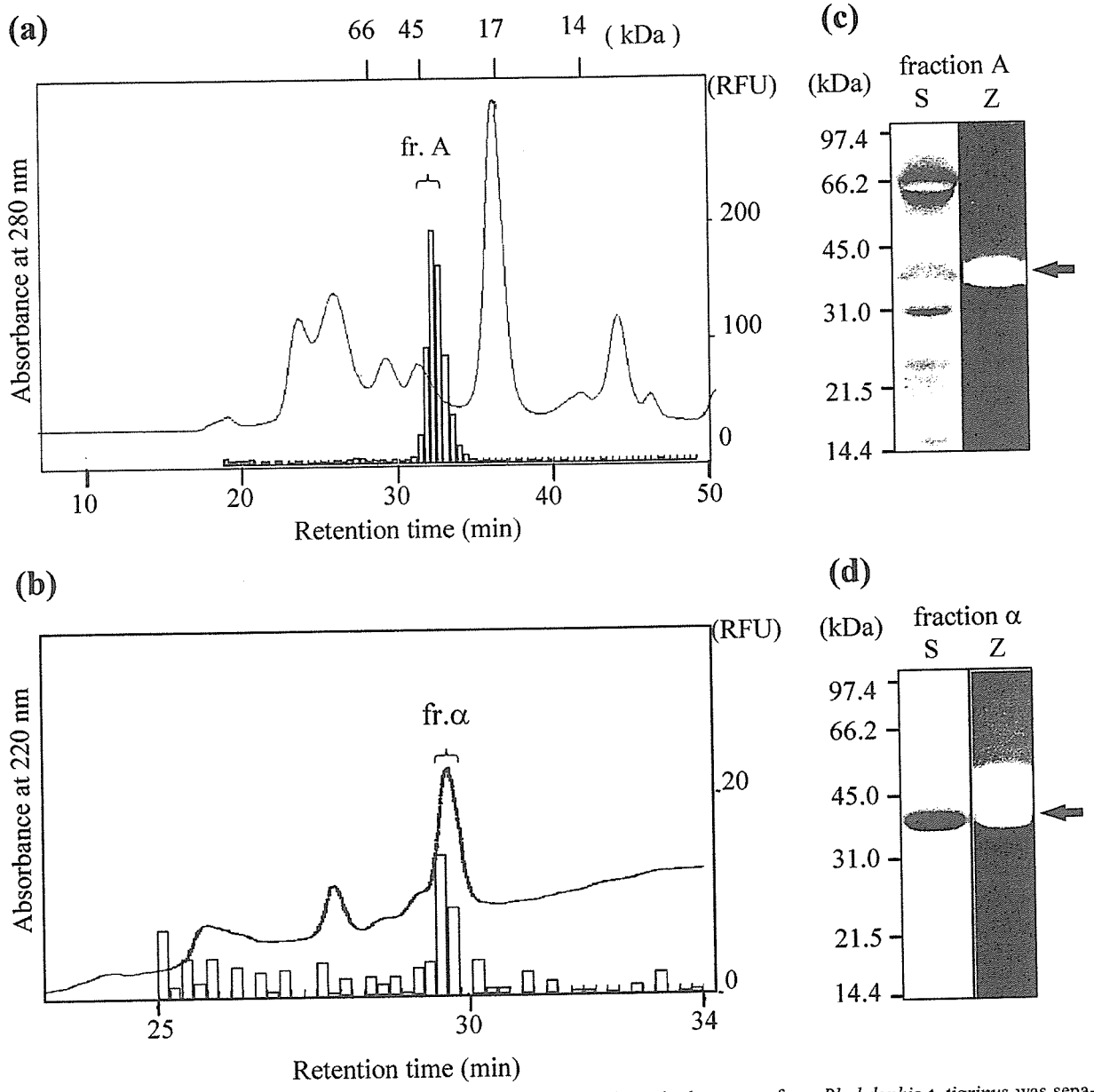

Fig. 2. Separation of 38 kDa metalloproteinase. The 38 kDa metalloproteinase in the venom from *Rhabdophis t. tigrinus* was separated by gel-filtration HPLC (a) and the active three fractions combined as "Fraction A". The 38 kDa metalloproteinase in Fraction A was next separated by column switch HPLC (b) and the active two fractions combined as "Fraction α". The proteolytic activity □ () was monitored by using 3163-v a fluorescence substrate. The 38 kDa metalloproteinase in Fraction A (c) and Fraction α (d) was then analyzed by SDS-PAGE with silver staining (lane S) and gelatin zymography (lane Z).

the 38 kDa metalloproteinase in crude venom, and compared it to those of r-MT1-MMP. As shown in Fig. 3, the enzyme in crude venom cleaved 3163-v strongly, 3167-v at medium and 3168-v very weakly. This proteolytic pattern was the same as that of r-MT1-MMP, the well-known metalloproteinase. r-MT1-MMP specifically cleaved the 3163-v between Gly and Leu residues with high sensitivity. In contrast, the MMP enzyme in crude venom of *Agkistrodon contortrix contortrix* showed a proteolytic activity restricted to 3168-v (data not shown).

The pH dependency of the 38 kDa metalloproteinase using 3163-v as a substrate was shown in Fig. 4. The 38 kDa enzyme in both crude venom and fraction A showed a maximum activity at pH ranging 7.0 ~ 7.5. The pH dependency is very similar to that of r-MT1-MMP (Fig. 4). The results of substrate specificity and pH dependency noted for this 38 kDa enzyme has amazing similarity to MT1-MMP.

Next, the requirement of essential cation ions for proteolytic activities of the metalloproteinase was examined. The patterns of cation ion requirement were very similar to each other. In the case of *Rhabdophis. t. tigrinus* and r-MT1-MMP, Ca^{2+} and Zn^{2+} ions were essential for expressing the proteolytic activity. The proteolytic activities of the enzyme in both crude venom, fraction A and r-MT1-MMP were decreased to 79, 80 and 43%, respectively, in the absence of Zn^{2+} ion. In addition, the proteolytic activities were almost lost in the absence of Ca^{2+} ion. These results revealed that the Ca^{2+} ion is required for the metalloproteinase to express full proteolytic activity (Fig. 5).

Effects of various inhibitors on the 38 kDa metalloproteinase

The effects of various protease inhibitors on the proteolytic activity of the 38 kDa metalloproteinase in both crude venom and fraction A were further examined (Table 2). The inhibitory effects on the crude venom were very similar to fraction A. The proteolytic activities in fraction A were inhibited by a series of metalloproteinase inhibitors such as EDTA, EGTA and 1,10-phenantholine, but not inhibited by PMSF, phosphoramidon and luepeptin, a series of serine protease inhibitors. Cysteine protease inhibitors such as antipain, E-64, and aspratic protease inhibitor pepstatin A showed a weak inhibitory effect on the enzyme. BS-10 had a high inhibitory effect on the metalloproteinase in fraction A as well as r-MT1-MMP. Interestingly, the responsibility of the inhibitory effects on the 38 kDa metalloproteinase in fraction A to various inhibitors was almost the same as that of r-MT1-MMP.

These results suggested that the properties and structure of the 38 kDa metalloproteinase are more similar to the MMP family than SVMP.

DISCUSSION

In the present study, we demonstrated for the first time that a novel metalloproteinase with molecular

Table 1. Comparison of N-terminal of catalytic domain and active site of 38 kDa metalloproteinase and various MMPs.

	N-ter of catalytic domain	Active site
38 kDa proteinase	FNTFPGDLK*	HExGHxxGxxHx
Human MMP-7	SLFPNSPK	HELGHSLGMGHS
Human MT1-MMP	YAIQGLK	HELGHALGLEHS
Chicken MMP-9	FLTFEGDLK	HEFGHSLGLDHS
Xenopus MMP-9	FNTFEGDLK	HEFGHALGLDHS
Fibrolase (P-I)	EQRFPQRYV	HEIGHNLGMNHD
Bilitoxin-1 (P-II)	ERYNPYKYI	HEIGHNLGMGHD
Jararhagin (P-III)	EQQRYDPYK	HEMGHNLGIHHD
RVVh (P-IV)	LVSTSAQFN	HELSHNLGMYHD
Mouse ADAM-17	RADPDPMKN	HELGHNFGAEHD

Human MMP-7 and MT1-MMP; Massova *et al.*, 1998,
Chicken MMP-9; Hahn-Dantona *et al.*, 2000, *Xenopus* MMP-9; Carinato *et al.*, 2000,
Fibrolase; Randolph *et al.*, 1992, Bilitoxin-1; Nikai *et al.*, 2000, Jararhagin; Paine *et al.*, 1992,
RVVh; Takeya *et al.*, 1992, Mouse ADAM-17; Black *et al.*, 1997.
* N-terminal of 38 kDa proteinase.

mass of 38 kDa exist in Duvernoy's gland of *Rhabdophis tigrinus tigrinus*. The snakes belonging to the Colubridae are classified as non-poisonous snakes such as *Cyclophiops herminae, Elaphe climacophora, Elaphe quadrivirgata* and *Amphiesma vibakari*, and poisonous snakes such as *Rhabdophis t. tigrinus, Molpolon monspessulanus, Spalerosophis diadema clifordi* and *Thamnophis sirtalis parietaris*, whose venoms have LD_{50} of 0.265 (i.v.), 6.5 (i.v.), 2.75 (i.v.) and 33.3 (i.p) mg/kg to mice, respectively (Sakai *et al.*, 1983; Rosenberg *et al.*, 1985). Although the lethal activity of *Rhabdophis t. tigrinus* is the most potent among poisonous Colubridae snakes, there are few reports of a fatal case by biting with this snake. However, once the venom was injected into an animal body, serious symptons were caused (Sakai *et al.*, 1984). Thus the antivenom was prepared for cure in Japan.

The hemorrhagic and nonhemorrhagic metalloproteinases in the snake venom have been reviewed by Takeya *et al.* (1993). Hemorrhage is a common mani-

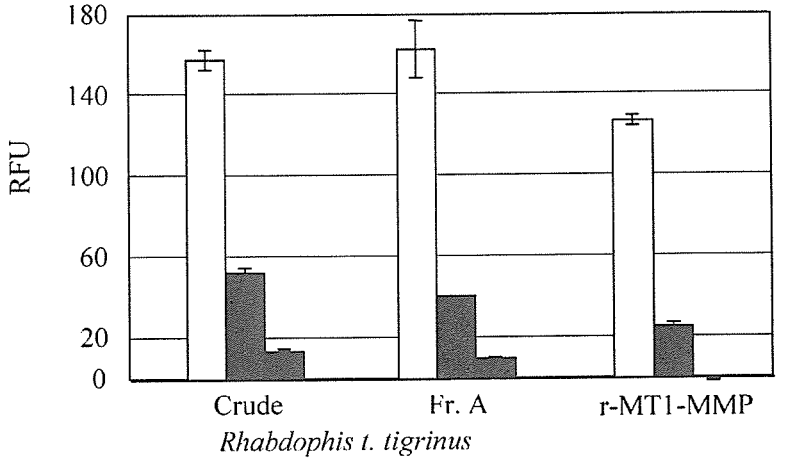

Fig. 3. Substrate specificity. The fluorescence substrates 3163-v □ (), 3167-■ () and 3168-v (■), were used to determine the proteolytic activity of 38 kDa metalloproteinase from *Rhabdophis t. tigrinus*, r-MT1-MMP.

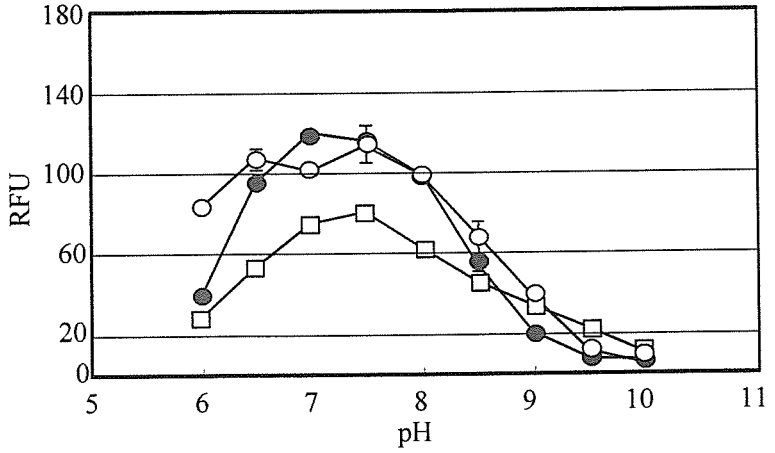

Fig. 4. pH dependency of r-MT1-MMP (□) and 38 kDa metalloprteinase in crude venom (●) and partially purified fraction A of *Rhabdophis t. tigrinus*. (○).

festation in the victim following the bite of crotalid and viperid snakes (Ohsaka, 1979). Various components, such as hemorrhagic factors and metalloproteinases (Tu, 1991), in the snake venoms would cause localized hemorrhage by direct actions on the blood vessel walls. In our preliminary experiments, when crude venom was intravenously injected, mice were dead with disseminated intravascular coagulation syndrome and hemorrhage in the tissues having rich capillary blood vessels such as the lungs and brain. This lethal effect was thought to be due to the hemorrhagic factor (Ogawa and Sawai, 1986).

The 38 kDa metalloproteinase in the Yamakagashi's venom could be separated from the components showing lethal activity and prothrombin activating activity which were eluted in the high molecular fraction (Morita *et al.*, 1988). These results suggest that the 38 kDa metalloproteinase is a nonhemorrhagic metalloproteinase and may act as a cofactor which can enhance lethal activity. In our screening test, the crude venom from 36 snakes belonging to *Trimeresurus, Agkistrodon, Crotalus* and *Naja* had high proteolytic activity to cleave 3168-v. However, the crude venom of *Rhabdophis. t. tigrinus* showed high specificity to cleave 3163-v, and the substrate specificity is very similar to the well-known MMPs (Fig. 3). Most of the MMPs are initially expressed as inactive zymogens, and activate subsequently by removing the N-terminal

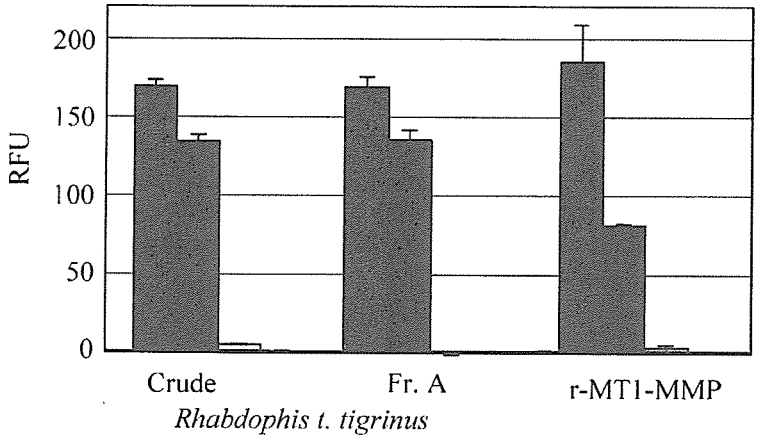

Fig. 5. Metal requirement. The proteolytic activity of 38 kDa metalloproteinase was determined in the presence of Zn^{2+} and Ca^{2+} (▩), Ca^{2+} (■), Zn^{2+} (□), and in absence of both Zn^{2+} and Ca^{2+} (□).

Table 2. Effect of various inhibitors on 38 kDa metalloproteinase.

Inhibitors	Concentration (mM)	Inhibition (%)		
		R. t. tigrinus		r-MT1-MMP
		Fr. A	Crude	
EDTA	5.0	100	100	98.6
EGTA	5.0	97.3	90.1	97.0
1,10-phenanthloline	0.5	98.5	100	100
PMSF	0.5	< 5.0	< 5.0	9.3
Phosphoramidon	0.5	7.7	6.5	5.4
Leupeptin	0.5	5.8	7.1	< 5.0
Antipain	0.5	13.0	19.7	21.9
E-64	0.5	8.5	29.6	13.3
Pepstatin A	0.5	39.4	31.8	28.1
BS-10	0.5	98.8	95.1	91.8

prodomain, which has a cysteine residue masking the zinc-binding site in catalytic domain (Van wart and Birkedal-Hansen, 1990). It is well known that zinc metalloproteinases such as MMPs and SVMPs possessing HExGHxxGxxHS motif and HExGHxxGxxHD motif, respectively, in their molecule (Hooper, 1994), are able to bind to BS-10 through a cysteine switch mechanism (Fig. 1). Indeed, the BS-10 column possessed a high affinity to HExGHxxGxxH motif through the cysteine switch mechanism with essential zinc ion and was available for purifying zinc metalloproteinases. As expected, the 38 kDa metalloproteinase could be purified by the column switch HPLC system using the BS-10 conjugating affinity column with higher recovery (Fig. 2). These results demonstrate that the 38 kDa metalloproteinase possesses HExGHxxGxxH sequence in its molecule which has been shown to be conserved in many reprolysins and MMPs.

The N-terminal amino acid sequence of the 38 kDa metalloproteinase shared a high homology to that of *Xenopus* MMP-9 in comparison with those of the human MMP, ADAM and SVMP (Table 1). The predicted molecular mass of MMP-9 identified from humans (Murphy and Cockett, 1995), chickens (Hahn-Dantona *et al.*, 2000) and *Xenopus* (Carinato *et al.*, 2000) was 92 kDa, 75 kDa and 75 kDa, respectively, and has been shown to consist of signal peptide domain, propeptide domain, catalytic domain, fibronectin type-II -like domain, hinge and hemopexin-like domain in its molecule. Judging from the molecular size, the 38 kDa metalloproteinase seemed to consist of catalytic domain only, and might be produced from latent pro-form and activated by a similar machanism as a proposition for many MMPs. The hemopexin-like domain of MMPs is known to play an important role in the recognition of substrate in tissue. If the 38 kDa metalloproteinase resulted from its precursor protein consisting of propeptide domain, catalytic domain, hinge and hemopexin-like domain similar to the structure of MMP-9 after removing the hemopexin-like domain by autodigestion during the preparation, a corresponding protein with larger molecular mass than 38 kDa should be detected in the fresh Yamakagashi venom. However, no protein with larger or smaller molecular mass than 38 kDa was detected to express proteolytic activity in the fresh Yamakagashi venom. In addition to this observation, the properties of the purified 38 kDa metalloproteinase were almost the same as that in crude venom of *Rhabdophis. t. tigrinus*, suggesting that the 38 kDa metallproteinase exists from the first.

The biochemical characteristics of the 38 kDa metalloproteinase obtained by gelatin zymography, pH dependency, metal requirements and responsibility to EDTA and EGTA were strongly similar to those of well-known MMPs. In addition, the substrate specificity and responsibility to various proteinase inhibitors of this 38 kDa metalloproteinase are also similar to the well-known MMPs

Based on the results obtained in the present study, we concluded that the 38 kDa metalloproteinase was a novel metalloproteinase whose proteolytic activity may be regulated by a cysteine switch mechanism and could be classified as one of the matrix metalloproteinases rather than snake venom metalloproteinases.

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Isolation and identification of proangiotensin-12, a possible component of the renin–angiotensin system

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Abstract

The renin–angiotensin (RA) system plays an important role in regulating blood pressure and fluid balance. In the search for bioactive peptides with an antibody binding to the N-terminal portion of angiotensin II (Ang II), we isolated a new angiotensinogen-derived peptide from the rat small intestine. Consisting of 12 amino acids, this peptide was termed proangiotensin-12 based on its possible role of an Ang II precursor. Proangiotensin-12 constricted aortic strips and, when infused intravenously, raised blood pressure in rats, while both the vasoconstrictor and pressor response to proangiotensin-12 were abolished by captopril and by CV-11974, an Ang II type I receptor blocker. Proangiotensin-12 is abundant in a wide range of organs and tissues including the small intestine, spleen, kidneys, and liver of rats. The identification of proangiotensin-12 suggests a processing cascade of the RA system, different from the cleavage of angiotensinogen to Ang I by renin.

Keywords: Proangiotensin-12; Renin–angiotensin system; Pressor peptide; Angiotensinogen; Processing

A number of mechanical or humoral mechanisms are involved in blood pressure and body fluid homeostasis in mammals. Among them, the renin–angiotensin (RA) system plays a pivotal role, regulating the vascular tone of resistant vessels and glomerular filtration or re-absorption of electrolytes in the kidneys. The protease enzyme renin secreted from kidneys cleaves specifically angiotensinogen circulating in the blood to produce angiotensin I (Ang I), a peptide consisting of 10 amino acid residues, which in the presence of angiotensin-converting enzyme (ACE), is in turn converted to Ang II, a potent pressor peptide mediating the major actions of the RA system as a circulating hormone [1,2]. On the other hand, recent research has revealed new aspects of the RA system. For example, the tissue RA system has been vigorously studied, and its activation is assumed to be regulated independently of the systemic RA system [3–5]. Another example is the identification of Ang II-derived peptides that are shorter than Ang II, such as Ang(1–7), Ang III, or Ang IV, having pharmacological properties different from those of Ang II [6–11]. Meanwhile, consisting of 10 amino acid residues, Ang I has been thought to be produced by renin directly from angiotensinogen, a protein of 452 amino acids for humans or 453 for rats [12,13], but there has been no report on the occurrence of angiotensinogen-derived peptides of amino acid sequences longer than Ang I. In the present study, on searching for peptides structurally related to Ang II, we purified an angiotensinogen-derived pressor peptide of 12 amino acids, which is thought to be one of the major components of the RA system.

Materials and methods

Reagents and animals. Ang I and Ang II were purchased from Peptide Institute Inc. (Osaka, Japan) and proangiotensin-12 was synthesized by Bex. Co., Ltd. (Tokyo, Japan). CV-11974 was kindly provided by Takeda

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Pharmaceutical Company Limited (Osaka, Japan). Wistar rats of 6–7 weeks of age were purchased from Charles River Laboratories (Kanagawa, Japan) and New Zealand white rabbits from Kyudo Co., Ltd. (Saga, Japan). The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2004-100-2, 2004-101-2).

Detection of immunoreactive N-terminal Ang II. To prepare antiserum against the N-terminal of Ang II, synthesized Ang II-Cys was conjugated with *keyhole limpet* hemocyanin at room temperature over 2 h. After dialyses repeated four times in 50 mmol/L PBS, the conjugate solution was emulsified with an equal volume of Titer Max Gold (Sigma–Aldrich, Tokyo, Japan) and injected subcutaneously into New Zealand white rabbits every two weeks over three months. A specific radioimmunoassay (RIA) was prepared with the antiserum obtained from an immunized rabbit, as previously described [14,15]. This RIA cross-reacted with Ang I and Ang III at levels of 50% and 12.5%, respectively, without cross-reactions with Ang IV and Ang(1–7). To characterize the immunoreactive N-terminal Ang II in tissues, 1.0 g of sample from various rat tissues was immediately boiled for 10 min and acidified with acetic acid to a final concentration of 1.0 mol/L to inactivate proteases. Then the samples were homogenized and centrifuged for 20 min at 12,000 rpm, and the supernatant was applied to Sep-Pak C18 cartridges. After elution with 60% CH_3CN in 0.1% trifluoroacetic acid, the peptide extracts were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) with an ODS-120A column, and the immunoreactive N-terminal Ang II of each fraction was measured by the RIA.

Purification procedure. The peptide extract was obtained with the above-described method from 380 g of small intestine, a rat tissue showing the largest peak of immunoreactive N-terminal Ang II at a position clearly different from that of Ang I or Ang II in the RP-HPLC analysis. The sample was applied to a SP Sephadex C-25 column eluted with 2.0 mol/L pyridine, and subjected to gel filtration with Sephadex G-50 and an affinity column (Affi-Gel 10 Active Ester Agarose, Bio-Rad) which had been prepared with the anti-N-terminal Ang II antiserum. These purification steps were done while monitoring immunoreactive N-terminal Ang II with the RIA. The extract was further purified by three steps of HPLC with columns of ODS-120A, diphenyl, and Chemco sorb3-ODS-H. The amino acid sequence of the finally purified peptide was analyzed by a time-of-flight mass spectrometric method.

Mass spectrometry. To determine the amino acid sequence and molecular weight of the purified peptide, a tandem mass spectrometric analysis was performed with positive electrospray ionization using a QTof-2 quadrupole time of flight mass spectrometer (Micromass, UK). The sample was dissolved in a solution of water/methanol/acetic acid mixed at 49:49:2 by volume and nanosprayed from off-line MS emitters (Proxeon, Denmark) with a capillary voltage of 1.5 kV and a cone voltage of 30 V. The mass spectrum was acquired from m/z 200 to 2000. For tandem mass spectrometry, the triply charged ion with m/z 524.97 was subjected to collision-induced dissociation with argon gas in the 30 eV collision energy range. Data acquisition and processing were performed using MassLynx v4.0 (Micromass). The resultant tandem mass spectrum was subjected to a Mascot MS/MS ion search (Matrixscience, UK) and also interpreted by PepSeq (Micromass).

Radioimmunoassay for the C-terminal portion of proangiotensin-12. To specifically detect proangiotensin-12 in tissues and plasma, we developed a RIA, as previously described, with antiserum raised against the C-terminal portion of the peptide [14,15]. Synthetic proangiotensin-12 was conjugated with bovine thyroglobulin at room temperature for 15 min by the glutaraldehyde method. New Zealand white rabbits were immunized with the dialyzed conjugate, according to the procedure described above. After the immunization, specific antibody was purified from the antiserum using an affinity column (NHS-activated Sepharose 4 Fast Flow) with the C-terminal peptide of proangiotensin-12, Ile-His-Pro-Phe-His-Leu-Leu-Tyr. The proangiotensin-12 levels in tissues and plasma of male Wistar rats were determined by RIA with the purified antibody, following extraction with a Sep-Pak C18 cartridge, as described previously [14,15]. This RIA detected 1.6 and 3.1% of angiotensinogen(1–14) and angiotensinogen(1–17), respectively, but showed no cross-reactivity with Ang I, Ang II, Ang III, Ang IV, or Ang(1–7). The Ang I or Ang II levels in tissues and plasma were similarly determined by RIAs with anti-C-terminal of Ang I and Ang II antisera purchased from Miles and Cortex Biochem, Inc. (San Leandro, USA), respectively [16,17]. To characterize the immunoreactive C-terminal proangiotensin-12 in the extract of rat small intestines, a RP-HPLC analysis was done with an ODS-120A column as described above.

Pharmacological studies ex vivo and in vivo. We examined the effects of proangiotensin-12 on vascular tone with perfused aorta isolated from rats as reported previously [18]. The aortic rings were mounted under a passive tension of 1.0 g in organ baths containing Krebs–Henseleit solution oxidized with 95% O_2 and 5% CO_2 at 37 °C, and left for 60 min for equilibration. The viability of the aortic rings was confirmed by exposing the samples to 60 mmol/L KCl, 10^{-7} mol/L phenylephrine, and 10^{-6} mol/L acetylcholine. Contractions of the aortic samples were recorded before and after Ang I, Ang II, or proangiotensin-12 was added to the perfusion solution at the indicated concentration. The responses to these peptides were also tested in the presence of 10^{-7} mol/L captopril, an ACE inhibitor, or 10^{-8} mol/L CV-11974, an Ang II type 1 (AT1) receptor antagonist. Next, the effects of proangiotensin-12 on blood pressure were examined *in vivo* with rats fed a normal rat chow, as previously described [19]. Male Wistar rats weighing 220–260 g were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Synthetic proangiotensin-12 dissolved in 100 μl saline was injected into the jugular vein at the bolus-dose indicated, and blood pressure and heart rate were monitored before and after the injection by a carotid artery catheter connected to a pressure transducer with the Power Lab system (MLT0699 Disposable BP Transducer, AD Instruments, Australia). The responses to proangiotensin-12 were similarly tested 2 min after the intravenous injection of 0.03 mg/kg captopril or 0.3 mg/kg CV-11974.

Statistical analysis. Comparisons of all data were made with an analysis of variance (ANOVA) followed by Scheffe's test. Values are presented as means ± SE and statistical significance was set at $P < 0.05$.

Results

Purification of proangiotensin-12

As a first step in the isolation of Ang II-related peptides, we analyzed immunoreactive N-terminal Ang II in peptide samples extracted from various tissues of rats with RP-HPLC and a RIA. In those analyses, we found three clear peaks of immunoreactive N-terminal Ang II in rat tissues including the small intestine (Fig. 1): the earliest peak and the next peak corresponded to Ang II and Ang I, respectively. Interestingly, the largest peak emerged later

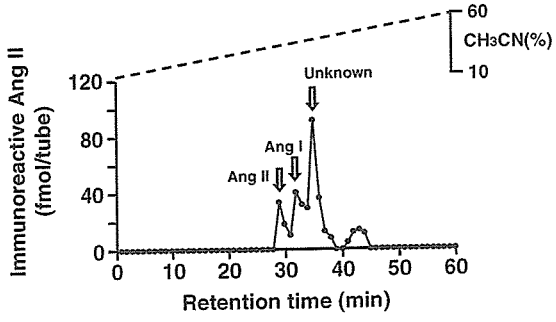

Fig. 1. Immunoreactive N-terminal Ang II in rat small intestine. An extract of the small intestine was subjected to RP-HPLC, where the immunoreactive N-terminal Ang II of each fraction was determined by specific radioimmunoassay, as described. An elution gradient of 10% to 60% CH_3CN was made in 0.1% trifluoroacetic acid over 60 min with a flow rate at 1 ml/min.

than Ang I in the small intestine, suggesting the presence of an unknown peptide similar in sequence to the N-terminal portion of Ang II. Therefore, we tried to purify this unknown peptide from 380 g of rat small intestine by several steps of ion-exchange, gel-filtration, and affinity chromatography, and RP-HPLC, as described in the Materials and methods section. Fig. 2A shows the final step of purification with RP-HPLC, where the unknown peptide was obtained as a single peak. The purified peptide sample was then subjected to the tandem mass spectrometry and found to consist of 12 amino acid residues (Fig. 2B). In comparison of the sequences of Ang I and the N-terminal portion of angiotensinogen, the unknown peptide was deduced to be a C-terminal extended form of Ang I (Fig. 3). We have termed this novel peptide proangiotensin-12, based upon the results of the tissue distribution and pharmacological experiments of the present study, suggesting a role for this peptide as a precursor of Ang II.

Measurement of tissue and plasma levels of proangiotensin-12

Next, we developed a radioimmunoassay specifically detecting the C-terminal portion of proangiotensin-12 to clarify the presence of this novel peptide in rat tissues including the small intestine. As shown in Table 1, proangiotensin-12 was abundantly detected in a variety of tissues, where its levels were higher than those of Ang I or Ang II, except for the lungs, adrenal glands, pancreas, and aorta. These tissue levels determined by the RIA specific to the C-terminal were found to be mostly identical to those estimated by an immunoreactive peak corresponding to proangiotensin-12 (Fig. 1) by RP-HPLC analyses with RIA detecting the N-terminal Ang II. In contrast to the tissue levels, the plasma concentration of proangiotensin-12 was lower than that of Ang I or

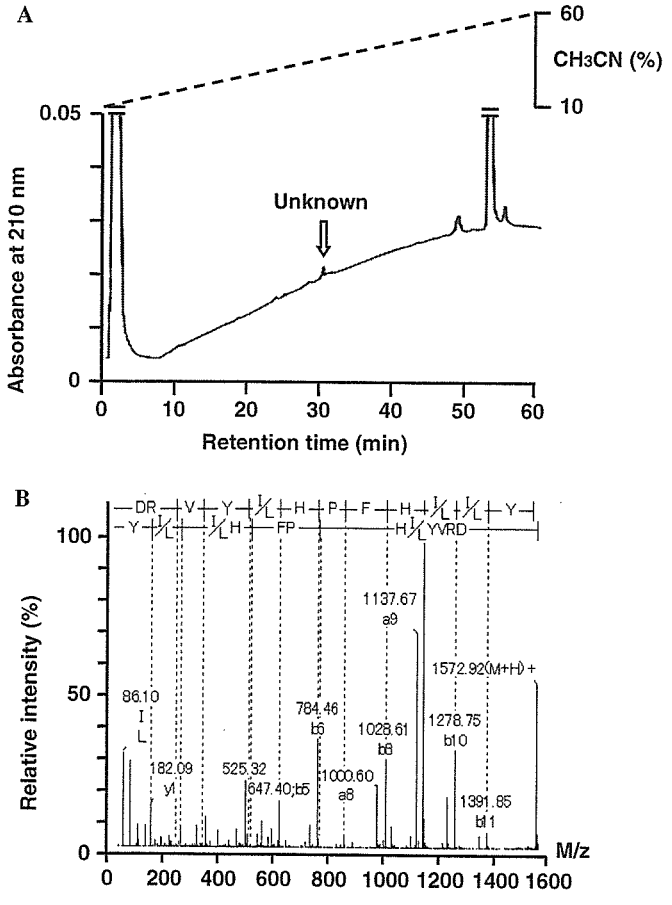

Fig. 2. Final purification by RP-HPLC (A) and tandem mass spectrometric analysis of the purified peptide (B).

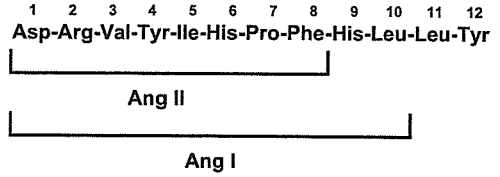

Fig. 3. Amino acid sequence of proangiotensin-12.

Table 1
Specific measurement of immunoreactive proangiotensin-12, Ang I, and Ang II levels in rat tissues and plasma

	Proangiotensin-12	Ang I	Ang II
Small intestine	663.1 ± 123.3	283.6 ± 43.0	254.9 ± 48.3
Spleen	419.1 ± 18.4	71.1 ± 4.0	302.0 ± 8.7
Kidneys	380.3 ± 27.0	365.2 ± 29.7	116.4 ± 6.9
Liver	252.0 ± 13.8	79.6 ± 2.6	87.9 ± 4.6
Stomach	241.7 ± 15.5	148.2 ± 23.0	81.5 ± 6.8
Lungs	227.8 ± 20.1	56.7 ± 5.7	236.2 ± 31.7
Adrenal glands	223.8 ± 8.2	121.4 ± 27.7	3049.2 ± 779.5
Heart	150.6 ± 11.3	84.9 ± 7.8	42.3 ± 7.3
Brain	147.4 ± 8.1	52.0 ± 2.7	29.6 ± 3.6
Pancreas	87.2 ± 13.8	37.8 ± 11.6	93.0 ± 17.6
Aorta	24.1 ± 7.5	39.6 ± 2.4	118.5 ± 6.6
Plasma	10.1 ± 2.7	382.5 ± 79.9	28.9 ± 8.8

The tissue or plasma levels of proangiotensin-12 were determined by a radioimmunoassay specifically detecting the C-terminal portion of the peptide as described in the Materials and methods section. The tissues are listed in order of immunoreactive proangiotensin-12 levels. The results are shown as means ± SE for eight rats examined (fmol/g tissue or fmol/ml plasma).

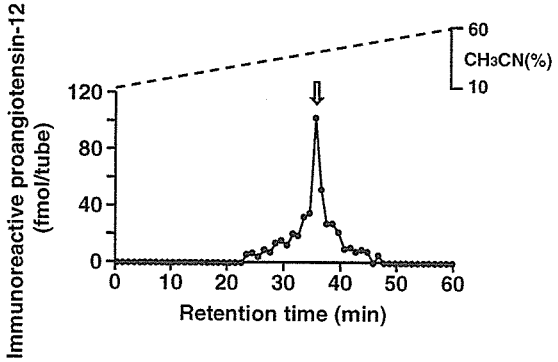

Fig. 4. Characterization of immunoreactive C-terminal proangiotensin-12 in rat small intestine by RP-HPLC and radioimmunoassay. RP-HPLC was conducted as described in the legend for Fig. 1. The arrow indicates the position of the full-length synthetic proangiotensin-12 peptide.

Ang II. The HPLC analysis revealed that immunoreactive proangiotensin-12 in the small intestine was eluted at the same position as the complete synthetic peptide (Fig. 4), further confirming the amino acid sequence and presence of proangiotensin-12.

Pharmacological studies ex vivo and in vivo

To study the biological actions of proangiotensin-12, we first looked at the effects on the vascular tonus of perfused rat aortic rings *ex vivo*. As shown in Fig. 5, proangiotensin-12 dose-dependently constricted the rat aorta as did Ang I or Ang II. Ang I and proangiotensin-12 had weaker vasoconstrictor effects than Ang II at concentrations of 3–30 nmol/L, but similar maximum contractions were obtained at 100 nmol/L of all three peptides. The constrictor action of proangiotensin-12 was mostly abolished in the presence of captopril, an ACE inhibitor, or CV-11974, an AT1 receptor blocker. Next, we examined the effects of proangiotensin-12 on blood pressure levels *in vivo* in anesthetized rats. A rise in arterial blood pressure was observed immediately after the intravenous injection of a bolus of proangiotensin-12 in rats, with a return to the basal level in 3–4 min (Fig. 6A). The pressor effects were dose-dependent and attenuated by pre-administration of captopril or CV-11974 (Figs. 6B and C), a result consistent with the *ex vivo* study.

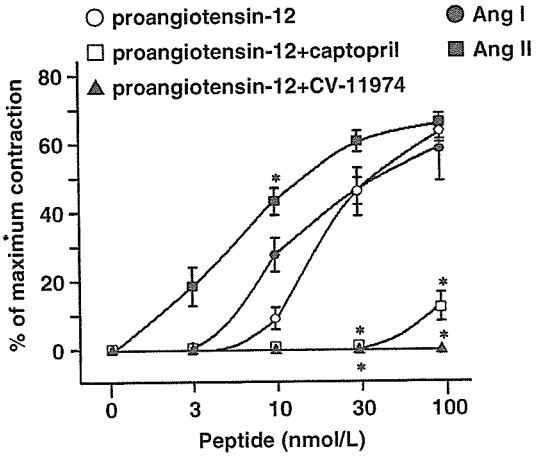

Fig. 5. Vasoconstrictor effects of Ang I, Ang II, and proangiotensin-12 on perfused rat aorta *ex vivo*. Captopril and CV-11974, an AT1 receptor antagonist, were used at 10^{-7} and 10^{-8} mol/L, respectively. The results are shown as means ± SE of % of maximum contraction induced by 60 mmol/L KCl for six to seven samples. $^{*}P < 0.05$ vs proangiotensin-12 alone.

Discussion

In this report, we describe the purification, sequence determination, tissue distribution, and vasoconstrictor properties of the novel angiotensinogen-derived peptide, Ang I-Leu-Tyr, which consists of 12 amino acid residues. We have termed this novel peptide proangiotensin-12, based upon the present results that suggest its role as a precursor of Ang II.

A concern may be raised over non-specific cleavage between Tyr-12 and Ser-13 of rat angiotensinogen, resulting in the occurrence of proangiotensin-12 as a non-specifically fragmented product during the extraction procedure; however, this possibility is unlikely based on the following reasons or findings. First, samples of rat small intestine were immediately boiled and acidified after resection in

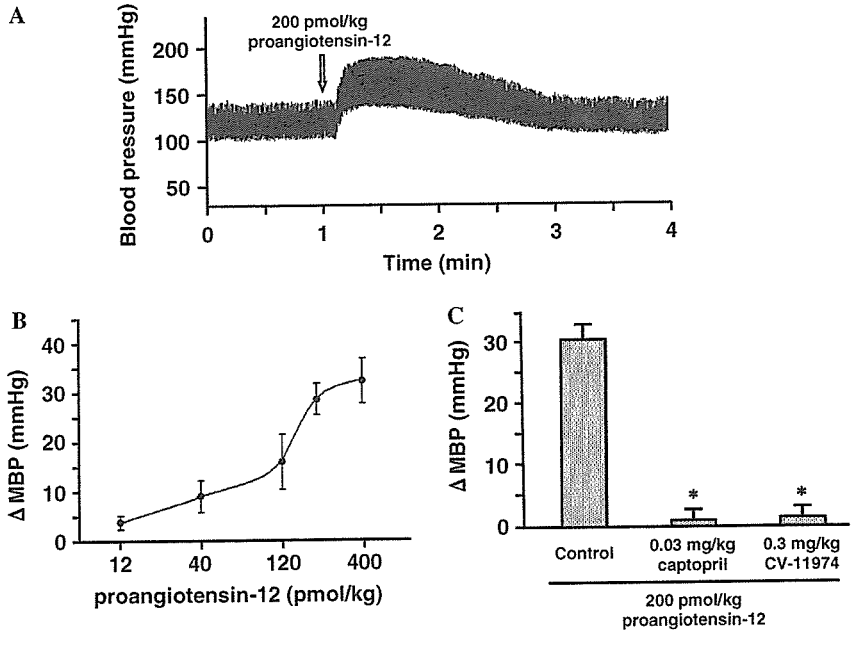

Fig. 6. (A) Representative blood pressure recording following the intravenous injection of a 200 pmol/kg bolus of proangiotensin-12 in anesthetized rats, (B) dose-dependent pressor effects, and (C) attenuation of the effects of proangiotensin-12 by captopril or CV-11974, an AT1 receptor blocker. ΔMBP indicates the maximum rise of mean blood pressure from the baseline. Either captopril or CV-11974 was intravenously injected at the indicated dose 2 min before the injection of proangiotensin-12. The results are shown as means ± SE for five rats examined. $^{*}P < 0.05$ vs proangiotensin-12 alone.

the extraction procedure. Non-specific cleavage of peptides can be avoided with this step which inactivates enzymes with proteolytic activity [20,21]. Second, the HPLC analysis of immunoreactive N-terminal Ang II (Fig. 1) showed three clear peaks, which correspond to Ang II, Ang I, and proangiotensin-12, without fragmentation of peptides. The specific radioimmunoassay (Table 1) also revealed that proangiotensin-12 is ubiquitously present in various organs and tissues, as are Ang I and Ang II. Last, the immunoreactive proangiotensin-12 in the small intestine was eluted at the position of the full-length peptide in the RP-HPLC analysis and radioimmunoassay detecting the C-terminal portion of proangiotensin-12 (Fig. 4). These findings are clearly indicative of the endogenous occurrence of proangiotensin-12.

A number of mechanisms, either mechanical or humoral, have been known to be involved in regulating blood pressure and body fluid balance in mammals. Among the humoral factors, the RA system plays a pivotal role: renin secreted mainly from kidneys cleaves angiotensinogen circulating in the blood to produce Ang I, which is then converted to Ang II exerting various effects particularly important for blood pressure and body fluid homeostasis. In the meantime, recent progress in research has revealed new aspects of the RA system. One example is identification of the Ang II-derived bioactive peptides with amino acid sequences shorter than Ang II such as Ang(1–7), Ang III or Ang IV [6–11]. Given this fact, it should be noted that proangiotensin-12 is a peptide longer than Ang I and such an angiotensinogen-derived peptide had not been identified. The second example is an active role of the tissue RA system probably independent of the systemic RA system [3–5]. In the present study, radioimmunoassay specifically detecting the C-terminal portion of proangiotensin-12 revealed ubiquitous presence of this novel peptide in various tissues and organs at the concentrations comparable with those of Ang I and Ang II, while the plasma concentration was lower than that of Ang I or Ang II. The relatively higher tissue levels suggest a significant role of proangiotensin-12 as a molecule of the tissue RA system.

In the present study, proangiotensin-12 exerted vasoconstrictor activity *ex vivo* on rat aortic rings, potency of which was similar to Ang I, while somehow weaker than Ang II at concentration of 3–30 nmol/L, showing the maximum contraction similar to Ang II. When injected intravenously in rats, proangiotensin-12 immediately raised blood pressure levels, and both the vasoconstrictor and pressor effects mostly disappeared following administration of captopril or CV-11974, an AT1 receptor blocker. These findings suggest prompt conversions of proangiotensin-12 to Ang I, and then Ang I to Ang II by ACE. Although the enzymes involved in the conversion to Ang I remain to be identified, the prompt cleavages of proangiotensin-12 to produce Ang II support the hypothesis of a significant role of proangiotensin-12 as an important molecule of the RA system.

Angiotensinogen is recognized to be produced and supplied to the blood mainly by the liver, though this precursor protein has been shown to be expressed widely in other tissues [22–25]. Consistent with this, according to our quantitative PCR, angiotensinogen mRNA was detected in various rat organs and tissues including the small intestine with the highest level in liver (data not shown). Because renin is an exclusively specific enzyme which cleaves angiotensinogen directly to produce Ang I, it is unlikely that renin is involved in the production of proangiotensin-12 [1,2]. An important question related to this is where the cleavage of angiotensinogen to proangiotensin-12 occurs: in tissues or in plasma. Clarifying the processing cascade of angiotensinogen would therefore provide us with information not only on the production of proangiotensin-12 but also on the mechanism activating the RA system and the role of proangiotensin-12 in blood pressure and body fluid homeostasis. Indeed, we are currently working on characterization and purification of enzymes that produce or cleave proangiotensin-12. Additionally, it should be clarified whether or not Ang I precursor peptides similar to proangiotensin-12 are present in other species including humans. Thus, the identification of proangiotensin-12 warrants future research on the RA system, which should be aimed at addressing a number of these unanswered questions.

Acknowledgments

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