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Flow injection analysis for measurement of activity of matrix metalloproteinase-7 (MMP-7)[1]

Michiyasu Itoh [a], Mamoru Osaki [a], Tadashige Chiba [a], Kazuto Masuda [a], Toshifumi Akizawa [a,*], Masanori Yoshioka [a], Motoharu Seiki [b]

[a] *Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-01, Japan*

[b] *Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920, Japan*

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Abstract

A simple and convenient method for measuring the activity of a recombinant human matrix metalloproteinase 7 (MMP-7, matrilysin) was developed by flow injection analysis (FIA). For this method, purified recombinant MMP-7 zymogen expressed in *E. coli* and the substrate peptide (MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2) were used. Following the incubation of substrate peptide with activated r-proMMP-7, the resulting fluorescent product peptide (MOCAc-Pro-Leu-Gly) was monitored with a fluorescence detector (λ_{ex} 328 nm, λ_{em} 393 nm) without chromatographic separation. In this FIA system, the analysis time is 2 min and the standard curve is linear from 5 to 100 pmol of the product peptide injected. In order to use this FIA system as a method for screening inhibitors against MMP-7, the effects of $CaCl_2$, EDTA and of the tissue inhibitor of metalloproteinase-1, and -2, were tested. A synthetic PRCGXPD-containing peptide (BS-10) was also observed to inhibit MMP-7 activity, with an IC_{50} value of 104 μM. Thus, it was concluded that the activity of r-MMP-7 can be reliably measured by the proposed system. Furthermore, to confirm the utility of this FIA system as a screening method, the inhibitory activity of the MMP-related substance in Joro spider (*Nephila clavata*) venom was measured by this method. This inhibitory activity was observed in an extract of a venom diluted 1000-fold. Thus, the FIA method is not only simple and quick, but also sensitive enough to screen and analyze the inhibitory properties of a large number of test compounds. © 1997 Elsevier Science B.V.

Keywords: Matrilysin; Matrix metalloproteinase-7; Flow injection analysis; Fluorogenic substrate peptide; Joro spider venom

1. Introduction

The family of matrix metalloproteinase (MMPs) are zinc-binding endopeptidases that degrade a variety of extracellular macromolecules

* Corresponding author.

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such as collagens, glycoproteins and proteoglycans [1]. These activities play an essential role in the tissue remodelling that occurs during various physiological processes such as morphogenesis, wound healing, ovulation, implantation and uterus involution [2–4]. On the other hand, MMPs have been implicated in disease states and cause tissue damage, as can be seen in many pathological processes such as tumor invasion [5–7], tissue ulceration and rheumatoid arthritis [8]. It is important to clarify the regulation of the enzyme activities to achieve a better understanding of the mechanisms of tissue remodelling and to aid development of inhibitors to control tissue damage. The screening of inhibitors against MMPs is beginning to take place on the basis of X-ray analysis of the catalytic domain in MMPs [9], and in this way many compounds are undergoing tests for their inhibitory activity against MMPs. To find inhibitors a simple and easy screening method is required, such as the microtiter plate method or the flow injection method, which can analyze large numbers of test compounds in a short period.

Human MMP-7 (matrilysin) was initially identified as a cDNA isolated from a mixed tumor library and was characterized as a MMP by expression in a eukaryotic system [10]. MMP-7 is the smallest in the family and is frequently expressed in malignant tumor cells [11–13]. In the course of studies on MMPs, the authors have established a simple means of preparing a large quantity of functional recombinant human proMMP-7 (r-proMMP-7) using an expression system in *E. coli* [14]. Briefly, MMP-7 cDNA was subcloned into a bacterial expression vector, which has tandemly repeated T7 prompters to produce the recombinant protein in *E. coli*. A histidine hexamer (H6-tag) was added to the C-terminus of the protein to facilitate purification of recombinant protein using Ni-NTA resin [15,16]. The plasmid was transfected into *E. coli* BL21 (DE3) cells that have a lysogenic T7 RNA polymerase gene under the control of the lac promoter [17]. Most of the r-proMMP-7 was detected in the precipitate as insoluble inclusion bodies. The precipitate was solubilized with 8 M urea containing 1% Triton X-100 and bound to Ni-NTA resin. The resin-bound r-proMMP-7 was eluted at pH 4.5 (6 M urea/1% Triton X-100/10 mM Tris–HCl/100 mM Na-phosphate), and dialyzed against TNCB buffer containing urea and Triton X-100. Refolding proceeded during dialysis against TNCB buffer with stepwise decreased concentrations of urea (3 to 0 M) and Triton X-100 (1 to 0.1%). It was observed that the r-MMP-7 cleaved the substrate DNP-peptide (DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg [18]) between Gly and Ile residues, by a reversed-phase HPLC method [14]. Preparation of a large quantity of pure r-MMP-7 may also aid the screening of inhibitors in the search for better therapeutic agents, and may also aid biochemical studies.

The use of peptide substrates to measure the activity of proteolytic enzymes has advantages as follows: (1) the structures or properties of the peptides are relatively insensitive to environmental parameters, rather than macromolecular protein substrates; (2) kinetic studies may be possible because the concentration of substrate is easily changed, which is essential for determining individual kinetic parameters, and for evaluating the mode of inhibition of inhibitors; (3) sequence specificity can be investigated by changing amino acid sequences; (4) a reporting probe required for assay monitoring, such as chromophores or fluorophores, can be easily incorporated.

Knight et al. [19] introduced the use of (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (MOCAc-Pro-Leu-A_2pr(DNP)-Ala-Arg-NH_2) as a general substrate for the assay of matrix metalloproteinases. This fluorogenic peptide substrate containing MOCAc [(7-methoxy-coumarin-4-yl)acetyl] as a fluorophore group and DNP (2,4-dinitrophenyl) as a quencher group is hardly fluorescent. A 190-fold increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm) is observed on hydrolysis of the peptide [19]. A fluorogenic substrate provides a particularly convenient enzyme assay method.

This paper describes the development of a simple method to measure MMP-7 activity by flow injection analysis (FIA) with the fluorogenic sub-

strate (MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2). This substrate is cleaved between Gly and Leu residues by the activated r-MMP-7, and results in an increase in fluorescence derived from the MOCAc-Pro-Leu-Gly, which is readily analyzed by FIA. The FIA method is not only easy and simple but is also quick and very useful for screening of inhibitors against MMP-7. In addition, an application of this method is shown to identify inhibitors and a MMP-7 related compound in the venom of Joro spider, *Nephila clavata*.

2. Experimental

2.1. Materials

Fluorogenic substrate (MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2) and its reference peptide (MOCAc-Pro-Leu-Gly) were purchased from the Peptide Institute, Inc., Osaka. The Ni-NTA resin was from QIAGEN, USA. The structure of BS-10 peptide, a decapeptide derived from the propeptide domain of MMP-7, is Met-Gln-Lys-Pro-Arg-Cys-Gly-Val-Pro-Asp. The BS-10[C^6-S] peptide has the same amino acid sequence, except that cysteine was replaced by serine. Syntheses of BS-10 and BS-10[C^6-S] peptides were performed by using solid-phase synthesis with the Fmoc method on a model 433A Applied Biosystems peptide synthesizer and deprotected in the usual manner. All the other chemicals were of reagent grade, was commercially available.

2.2. Preparation of recombinant proMMP-7 (matrilysin), expressed in E. coli

Recombinant human proMMP-7 (promatrilysin) was expressed and purified as described by Itoh et al. [14]. This r-proMMP-7 protein was shown to be identical to authentic native MMP-7 in all activity assayed. The r-proMMP-7 (28.6 μM) was diluted with 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, and was activated by trypsin. Trypsin was added to give a final concentration of 0.2 μM, using a stock solution of 40 μM in 1 mM HCl, 10 mM $CaCl_2$. After incubation at 37°C, diisopropylphosphofluoridate (DIFP) (500 mM in 2-propanol) was added to give a final concentration of 20 mM to remove trypsin activity prior to analysis.

2.3. Enzyme reaction and flow injection analysis

The fluorescent substrate, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2), as described by Knight et al. [19], was used throughout. The assay was performed at 37°C using 20 μM substrate and 26.7 nM enzyme in an assay buffer comprised of 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 0.05 mM $ZnCl_2$, 0.05% Brij 35, and 0.02% NaN_3. Substrate solutions were stored in dimethyl sulfoxide (DMSO); the final DMSO concentration was 2%. After 4 h incubation, 50-μl aliquots were drawn and added to 50 μl of 100 mM EDTA to stop the reaction. Then, 10 μl of reaction mixture was injected into a flow injection analysis system which was based on Jasco fluorescence HPLC equipment (Jasco, Tokyo): HPLC pump model 880-PU; Auto Sampler model AS-950; Fluorescence Detector model FP-920; and Integrator model 807-IT. A sample was injected every 2 min with 30% CH_3CN/0.06% Brij 35 (carrier solvent). The flow rate was set at 1.0 ml/min, and fluorescence was detected at 328 nm (excitation) and 393 nm (emission), at which there should be no interference from the 7-methoxycoumarin group.

2.4. Aqueous extraction of Joro spider venom

The preparation of the venom extract was performed in the same manner as previously reported [20]. Briefly, 500 Joro spiders were collected in the Kinki district, central Japan. Approximately five hundred pairs of venom glands were put into a test tube and were immediately boiled with 30 ml of water for 10 min. After boiling, the venom glands were homogenized with a glass rod. The

homogenate was centrifugated at 1000 × g for 15 min. The remaining venom were treated in the same manner. The supernatant was lyophilized and stored at −80°C before use. Preparative HPLC (HPLC system of Jasco, Tokyo) was carried out using a column of Capcellpak C18 (10 mm I.D. × 250 mm, Shiseido, Tokyo) at 40°C; the flow rate of each eluent was set at 3.0 ml/min using a linear gradient from 0.1% TFA to 50% CH_3CN containing 0.1% TFA for 30 min, and then continuously for an additional 10 min. Elution was monitored by UV at 220 nm and eluate collected every 1 min. Each fraction collected was concentrated to dryness by lyophilized. A gland unit (GU) was defined as the activity corresponding to one venom gland extract in the assay.

3. Results

3.1. Cleavage of the fluorogenic peptide with recombinant proMMP-7

Preparation of recombinant MMP-7, expressed in *E. coli*, was described previously [14]. The latent form of r-proMMP-7 can be prepared with a simple one-step purification by using a molecular biological technique. The treatment of proMMP-7 with agents or conditions which resulted in the activation of other metalloproteinases confirmed that the trypsin was able to activate proMMP-7 (data not shown). The purified and refolded r-proMMP-7 was incubated at 37°C with trypsin. Maximal activity was achieved within 60 min in the presence of 0.2 μM trypsin. Analysis of the activation process by casein zymography indicated that the increase in activity was concomitant with a shift of caseinolytic activity by r-MMP-7 from M_r 31 000 to M_r 22 000 (data not shown).

The trypsin-activated r-proMMP-7 was incubated with the fluorogenic substrate peptide, MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2. After 1 h incubation at 37°C, the reaction mixture was analyzed by reversed-phase HPLC. As shown in Fig. 1, two extra peaks (peak 1 and 2) appeared with the original peak of the substrate peptide (peak 3). FAB-mass spectrometry with a glycerin matrix (using JMX-DX300 mass spectrometer, JOEL, Tokyo), showed the molecular weight of the compound in peak 1 to be 609, and that in peak 2 to be 501. Thus, peaks 1, 2, and 3 were identified as Leu-A_2pr(DNP)-Ala-Arg-NH_2, MOCAc-Pro-Leu-Gly, and MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2, respectively. Only the compound in peak 2 was fluorescent (328 nm excitation, 393 nm emission), and no peaks other than peaks 1, 2 and 3 appeared after incubation for 12 h with r-MMP-7 (data not shown). Finally it was concluded that r-MMP-7 cleaves the substrate peptide between Gly and Leu residues, and that the entire fluorescence of the reaction mixture was derived from the MMP-7 cleaved peptide, MOCAc-Pro-Leu-Gly. This hydrolysis reaction seems to be applicable to flow injection analysis, because there is no need to separate the products from substrate.

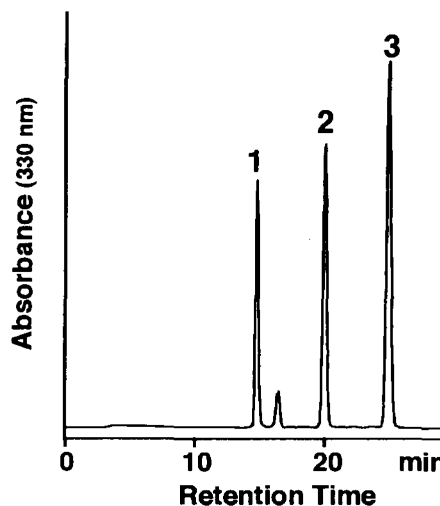

Fig. 1. Digestion of the fluorogenic substrate peptide with r-MMP-7. The fluorogenic substrate peptide (MOCAc-Pro-Leu-Gly-Leu-A_2pr(DNP)-Ala-Arg-NH_2) was incubated with trypsin-activated r-proMMP-7 in 50 μl of 50 mM Tris–HCl (pH 7.5)/150 mM NaCl/10 mM $CaCl_2$/0.05% Brij 35/0.02% NaN_3 at 37°C for 2 h (for details see text). Peaks 1, 2, and 3 indicate the cleaved peptide (Leu-A_2pr(DNP)-Ala-Arg-NH_2), the cleaved fluorescent peptide (MOCAc-Pro-Leu-Gly), and the fluorogenic substrate peptide, respectively (as confirmed by amino acid analysis and mass spectrometry).

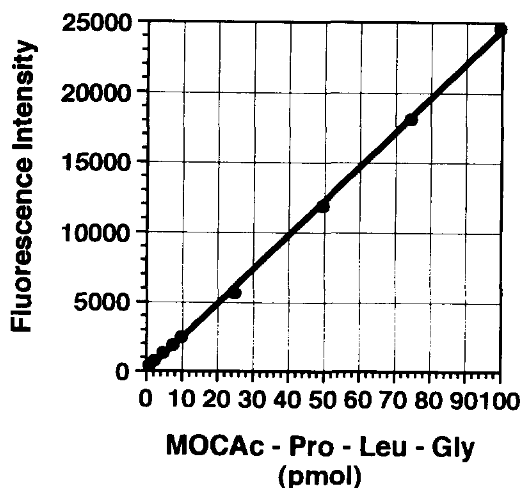

Fig. 2. The titration curve for MOCAc-Pro-Leu-Gly peptide. The fluorescence intensity of the MOCAc-Pro-Leu-Gly peptide was measured at 328 nm excitation and at 393 nm emission. The fluorescence intensities were plotted against the amounts of the peptide in 10 µl of injection.

3.2. Flow injection analysis of r-MMP-7

To quantify the amount of cleaved peptide, the fluorescence intensity of 7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly (MOCAc-Pro-Leu-Gly) peptide, which is the putative cleaved product peptide, was measured. As shown in Fig. 2, the relationship between the fluorescence intensity and the amount of MOCAc-Pro-Leu-Gly peptide (from 5 to 100 pmol per 10 µl injection) was linear:

Amount of peptide (pmol)

$= 3.327 \times 10^{-3} \times$ *Fluorescence intensity*

The amounts of the cleaved peptide in the following experiments were calculated by using this formula.

The cleavage reaction of fluorogenic substrate peptide by activated r-MMP-7 was performed by changing the enzyme or substrate concentration. The peptide cleavage reaction was performed with 12.5 ng to 6.4 µg for r-MMP-7 for 1 h at 37°C. The amount of MOCAc-Pro-Leu-Gly increased with the amount of r-MMP-7 up to 6.4 µg in the reaction (Fig. 3A). Hydrolysis of the substrate peptide with 32 ng of r-MMP-7 continues until 9 h at concentrations of substrate from 2.5 µM to 20 µM (Fig. 3B). It is suggested that the activity of r-MMP-7 can be measured by this FIA system.

To confirm that the activity of r-MMP-7 is measured by this FIA system, the effects were analyzed of $CaCl_2$, EDTA, and tissue inhibitors

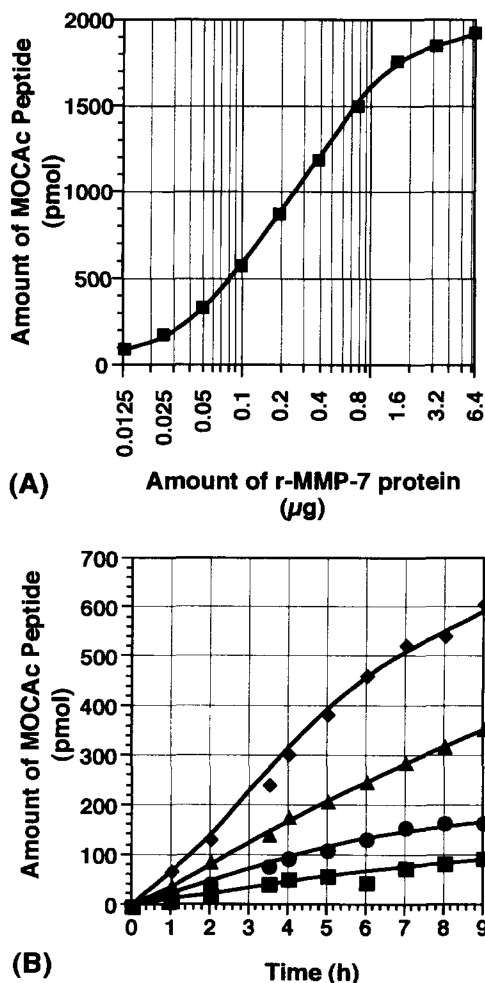

Fig. 3. Digestion of the fluorogenic substrate peptide by r-MMP-7. (A) One nmol of the substrate peptide was incubated with the indicated amount of r-MMP-7 at 37°C for 1 h. After termination of the reaction, the samples were analyzed by FIA. (B) 32 ng of r-MMP-7 was incubated with 20 µM (◆), 10 µM (▲), 5 µM (●), and 2.5 µM (■) of the fluorogenic substrate peptide at 37°C for the period indicated, and analyzed by FIA.

of metalloproteinase(TIMP)-1 and -2, MMP-specific natural inhibitors, on r-MMP-7 activity. It is reported that MMP-7 is a zinc-dependent enzyme that also requires 1 mM $CaCl_2$ to maintain its optimal activity [21]. As shown in Fig. 4A, concentration of $CaCl_2$ clearly affects MMP-7 activity. When no $CaCl_2$ was added to the reaction mixture, no hydrolysis was detected. Cleavage of peptide was increased by addition of $CaCl_2$ up to 1 mM. MMP-7 is inhibited by a wide variety of chelating agents including EDTA, 1,10-phenanthrolin, and dithiothreitol. Enzyme reaction was performed by addition of EDTA from 0 to 5 mM (reaction mixture contains 50 μM $ZnCl_2$ and 5 mM $CaCl_2$). The r-MMP-7 was inhibited by addition of EDTA, and no activity was detected at more than 4 mM EDTA addition (Fig. 4B). The tissue inhibitors TIMP-1 and TIMP-2 inhibit MMP-7 in stoichiometric fashion [22]. TIMP-1, -2, and bovine serum albumin (BSA; as negative control) were added to the reaction mixture, and the activity of r-MMP-7 measured by this FIA system. The initial velocities of hydrolysis were plotted against molar ratio of inhibitor protein to r-MMP-7 (Fig. 5). Although, BSA does not affect r-MMP-7 activity, both TIMP-1 and TIMP-2 inhibit it. Furthermore, these results showed that TIMP-1 binds to r-MMP-7 in a 1:1 ratio and that TIMP-2 binds r-MMP-7 in a ratio of 0.8:1. According to these results, it is concluded that this FIA system, using r-MMP-7 and fluorogenic peptide, is able to measure the activity of r-MMP-7 and should be applicable as an inhibitor screening system for MMP-7.

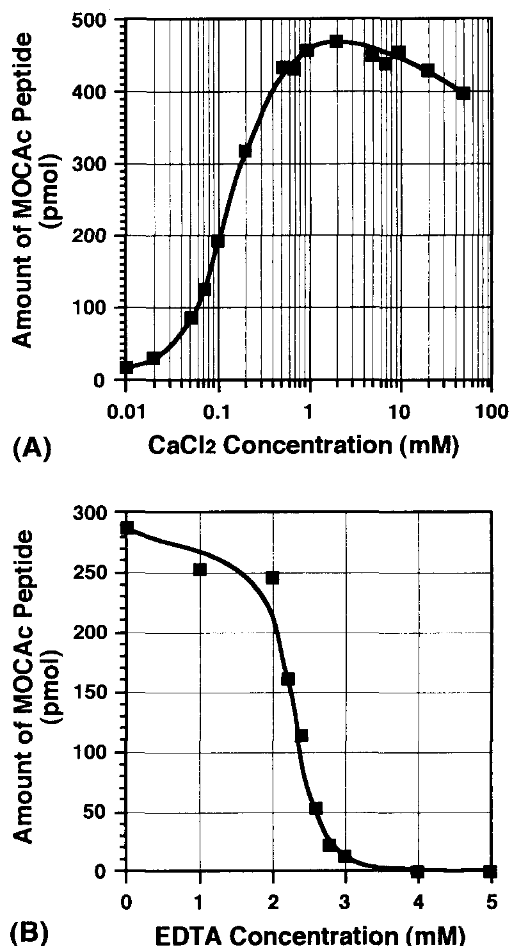

Fig. 4. Requirement of calcium for cleavage of substrate peptide by r-MMP-7. Hydrolysis of the fluorogenic peptide by r-MMP-7 were performed with various concentrations of $CaCl_2$ (A) and EDTA (B). After 4 h incubation at 37°C, the reactions were terminated and analyzed by FIA.

3.3. A synthetic peptide inhibitor against MMP-7

The latency of MMP zymogens is maintained by a mechanism called 'the cysteine switch' [23]. In latent enzymes, the cysteine residue in the PRCGXPD masks the zinc atom at the catalytic center. It may be possible that the PRCGXPD-containing peptide inhibits the MMP activity even in separated molecules (i.e. intermolecular interaction). The peptides containing PRCGXPD in MMP-3 (stromelysin) have been synthesized and their inhibition properties against MMP-3 reported [24]. The authors synthesized a 10 amino acid PRCGXPD-containing peptide derived from the propeptide domain of MMP-7 (BS-10; Met-Gln-Lys-Pro-Arg-Cys-Gly-Val-Pro-Asp) and analyzed its inhibitory property against MMP-7. Trypsin activated r-MMP-7 and BS-10 peptide were mixed and incubated at 37°C for 15 min. The reaction was started by addition of fluorogenic substrate peptide and incubated at 37°C for 4 h. The amounts of cleaved peptide (MOCAc-Pro-Gly-Leu) were plotted against concentration of BS-10 peptide (Fig. 6). BS-10 peptide inhibits

r-MMP-7 with an IC_{50} value of 104 μM. To confirm that the inhibition by BS-10 was caused by cysteine residue masking the zinc atom at the catalytic site, BS-10 [C^6-S] (which has a serine instead of cysteine in the BS-10 peptide) was tested. As shown in Fig. 6, BS-10 [C^6-S] does not inhibit r-MMP-7 at all ($IC_{50} > 1$ mM), as well as cysteine itself. These results indicate that the FIA method is able to analyze the inhibitory properties of high-molecular weight protein and that of low-molecular weight peptide. Furthermore, this method is not only simple and quick, but also sensitive enough to screen and analyze the inhibitory properties.

3.4. *Screening of MMP-related substances in the venom of Joro spider (Nephila clavata)*

As an application of this inhibitor screening system for MMP-7, water-extracted compounds from the venom of Joro spider (*Nephila clavata*) were tested. Various concentrations of the venom extract were added to the reaction mixture and the activity of r-MMP-7 was measured. Inhibition of hydrolysis was observed, depending on the concentration of the extract added (data not shown). These results suggest that there may be inhibitors against MMP-7 in the spider venom. The lower molecular weight fraction (< 100 kDa) showed the inhibitory activity. In contrast, the higher molecular weight fraction (> 100 kDa) stimulated the degradation activity of r-MMP-7 (data not shown). The extract was applied to preparative HPLC and fractions were collected every 1 min (Fig. 7). The earlier fractions in which polyamine toxins were expected to be eluted did not show the inhibitory activity even in higher concentration. However, the later eluting fractions (Nos. 21–30, 31–35, and 36–40) showed inhibitory activity against r-MMP-7. Especially in fraction 31–35, marked inhibitory activity was observed even at 1000-fold dilution (Fig. 8). These results suggest that there may be a specific inhibitor in the venom of Joro spider.

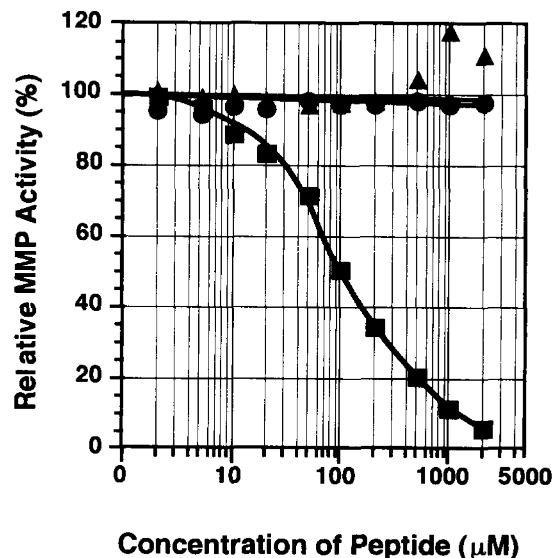

Fig. 6. Inhibition of r-MMP-7 by BS-10 peptide. Hydrolysis of the fluorogenic peptide by r-MMP-7 was performed by addition of BS-10 (■), BS-10[C^6-S] (●), and cysteine alone (▲). After 4 h incubation at 37°C, the reactions were terminated and analyzed by FIA.

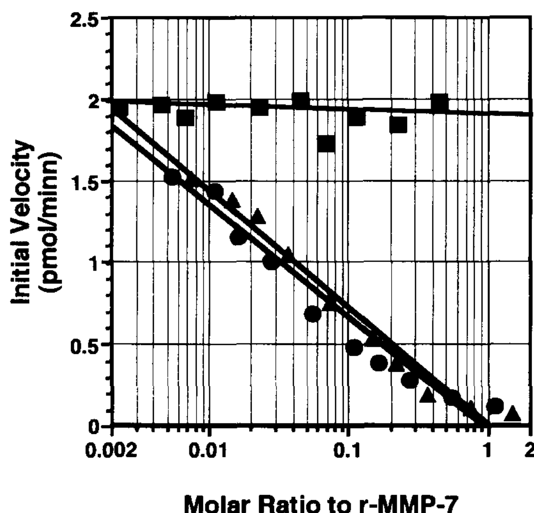

Fig. 5. Inhibition of r-MMP-7 by TIMPs. Hydrolysis of the fluorogenic peptide by r-MMP-7 was performed by addition of TIMP-1 (▲), TIMP-2 (●), and BSA (■). After 2 h incubation at 37°C, the reactions were terminated and analyzed by FIA.

4. Discussion

In this paper, a simple FIA method is described to measure the activity of r-MMP-7, which is appli-

cable for use as an inhibitor screening system. The key feature of this method is the use of pure and functional r-MMP-7 zymogen and that of a fluorogenic substrate peptide.

As various MMPs are expressed in the same type of cell, the culture media contains several kinds of MMPs [1], a highly purified enzyme is necessary to test for specific inhibitors against the individual enzymes.

The MMPs are a gene family of zinc-binding endopeptidases that degrade a variety of extracellular macromolecules. Each MMP is distinguished by its ability to degrade specific protein substrates, such as collagens, proteoglycans, laminin, fibronectin, and others [1,21]. However, it is impractical for several reasons to use these macromolecules as substrates over a wide range of conditions. First, the structures and properties of proteins are sensitive to environmental parameters. Second, it can be difficult to vary the concentration of macromolecular substrates without encountering aggregation, and that makes it impossible to study kinetic parameters of enzyme reaction. For the reasons enumerated above, it is highly desirable to have convenient assays that utilize small synthetic peptides as the substrate. It is reported that r-MMP-7 cleaves the substrate DNP-peptide (DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg) between Gly and Ile residue, which can be detected using reversed-phase HPLC taking over 60 min (30 min for gradient separation and 30 min for regeneration of the column) for each assay [14]. It was too time-consuming and insensitive an assay method to use for the screening of inhibitors against MMP-7. The fluorogenic peptide substrate generates a cleaved product MOCAc-Pro-Leu-Gly and only this was fluorescent at 328 nm excitation and 393 nm emission, suitable

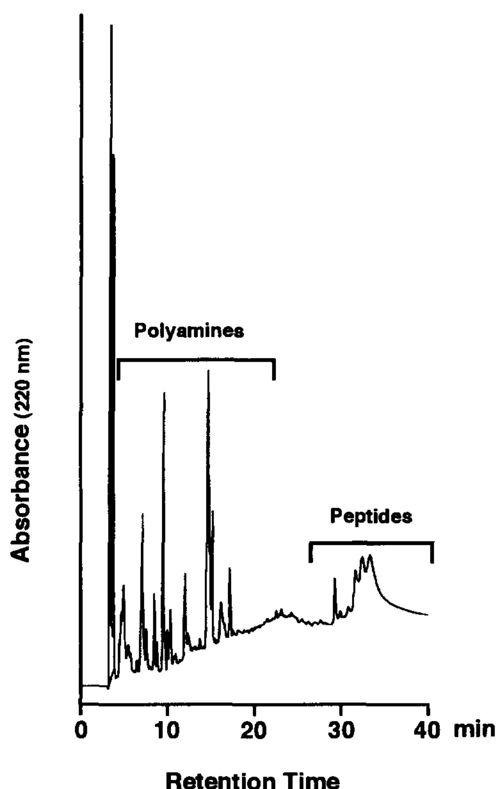

Fig. 7. Chromatogram of crude extract from venom of joro spider, *Nephila clavata*. Two gland units (GU) of crude extract of venom were separated by reversed-phase HPLC column (Capcellpak C18, 10 mm I.D. × 250 mm) for 30 min with a programmed gradient at 40°C. The compounds were detected by UV 220 nm. The earlier and later fractions, containing polyamine toxins and proteins (or polypeptides), respectively, were separated under these conditions.

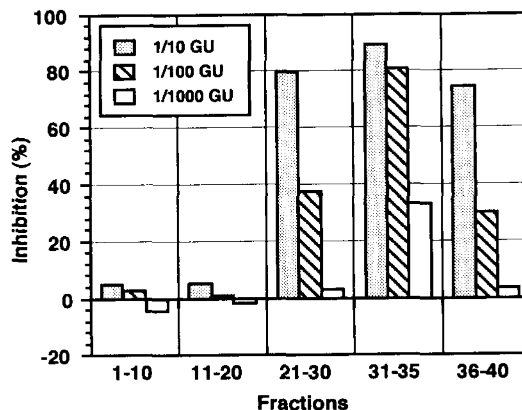

Fig. 8. Inhibition of r-MMP-7 by the venom extract of joro spider. Eluate fractions from the preparative HPLC (shown in Fig. 7) were collected every 1 min. Fractions 1 to 10, 11 to 20, 21 to 30, 31 to 15, and 35 to 40 were combined. The indicated amount (GU) of each combined fraction was incubated with trypsin-activated r-proMMP-7 at 37°C for 15 min. The reaction was started by addition of the substrate and incubated at 37°C for 4 h. The rate of inhibition were calculated from the values of fluorescence intensity obtained in the presence and absence of venom extract in the reaction mixture.

for the flow injection analysis (FIA) system. This application reduces the assay time for one sample from 60 min to 2 min. It is a significant advantage for the screening method, where large numbers of test compounds have to be analyzed in short period.

In this FIA system, the relationship between the fluorescence intensity and the amount of product peptide was linear, and the enzymatic hydrolysis was shown to be quantitative (Figs. 2 and 3). As shown in Fig. 4, r-MMP-7 requires 1 mM $CaCl_2$ for maximal activity and is inhibited by EDTA. The stoichiometric inhibition by TIMPs was shown in the titration experiments of active r-MMP-7 with TIMP-1 and -2 (Fig. 5). Moreover, BS-10 peptide, which is derived from the propeptide domain of MMP-7, inhibits r-MMP-7 with an IC_{50} value of 104 μM. According to these results, the increase in the fluorescence obtained from the FIA system described here reflects the activity of r-MMP-7. Thus, this FIA method is suitable as a method for screening of inhibitors against MMP-7.

Fig. 6 shows that BS-10 peptide inhibits r-MMP-7, although BS-10[C^6-S] peptide and cysteine itself do not. It is suggested that not only the cysteine residue, but also the amino acid sequence surrounding PRCGVPD, affects the inhibitory property against MMP-7, through the specific conformation for ligation to the zinc-binding catalytic center of the enzyme. The longer fragment peptide of the propeptide domain may inhibit hydrolysis activity more effectively. However, although the inhibition mode by BS-10 peptide seems to be 'predominantly competitive' against MMP-7, a more detailed kinetic characterization should be carried out.

The utility of this inhibitor screening FIA system for r-MMP-7 was confirmed by the screening of the inhibitors in the venom of Joro spider, *Nephila clavata*. As shown in Fig. 7, the fraction containing polyamine toxins did not show the inhibitory activity as well as synthetic joramine and spidamine which are biological active polyamine toxins [25]. In contrast, the inhibitory fraction seemed to contain proteins and polypeptides, according to the results of SDS-polyacrylamide gel electrophoresis and amino acid analysis (data not shown). By using this FIA method, the activity in only 1/1000 of spider venom can be measured. In addition, even in the fraction obtained from a reversed-phase column which contained organic solvent, the activity can be tested directly, if the sample volume is small. Moreover, in the case of a gel filtration or ion-exchange column, in which aqueous buffer solution is used as a mobile phase, the fractions can be applied to this FIA method with ease. This advantage is very useful for the purification of the novel MMP proteins even if unstable, as well as the active enzyme.

A simple FIA system is reported to measure r-MMP-7 activity, which is applicable to the screening of inhibitors against MMP-7. This system is easily applied to the characterization of MMP-related substances, and the other MMPs by preparing pure and functional enzymes. Furthermore, the authors are now developing a more convenient and useful microtiter plate assay system, which may facilitate a more systematic survey of inhibitors for MMPs in natural and synthetic compounds.

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