Use of Edman degradation sequence analysis and matrix-assisted laser desorption/ionization mass spectrometry in designing substrates for matrix metalloproteinases

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Abstract

The matrix metalloproteinase (MMP) family has been implicated in the process of a variety of diseases such as arthritis, atherosclerosis, and tumor cell metastasis. We have been designing single-stranded peptides (SSPs) and triple-helical peptides (THPs) as potential discriminatory MMP substrates. Edman degradation sequence and matrix-assisted laser desorption/ ionization mass spectrometric (MALDI-MS) analyses of proteolytic activity have been utilized to aid in further substrate design. THP models of the $\alpha 1(I)772-786$ sequence from type I collagen were synthesized to examine the triple-helical substrate specificity of MMP family members. Sequence and MALDI-MS analyses were used in conjunction with a fluorometric assay to determine the exact point of cleavage by each MMP. MMP-1 (interstitial collagenase) cleaved the substrates at a single Gly–Ile bond, analogous to the cleavage site in type I collagen. MMP-2 (M_r 72 000 type IV collagenase; gelatinase A) was found to cleave the substrates at two sites, a Gly–Ile bond and a Gly–Gln bond. MMP-3 (stromelysin 1) was found to cleave only one of the substrates after reaction for 48 h. Ultimately, sequence and MALDI-MS analyses allowed us to detect an additional cleavage site for MMP-2 in comparison to MMP-1, while MMP-3 was found to cleave a substrate after an extended time period. The second cleavage site would cause the kinetic parameters for MMP-2 to be overestimated by the fluorometric assay. Further design variations for these substrates need to consider the presence of more stable triple-helical conformation (to eliminate MMP-3 binding) and the removal of Gly–Gln bonds that may be susceptible to MMP-2. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The matrix metalloproteinase (MMP) family plays an integral role in both normal and pathological connective tissue remodeling. This accelerated local turnover of the extracellular matrix can be found in such diverse diseases as arthritis, glomerulonephritis, periodontal disease, and tumor cell invasion and metastasis [1,2]. In particular, MMPs are believed to initiate interstitial collagen catabolism and participate in denatured collagen (gelatin) degradation. Interstitial collagens (types I–III) are hydrolyzed at a single locus by MMP-1 (collagenase 1) [3], MMP-8 (collagenase 2) [3], MMP-13 (collagenase 3) [4], and MMP-18 (collagenase 4) [5]. MMP-2 hydrolyzes type I collagen with a similar k_{cat} and a slightly higher K_M values than those of MMP-1 at the same single locus [6]. MMP-3 binds to type I collagen, but

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does not cleave the triple-helical domain [7,8]. Recent studies by Ohuchi et al. [9] showed that membrane type-1-MMP (MMP-14) also cleaves interstitial collagens.

One approach for better defining the mechanisms by which MMPs hydrolyze native interstitial collagens is the utilization of triple-helical peptide (THP) model substrates. Substrates containing only potential cleavage sites would be much smaller than native collagens, and thus could be used in combination with mutagenesis to distinguish MMP domains responsible for binding and orienting native collagens, as opposed to domains responsible for unwinding the triple-helix and cleaving individual collagen strands. Mechanistic information could be obtained via studies of MMP kinetics and X-ray crystallographic analysis of MMP/THP co-crystals. Also, THP substrates could be used to develop selective substrates for MMP family members, which in turn would allow for the design of specific inhibitors. Along these lines, we have previously described a fluorogenic synthetic substrate that is selectively hydrolyzed by MMP-3 [10]. Based on collagenolytic activities, it is possible that THPs may be used to design selective substrates for MMP-1, MMP-2, MMP-8, MMP-13, MMP-14, and MMP-18.

Our laboratory has developed a solid-phase THP synthetic method which features a C-terminal Lys covalent branch [11–13]. A variety of THPs have been constructed for the study of cellular recognition of collagen [12–15], including one that incorporates the collagenase cleavage site in type I collagen [13]. We have also described an approach by which the non-covalent association of lipophilic molecules, N-terminally linked to a peptide, can be used to form stable ''peptide-amphiphile'' triple-helices [16–18]. For the present study, we have used both the covalent branch and peptide-amphiphile approaches to construct THP models of the collagenase cleavage site in type I collagen. The THPs incorporate the α1(I)772–786 sequence Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp. We have compared the susceptibility of THPs to the MMP family members MMP-1, MMP-2, and MMP-3, using Edman degradation sequence, matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS), and fluorometric analyses.

2. Experimental

2.1. Materials

All standard peptide synthesis chemicals were analytical reagent grade or better and purchased from Perkin-Elmer–Applied Biosystems. (Foster City, CA, USA) or Fisher. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was from Richelieu Biotechnologies (St.-Hyacinthe, Canada). 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives were obtained from Novabiochem (San Diego, CA). Amino acids are of the L-configuration (except for Gly). The monoalkyl chains used in this study, hexanoic acid [CH_3–$(CH_2)_4$–CO_2H, designated C_6] and decanoic acid [CH_3–$(CH_2)_8$–CO_2H, designated C_{10}], were purchased from Aldrich. Peptide-amphiphile THPs were synthesized and purified by methods previously described in our laboratory [17]. The synthesis and characterization of the branched α1(I)772–786 THP has been published [13].

2.2. Peptide analyses

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Hewlett-Packard 1090 or 1100 liquid chromatograph equipped with a Hypersil small-pore, narrow-bore C_{18} RP column (5 μm particle size, 120 Å pore size, 100×2.1 mm). Eluants were 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was 0–50% B in 30 min with a flow of 0.3 ml/min. Detection was at 229 nm. Edman degradation sequence analysis was performed on an Applied Biosystems 477A protein sequencer/120A analyzer as described [19] for ''embedded'' (noncovalent) sequencing. MALDI time-of-flight (TOF) MS was performed on a Hewlett-Packard G2025A LD-TOF mass spectrometer using either a sinapinic acid or 2,5-dihydroxybenzoic acid-2-hydroxy-5-methoxy benzoic acid (9:1, v/v) matrix [20]. Matrix solution [1 μl of 20 mg/ml in 0.1% aqueous TFA–acetonitrile (2:1)] was applied to the probe and air dried. A 1 μl volume of peptide solution (20–40 μM) was applied and air dried. Water (1 μl) was added and wicked off, and 1 μl of

matrix solution was reapplied. The air dried probe was irradiated with a N_2 laser at 337 nm using a laser energy of ~3.90 μJ and pulses of 3 ns in duration. Between 110 and 160 single shots were run with each sample, summed, and averaged.

2.3. Matrix metalloproteinases

ProMMP-1 [21], proMMP-2 [22], and proMMP-3 [23] were purified from the culture medium of human rheumatoid synovial cells stimulated with rabbit macrophage-conditioned medium. ProMMP-2 was activated by reacting with 1 mM 4-amino-phenylmercuric acetate at 37°C for 45 min. ProMMP-1 was activated by reacting with 1 mM 4-aminophenylmercuric acetate and an equimolar amount of MMP-3 at 37°C for 6 h. After activation, MMP-3 was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. ProMMP-3 was activated by reacting with 5 μg/ml chymotrypsin at 37°C for 2 h. Chymotrypsin was inactivated with 2 mM diiso-propylfluorophosphate. The amounts of active MMP-

1, MMP-2, and MMP-3 were determined by titration with recombinant TIMP-1 [24] over a concentration range of 0.1–3 μg/ml.

2.4. Assays

Two different assay methods were utilized, the first for Edman degradation sequence analysis and the second for mass spectrometric and fluorometric analyses. For the first assay method, THPs were prepared as 260 μM stock solutions in phosphate-buffered saline (PBS). Initial MMP assays were carried out by incubating 40 μM substrate in either PBS or TNC buffer (50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, 0.02% NaN$_3$) with 67 nM enzyme for 24 h at 37°C. These reactions were stopped by adding 130 μl of 3% (v/v) glacial acetic acid. The fluorogenic substrates NFF-1 [Mca-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Lys-(Dnp)-Gly, where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is 2,4-dinitrophenyl] and NFF-3 [Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys-(Dnp)-NH$_2$, where Nva is norvaline] were used for
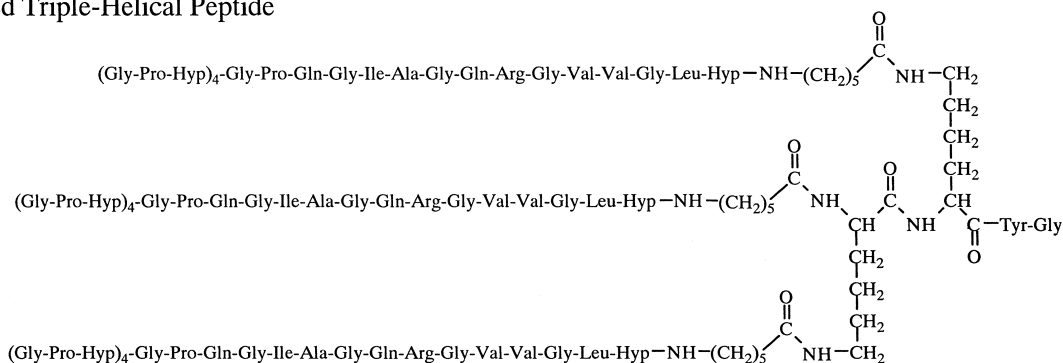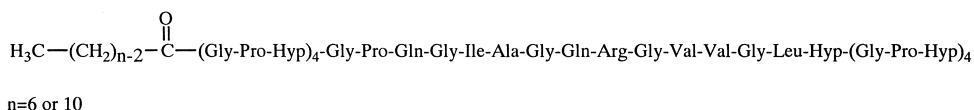

Fig. 1. Structures of THPs used in this study. Ahx is 6-aminohexanoic acid and Hyp is 4-hydroxy-L-proline.

assays of MMP-2 and MMP-3, respectively, as described [10].

For the second assay method, THPs were prepared as 270 μM stock solutions in ''fluorometric assay'' buffer (50 mM tricine, pH 7.5, 0.20 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35). MMP assays were carried out in assay buffer by incubating a range of substrate concentrations with 40 nM enzyme at 30°C. Enzymatic activity was terminated by the addition of 20 μl of the enzyme–substrate solution to 30 μl of *o*-phenanthroline (20 mM) at appropriate times. Rates of hydrolysis were monitored by the addition of 200 μl fluorescamine solution. Fluorescamine solution was prepared by first dissolving fluorescamine in acetone at a concentration of 40 mM, then diluting the fluorescamine concentration to 5 mM with assay buffer minus Brij 35. Fluorescamine reacts with free amino groups, resulting in a fluorophore with $\lambda_{excitation}=387$ nm and $\lambda_{emission}=480$ nm. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini Dual-Scanning Microplate spectrofluorometer.

2.5. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded over the range $\lambda=190$–250 nm on a Jasco J-600 instrument using either a 0.1 or 1.0 cm path-length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity ([Θ]) at $\lambda=225$ nm while the temperature was continuously increased in the range of 5–80°C at a rate of 0.2°C/min. Temperature was controlled using a Jasco PTC-348WI temperature control unit. For samples exhibiting sigmoidal melting curves, the reflection point in the transition region (first derivative) is defined as the melting temperature (T_m). Alternatively, T_m was evaluated from the midpoint of the transition.

3. Results and discussion

Three different THPs were constructed as possible MMP substrates (Fig. 1). The first incorporated the $\alpha1(I)772$–786 sequence between an N-terminal

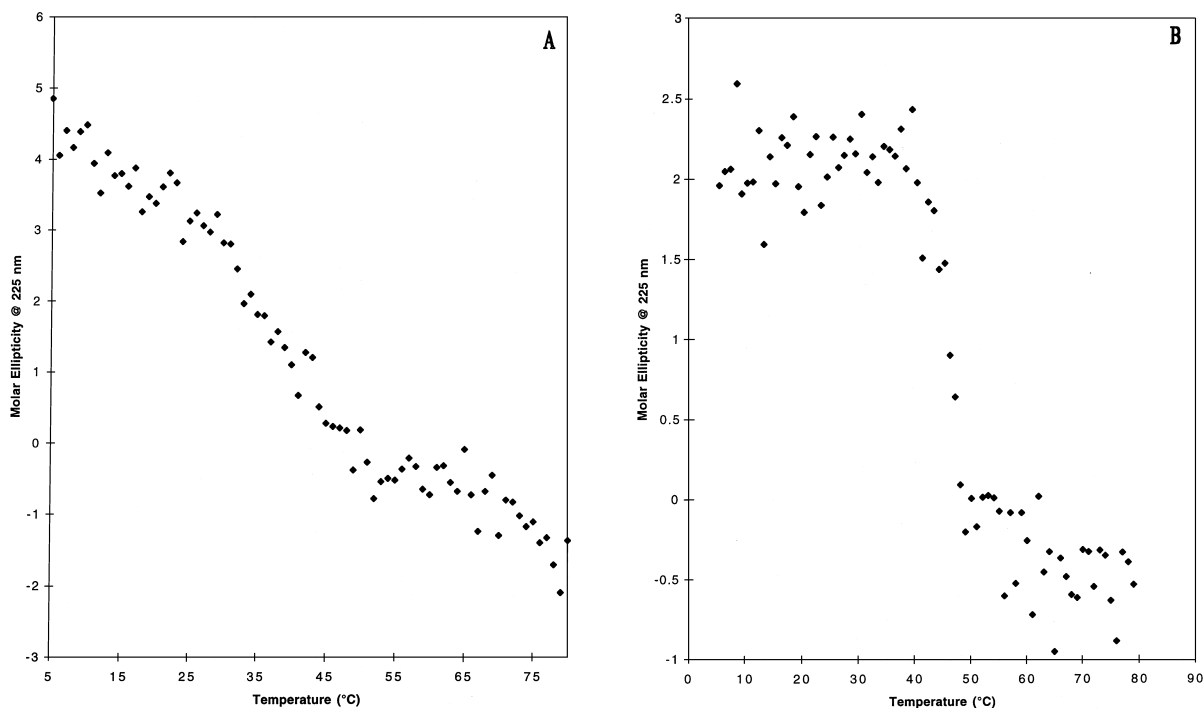Fig. 2. Temperature dependence of molar ellipticity ([θ]) at $\lambda=225$ nm for C$_6$-[$\alpha1(I)772$–786] (A) and C$_{10}$-[$\alpha1(I)772$–786] (B) peptide-amphiphiles. Both of the peptide-amphiphiles display thermal denaturation curves typical for collagen-like triple-helices.

(Gly-Pro-Hyp)$_6$ domain and a C-terminal covalent branching structure, and is designated "α1(I)772–786 THP". It forms a triple-helix with T_m ~43°C [13]. The other two THPs are peptide-amphiphiles which contain either a C$_6$ or C$_{10}$ alkyl chain. The C$_6$-[α1(I)772–786] and C$_{10}$-[α1(I)772–786] peptide-amphiphiles form triple-helices with T_m values of 35 and 46°C, respectively (Fig. 2). Thus, all three THPs possess sufficient thermal stabilities to be utilized as MMP substrates.

Several different buffers were considered for MMP assays. PBS, the simplest buffer system, did not interfere with Edman degradation sequence analysis and minimally suppressed sample ionization during MALDI-MS analysis. However, we found that MMP-2 was not active in PBS. All MMPs were active in TNC buffer, but the free amines from

Tris·HCl interfered with Edman degradation sequence analyses and reacted with fluorescamine in the fluorometric assay. Since fluorescamine reacts with primary but not secondary amines, substitution of Tris·HCl by tricine (which contains secondary amino groups) provided a buffer which was compatible with the fluorometric assay.

The THPs were first tested as potential substrates for MMP-1. THPs were found to be completely stable after overnight incubation in either PBS, TNC, or fluorometric buffer (see Fig. 3A). Following overnight reaction with MMP-1, RP-HPLC analysis indicated that the THPs were cleaved by MMP-1 (data not shown). Edman degradation sequence analysis of the α1(I)772–786 THP cleavage products (Fig. 3B) showed that MMP-1 hydrolysis occurred exclusively at the Gly–Ile bond, as the only amino
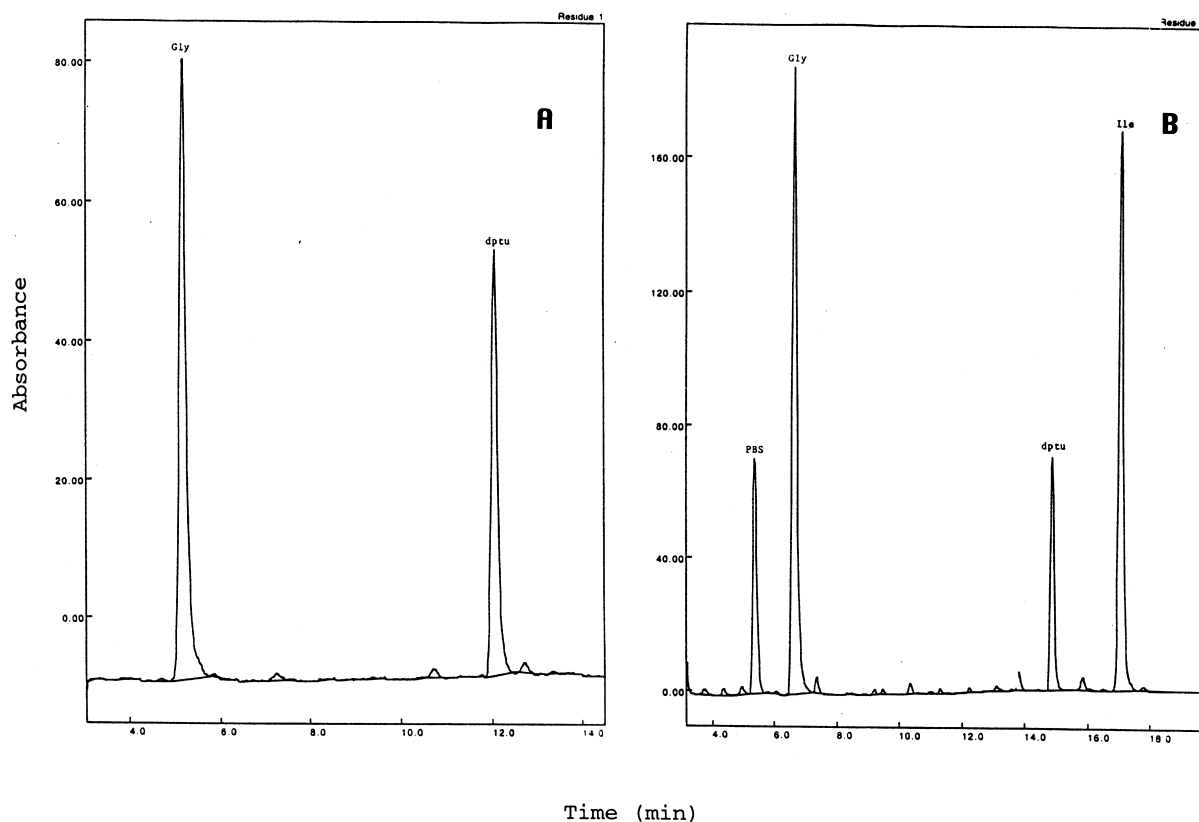

Time (min)

Fig. 3. Edman degradation sequence analysis of α1(I)772–786 THP following treatment with MMP-1. THP (40 μ*M*) was incubated with 67 n*M* MMP-1 in PBS for 24 h at 37°C. The reaction was stopped by addition of 3% (v/v) glacial acetic acid, and the reaction mixture was subject to Edman degradation sequence analysis. First cycle phenylthiohydantoin (PTH)-amino acid profiles are given for (A) THP and (B) THP hydrolysis by MMP-1. DPTU is diphenylthiourea.

acids seen in first cycle are PTH-Gly (emanating from the N-terminus of the intact THP or the N-terminal fragment of the cleaved THP) and PTH-Ile (emanating from the C-terminal fragment of the cleaved THP). The molar ratio of PTH-Ile to PTH-Gly was about 1:1, indicating complete cleavage of the THP. MMP-1 hydrolysis of the $\alpha 1(I)772$–786 THP was also examined by MALDI-MS. The intact $\alpha 1(I)772$–786 THP has a molecular mass of 9844. If the Gly–Ile bond is cleaved, the two products generated are the single-stranded N-terminal peptide (Gly-Pro-Hyp)$_6$-Gly-Pro-Gln-Gly ($[M+H]^+ =$ 1961.1 u) and the branched C-terminal peptide [Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp]$_3$-(Lys,Lys)-Tyr-Gly ($[M+3H]^+ =4033.7$ u). Mass spectrometric analysis of MMP-1 hydrolysis showed two products of $[M+H]^+ =1965.4$ u and $[M+3H]^+ =4039.8$ u (Table 1). Thus, both Edman degradation sequence analysis and MALDI-MS analyses indicated that MMP-1 cleaved the $\alpha 1(I)772$–786 THP exclusively at the Gly–Ile bond. This is the analogous bond cleaved by MMP-1 in the native $\alpha 1(I)$ collagen chain [3,25]. In similar fashion, MMP-1 cleaved the C_6-[$\alpha 1(I)772$–786] and C_{10}-[$\alpha 1(I)772$–786] peptide-amphiphiles exclusively at the Gly–Ile bond (Fig. 4 and Table 1). The rate of THP cleavage could be evaluated from fluorometric

analysis (Fig. 5). Based on the shapes of the hydrolysis curves, it appears that all three chains in the THP are cleaved in rapid succession. This result is consistent with that for MMP hydrolysis of another triple-helical peptide [26,27] and triple-helical collagens, in that all three strands of the triple-helix are cleaved rapidly and no intermediates are detected.

The three THPs were then used to study the triple-helical activity of other MMPs. Sequence and MALDI-MS analyses indicated that MMP-2 cleaved the THP at two loci, Gly–Ile and Gly–Gln (Table 1). The ratio of cleavage was 3:1, determined by comparing PTH-Val to PTH-Leu in cycle 7 of sequence analysis (data not shown). Fluorometric analysis of THP hydrolysis by MMP-2 would thus need to take into account the additional amino groups produced in order to compare kinetic parameters to MMP-1. In contrast to MMP-1 and MMP-2, neither sequence nor MS analysis could detect any MMP-3 cleavage of the C_6-[$\alpha 1(I)772$–786] and C_{10}-[$\alpha 1(I)772$–786] peptide-amphiphiles, while extended treatment (48 h) of the $\alpha 1(I)772$–786 THP with MMP-3 did result in some hydrolysis of the Gly–Ile bond (Table 1). Simultaneous studies using the fluorogenic substrate NFF-3 indicated that MMP-3 was highly active. For example, treatment of NFF-3 with MMP-3 resulted

Table 1
MALDI-MS analysis of THP hydrolysis by MMPs[a]

Enzyme	Substrate	Products (Da)	Nature of product
MMP-1	$\alpha 1(I)772$–786 THP	1965.4 (major)	N-terminal fragment, Gly–Ile cleavage
		4039.8 (major)	C-terminal fragment, Gly–Ile cleavage
MMP-1	C_6-[$\alpha 1(I)772$–786]	2175.4 (major)	C-terminal fragment, Gly–Ile cleavage
		3675.3 (minor)	Intact substrate
MMP-1	C_{10}-[$\alpha 1(I)772$–786]	1602.5 (minor)	N-terminal fragment, Gly–Ile cleavage
		2180.9 (minor)	C-terminal fragment, Gly–Ile cleavage
		3743.8 (major)	Intact substrate
MMP-2	$\alpha 1(I)772$–786 THP	1983.0 (Na$^+$ salt, major)	N-terminal fragment, Gly–Ile cleavage
		2224.7 (Na$^+$ salt, major)	N-terminal fragment, Gly–Gln cleavage
MMP-2	C_6-[$\alpha 1(I)772$–786]	1588.5 (major)	N-terminal fragment, Gly–Ile cleavage
		1933.6 (major)	C-terminal fragment, Gly–Gln cleavage
		2171.2 (major)	C-terminal fragment, Gly–Ile cleavage
MMP-2	C_{10}-[$\alpha 1(I)772$–786]	1601.0 (minor)	N-terminal fragment, Gly–Ile cleavage
		1843.1 (minor)	N-terminal fragment, Gly–Gln cleavage
		3732.0 (major)	Intact substrate
MMP-3	$\alpha 1(I)772$–786 THP	1978.2 (Na$^+$ salt)	N-terminal fragment, Gly–Ile cleavage
MMP-3	C_{10}-[$\alpha 1(I)772$–786]	3728.5	Intact substrate

[a] Hydrolysis was allowed to proceed for 45–72 h at 30–37°C.

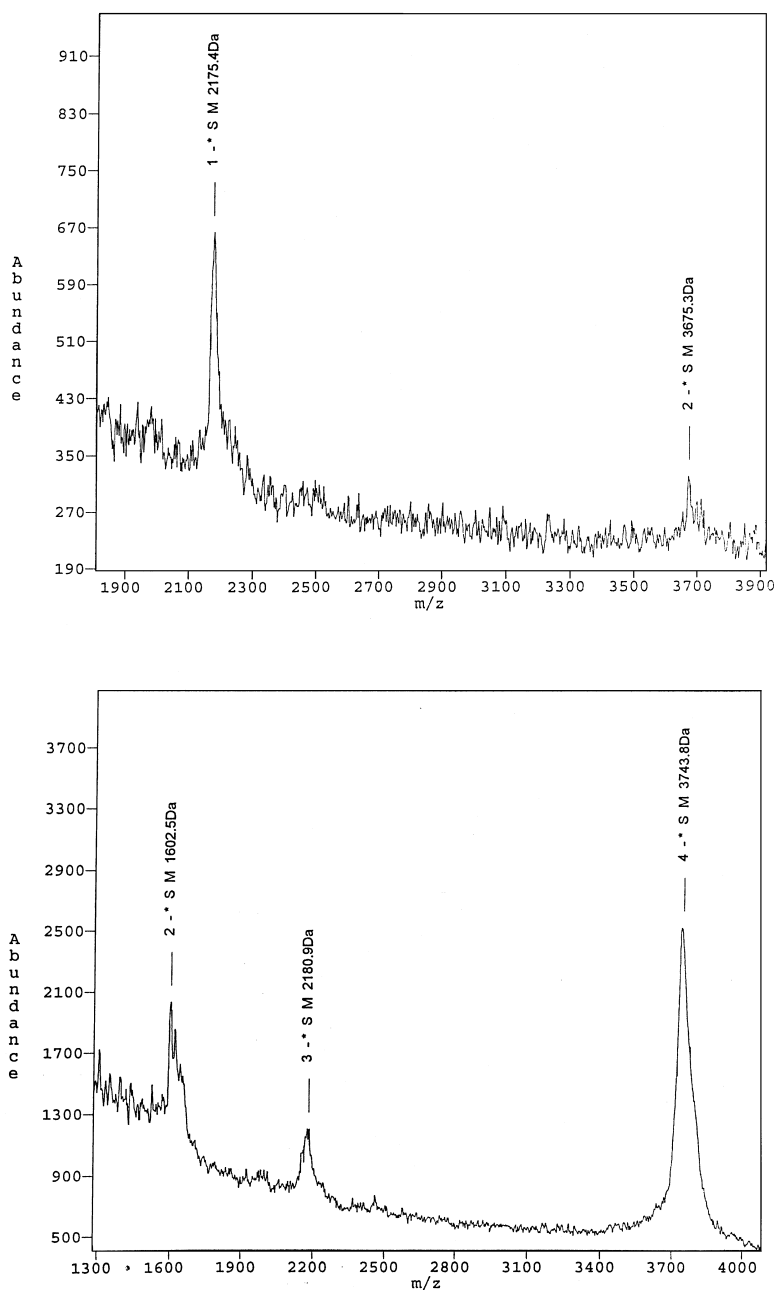Fig. 4. MALDI-TOF-MS analysis of C$_6$-[α1(I)772–786] (top) and C$_{10}$-[α1(I)772–786] (bottom) peptide-amphiphiles following treatment with MMP-1. C$_6$-[α1(I)772–786] or C$_{10}$-[α1(I)772–786] (20–40 μ*M*) was incubated with 40 n*M* MMP-1 in fluorometric assay buffer for 45–72 h at 30°C. See Table 1 for correlation of masses to peptide fragments.
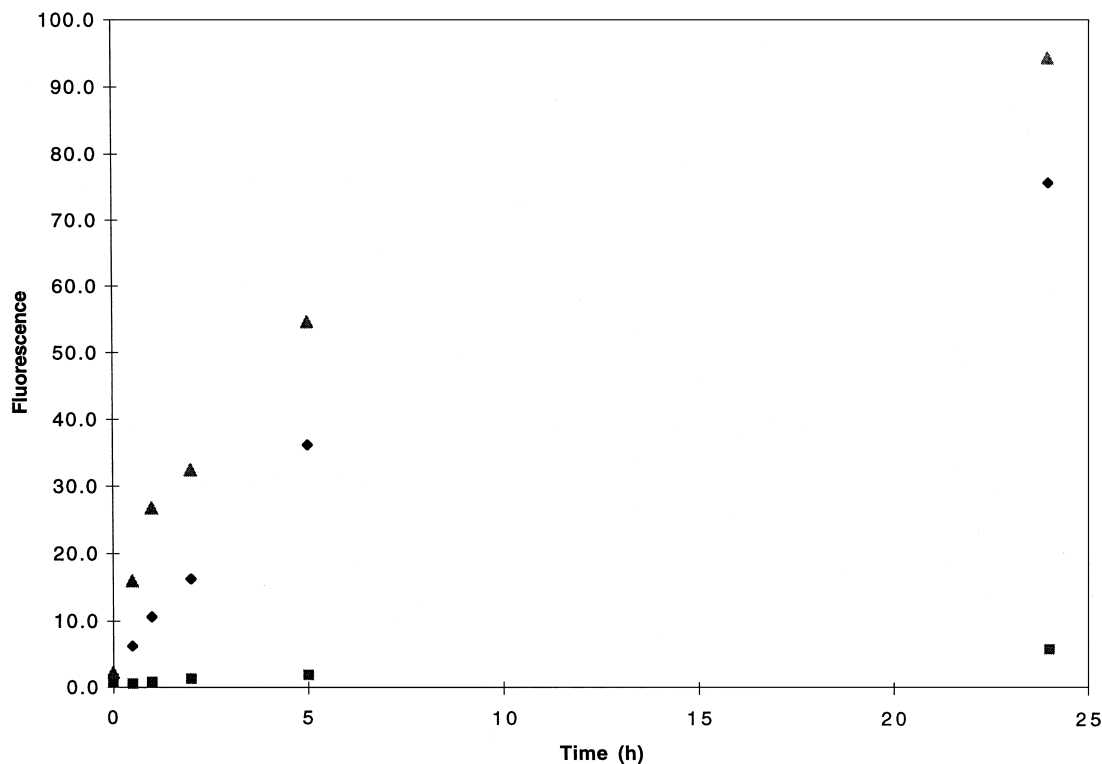
Fig. 5. Fluorometric analysis of $\alpha1(I)772$–786 THP (triangles), C_6-[$\alpha1(I)772$–786] (diamonds), and C_{10}-[$\alpha1(I)772$–786] (squares) following treatment with MMP-1. Each THP (40 μM) was incubated with 40 nM MMP-1 in fluorometric assay buffer at 30°C. Enzymatic activity was terminated by the addition of 20 μl of the enzyme–substrate solution to 30 μl of o-phenanthroline (20 mM). Rates of hydrolysis were monitored at $\lambda_{\text{excitation}}=387$ nm and $\lambda_{\text{emission}}=480$ nm following the addition of 200 μl of 5 mM fluorescamine solution.

in a rapid increase in substrate fluorescence and MS detection of two peptide products corresponding to Mca-Arg-Pro-Lys-Pro-Val-Glu and Nva-Trp-Arg-Lys(Dnp)-NH$_2$ (data not shown).

4. Conclusions

A variety of structural analyses have been performed previously on MMP family members, and the functions of the enzyme domains have been proposed based on their behavior towards native collagen [28]. In the present study we examined the suitability of synthetic THPs as peptide models of native collagens. THPs would allow for a better understanding of the specific functions of MMP domains, and could be used as selective substrates and subsequently specific inhibitors of these enzymes. We found that cleavage of the THPs did

occur with MMP-1, and only at the analogous Gly$_{775}$–Ile$_{776}$ bond found with native collagen. Ottl et al. [26,27] have demonstrated that a triple-helical peptide model of the [$\alpha1(I)$]$_2\alpha2(I)772$–785 region is cleaved by MMP-8 at a single bond. We can thus assume that THPs contain all the necessary information to direct the MMP binding and proteolysis. MMP-2 was also found to cleave the THPs. Cleavage occurred at two sites, the Gly–Ile bond and the Gly–Gln bond. MMP-3 had little or no catalytic activity towards the three THPs. Since MMP-3 has been found to cleave sequences analogous to $\alpha1(I)772$–786 in single-stranded form [25], the minimal cleavage of the $\alpha1(I)772$–786 THP suggested that triple-helical structure could be used to construct substrates and inhibitors that discriminate among MMP family members. The reduced MMP-3 activity also confirms the triple-helical nature of the substrate. Finally, development of the fluorometric

assay presented here will allow for the evaluation of kinetic parameters for MMP hydrolysis of THPs.

Acknowledgements

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