Anomalous pH-dependence of the activity of human matrilysin (matrix metalloproteinase-7) as revealed by nitration and amination of its tyrosine residues

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Matrilysin activity exhibits a broad bell-shaped pH-dependence profile, with pK_a values of 4.0 and 9.8. A maximum of five out of eight tyrosine residues in matrilysin were nitrated with tetranitromethane. On nitration of between one and five tyrosines, pK_a at the alkaline side (pK_{e2}) was shifted from 9.8 to 10.3–10.6, while that at the acidic side (pK_{e1}) was not altered. The pK_{e2} that was shifted by nitration to 10.3–10.6 was restored to 9.4–9.7 by subsequent amination, suggesting that the shift in pK_{e2} is induced by a negative charge introduced on the most reactive tyrosine, Tyr-150. The Michaelis constant (K_m) observed at pH 10 was decreased by nitration as a result of the increase

in pK_{e2} , suggesting that the residue with pK_{e2} may play a role in the recognition of substrate. When four or five tyrosines were nitrated, the activity at pH < 7 decreased significantly, while that at pH 7–10 was unchanged, and thus the pH-dependence was not bell-shaped, but anomalous, with a third pK_a (pK_{e3}) of 6.2–6.4 in addition to pK_{e1} and pK_{e2} . This suggests the possibility that a newly introduced nitrotyrosine residue has a strong influence on the activity as an ionizable group.

Key words: enzyme activity, matrix metalloproteinase, matrilysin, nitrotyrosine, pH-dependence, tyrosine residue.

INTRODUCTION

The matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that are believed to degrade extracellular matrix components and take part in both normal and pathological processes, including development, reproduction, maintenance, tissue destruction, fibrotic diseases and weakening of the matrix [1,2]. The activities of MMPs are strictly regulated at multiple steps, e.g. gene expression, activation of precursor zymogens, and inhibition by endogenous inhibitors [3]. Matrilysin (MMP-7; EC 3.4.24.23) is one of the smallest MMPs, consisting of a single peptide, a propeptide involved in enzyme latency, and a catalytic domain with a zinc-binding site. The molecular mass of the latent pro-form of matrilysin is 28 kDa, and that of its mature form is 19 kDa [4,5]. According to X-ray crystallographic analysis of recombinant human matrilysin, it is composed of a five-stranded β -sheet and three α -helices, and contains a zinc ion that is essential for activity, as well as another zinc and two calcium ions that are regarded as necessary for stability [6]. Matrilysin is overexpressed in lesions of various tissues [7], and degrades extracellular matrix components [8], suggesting that matrilysin plays a critical role in tumour invasion and metastasis. In fact, matrilysin enhances the metastatic potential of tumour cells [7,9–11]. From this viewpoint, understanding the mechanistic aspects of this enzyme should be therapeutically beneficial. We have reported the high stability and denaturation properties of matrilysin [12,13]. The interaction of matrilysin with synthetic inhibitors has been studied, and the significance of hydrophobic interactions has been suggested in the recognition of the inhibitors at the active site of matrilysin [14]. Green tea catechins, especially those with a galloyl group, inhibit matrilysin in non-competitive manner with a K_i of 0.47–1.65 μ M [15].

The pH-dependence of matrilysin activity exhibits an extraordinarily broad bell-shaped curve, indicating the involvement

of at least two ionizable groups in its catalytic mechanism [14,16]. Glu-198 (the numbering of the amino acid residues of pro-matrilysin is applied in this paper to activated matrilysin, beginning at number 78 for the N-terminal tyrosine residue [17]) or a water molecule bound to the active zinc (Lewis acid) is considered to be the ionizable group of the acidic side with pK_{e1} [18–20]. (In this paper, the pK_a of the ionizable group on matrilysin is designated pK_e , and that on the enzyme–substrate complex is pK_c ; the suffixes 1 and 2 indicate that the respective pK_a s are on the acidic and alkaline sides respectively.) On the other hand, Tyr-219, which is conserved in all MMPs [21], is thought to control activity as the group responsible for pK_{e2} [14,16], although no decisive evidence for this has been reported. The objectives of the present study were to elucidate the functional groups and to provide insights into the reaction mechanism of matrilysin. Matrilysin contains eight tyrosine residues, at positions 78, 94, 99, 150, 193, 216, 219 and 241. Here we demonstrate the effects of the nitration and amination of these tyrosine residues on the pH-dependence of matrilysin activity. The pK_a value for a phenolic hydroxy group of tyrosine is 9.7–10.1 [22,23], and those of 3-nitrotyrosine and 3-aminotyrosine are 7.2 and 10.0 respectively [24]. Therefore chemical modification might be useful for changing the electrostatic features of the residues, and also the electrostatic states of proteins. We also demonstrate the possibility that at least three functional groups are involved in the activity of matrilysin. The present study provides us with information on the group responsible for pK_{e2} , and also new insights into the catalytic mechanism of matrilysin.

EXPERIMENTAL

Materials

Recombinant human pro-matrilysin was prepared and converted into mature matrilysin according to the method reported

Abbreviations used: L(Dpa)AR, L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂; MMP, matrix metalloproteinase; MOCAC-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAC-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂; TNM, tetranitromethane.

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Figure 1 Nitration of tyrosine residues in matrilysin with TNM

(A) Absorption spectrum of matrilysin containing 3.7 nitrotyrosine residues measured in buffer A at pH 7.5 and 25 °C. (B) Dependence of the number of tyrosine residues nitrated on the TNM concentration. The number of tyrosine residues nitrated increased up to 5.1 with increasing [TNM]/[matrilysin] ratios (mol/mol).

previously [25]. The concentrations of pro-matrilysin and matrilysin were determined using molar absorption coefficients at 280 nm (ϵ_{280}) of 41.0 and 31.8 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ respectively [4]. MOCac-PLGL(Dpa)AR {(7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂; lot 490418} and MOCac-PLG [(7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; lot 490908] were purchased from the Peptide Institute (Osaka, Japan). The substrate is cleaved at the peptide bond between glycine and leucine residues on hydrolysis by matrilysin [26]. The concentrations of MOCac-PLGL(Dpa)AR and MOCac-PLG were determined using the molar absorption coefficients $\epsilon_{410} = 7.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{324} = 12.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ respectively [14,26]. A pUC19 plasmid containing the entire matrilysin cDNA was kindly provided by Dr K. Tanzawa (Sankyo, Tokyo, Japan). *Escherichia coli* BL21 (DE3) cells and pET-22b(+) vector were obtained from Novagen, Inc. (Madison, WI, U.S.A.). TNM (tetranitromethane; lot 04218CS) was from Aldrich (Steinheim, Germany). Pepsin (lot 118H7560) was from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). All spectrophotometric measurements were done with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan).

Nitration and amination of tyrosine residues in matrilysin

The nitration of matrilysin was performed by adding an appropriate volume of 1 or 10% (w/v) TNM solution in methanol to matrilysin solution (8.6–11 μM) in 50 mM Hepes buffer (pH 7.5) containing 10 mM CaCl₂ and 0.05% Brij-35 (buffer A) at 25 °C. The reaction mixture was maintained at 25 °C for 1 h and dialysed thoroughly against buffer A at 4 °C. The dialysed solution was subjected to gel filtration on a Sephadex G-25 (PD-10) column [1.5 cm (inner diameter) $\times$ 5 cm] equilibrated with buffer A at 25 °C. The number of tyrosine residues nitrated and the concentration of nitrated matrilysin were determined spectrophotometrically from the absorbance at 275 nm and that at 381 nm (a nitrophenol–nitrophenolate isosbestic point of 3-nitro-L-tyrosine) (Figure 1A) [23,27,28], using the following equations [28]:

$$A_{275} = 31\,900t + (4000 - 1360)m \quad (1)$$

$$A_{381} = 2200m \quad (2)$$

where t and m represent the molar concentrations of total matrilysin and nitrated tyrosine residues respectively. The molar absorption coefficients employed were 31 900 $\text{M}^{-1} \cdot \text{cm}^{-1}$ for native matrilysin, 4000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ for *N*-acetyl-3-nitrotyrosine [27],

Scheme 1 Reaction scheme for the pH-dependence of matrilysin activity with two ionizable groups involved in the enzymic activity

See the text for details.

1360 $\text{M}^{-1} \cdot \text{cm}^{-1}$ for *N*-acetyl-tyrosine (all at 275 nm) [27], and 2200 $\text{M}^{-1} \cdot \text{cm}^{-1}$ for *N*-acetyl-3-nitrotyrosine at 381 nm [24]. The nitrated matrilysin was reduced to the aminated enzyme with a 270–550-fold molar excess of sodium hydrosulphite in 50 mM Hepes buffer containing 10 mM CaCl₂ at pH 7.5 and 25 °C [24,29]. The end of amination was confirmed spectrophotometrically by disappearance of the absorbance of 3-nitrotyrosine at 381 nm. The reaction was completed within 2 min.

Fluorimetric analysis of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

The hydrolysis of MOCac-PLGL(Dpa)AR by matrilysin was initiated by mixing 1222 μl of assay buffer (see below), 20 μl of matrilysin solution (300–530 nM) and 8 μl of MOCac-PLGL(Dpa)AR (234 μM) dissolved in DMSO at 25 °C, and measured by following the increase in fluorescence emission intensity at 393 nm following excitation at 328 nm with a Jasco FP-777 fluorescence spectrophotometer (Jasco, Tokyo, Japan) equipped with a cell holder at a constant temperature. The assay buffers were 50 mM sodium acetate buffer (pH 3.6–5.8), 50 mM Mes buffer (pH 5.6–7.0), 50 mM Hepes buffer (pH 6.8–8.6) and 50 mM AMPPO {3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane sulphonic acid} buffer (pH 8.4–10.4), each containing 10 mM CaCl₂ at 25 °C. The reaction was carried out under pseudo-first-order conditions, whereby the substrate concentration, $[S]$, is much lower than the Michaelis constant, K_m , in order to avoid absorptive quenching effects of the substrate. Under these conditions the reaction velocity, v , is expressed as $k_{\text{cat}}[E]_0[S]/K_m$, where k_{cat} is the catalytic constant and $[E]_0$ is the initial enzyme concentration in the reaction mixture, and the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m .

Kinetic analysis of the pH-dependence of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

At first, the pH-dependence of k_{cat}/K_m for each of native, nitrated and aminated matrilysins was analysed by modelling an enzyme (EH_2) with two dissociable groups (Scheme 1) [30,31]. The proton dissociation constants (K_{e1} , K_{e2} , K_{es1} and K_{es2}), the substrate constants (K_s , K_s' and K_s'') and the catalytic constants (k_{cat}) are defined as shown in Scheme 1. The apparent k_{cat}/K_m [$k_{\text{cat}}/K_m(\text{app})$] is expressed as:

$$(k_{\text{cat}}/K_m)(\text{app}) = \frac{(k_{\text{cat}}/K_m)_0}{1 + \frac{[H]}{K_{e1}} + \frac{K_{e2}}{[H]}} \quad (3)$$

Scheme 2 Reaction scheme for the pH-dependence of matrilysin activity with three ionizable groups involved in the enzymic activity

The product, P, is formed not only from the EHS complex, but also from the EH_2S complex. See the text for further details.

where $[\text{H}]$ is the proton concentration, and $(k_{\text{cat}}/K_m)_o$ is the intrinsic or pH-independent k_{cat}/K_m derived from the EHS complex (see Scheme 1). The kinetic parameters $(k_{\text{cat}}/K_m)_o$, $\text{p}K_{e1}$ and $\text{p}K_{e2}$ for the pH-dependence of k_{cat}/K_m for each enzyme were determined from eqn (3) using the non-linear least-squares method [32].

However, Scheme 1 was not applicable to the pH-dependence of k_{cat}/K_m for matrilysin containing between 3.7 and 4.8 nitrotyrosines. Instead, Scheme 2, which illustrates an enzyme (EH_3) with three dissociable groups and in which the ES complexes of EHS and EH_2S give products, was applied to determine the kinetic parameters of these nitrated enzymes [30,31]. The proton dissociation constants (K_{e1} , K_{e2} , K_{e3} , K_{es1} , K_{es2} and K_{es3}), the substrate constants (K_s , K_s' , K_s'' and K_s''') and the catalytic constants (k_{cat} and $k_{\text{cat}'}$) are defined in Scheme 2. The reaction rate, v , is expressed as:

$$v = k_{\text{cat}}[\text{EHS}] + k_{\text{cat}'}[\text{EH}_2\text{S}] \quad (4)$$

Under pseudo-first-order conditions, the values of K_{es1} , K_{es2} and K_{es3} are considered to approximate to those of K_{e1} , K_{e2} and K_{e3} respectively. Then k_{cat}/K_m (app) is expressed as:

$$\begin{aligned}
 (k_{\text{cat}}/K_m)_{\text{(app)}} = & \frac{(k_{\text{cat}}/K_m)_o}{1 + \frac{K_{e2}}{[\text{H}]} + \frac{[\text{H}]}{K_{e3}} + \frac{[\text{H}]^2}{K_{e3}K_{e1}}} \\
 & + \frac{(k_{\text{cat}}/K_m)_o'}{1 + \frac{[\text{H}]}{K_{e1}} + \frac{K_{e3}}{[\text{H}]} + \frac{K_{e2}K_{e3}}{[\text{H}]^2}} \quad (5)
 \end{aligned}$$

where $(k_{\text{cat}}/K_m)_o$ and $(k_{\text{cat}}/K_m)_o'$ are the intrinsic k_{cat}/K_m values derived from the EHS and EH_2S complexes respectively. The kinetic parameters $(k_{\text{cat}}/K_m)_o$, $(k_{\text{cat}}/K_m)_o'$ and $\text{p}K_e$ for the pH-dependence of k_{cat}/K_m for matrilysin containing 3.7–4.8 nitrotyrosines were determined from eqn (5).

HPLC analysis of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

The hydrolysis of MOCac-PLGL(Dpa)AR was initiated by adding 30 μl of MOCac-PLGL(Dpa)AR (0.11–3.0 mM) dissolved

in DMSO to 970 μl of a matrilysin solution dissolved in assay buffer at pH 5.0–10.0. The initial concentrations of matrilysin and DMSO in the reaction mixture were 6.0–8.5 nM and 3.0% (v/v) respectively. The reaction was stopped at appropriate times by mixing 100 μl of the reaction solution with 400 μl of 1% (v/v) trifluoroacetic acid. The mixture (100 μl) was applied to reverse-phase HPLC performed on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) $\times$ 150 mm; Tosoh, Tokyo, Japan] equilibrated with 0.1% trifluoroacetic acid. A linear gradient was generated from 20% to 70% (v/v) acetonitrile at a retention time of 5 min over 15 min at a flow rate of 1.0 ml/min, and the absorbance of the eluate was detected at 335 nm. The substrate and its two products, MOCac-PLG and L(Dpa)AR {L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂}, were separated [13]. The initial rate, v , was determined from the time course of the amount of MOCac-PLG produced, which was evaluated from its peak area. The kinetic parameters k_{cat} and K_m were determined based on the Michaelis–Menten equation using non-linear least-squares methods [32]. The HPLC apparatus, consisting of a CCPM solvent delivery system, a UV-8010 UV monitoring system, a PX-8010 computer control system, and a Chromatocoder 21 integrator, was purchased from Tosoh.

CD analysis

A Jasco J-720 spectropolarimeter equipped with a Peltier system for cell temperature control was used for CD measurements. Calibration of the system and spectropolarimeter conditions were performed as described previously [33].

Determination of the nitrated tyrosine residue

Nitrated matrilysin was dialysed thoroughly against 100 mM Gly/HCl buffer (pH 3.0) at 4°C. The dialysed enzyme was digested with pepsin at an enzyme/substrate ratio of 1:50 (w/w) in 100 mM Gly/HCl (pH 3.0) at 37°C for 24 h. Then the peptide fragments were separated by reverse-phase HPLC with a TSKgel ODS-80Ts column [4.6 mm (inner diameter) $\times$ 250 mm] using the same system as described above. Peptides containing nitrotyrosine residues were sequenced by automated Edman degradation with a model 477A pulse-liquid sequencer system (Applied Biosystems, Foster City, CA, U.S.A.).

Matrilysin assay with azocoll substrate

Azocoll substrate (< 50 mesh; lot B10515) was purchased from Calbiochem (La Jolla, CA, U.S.A.). The assay was performed according to the method described previously [12]. Azocoll (3.0 mg) was suspended in 2.0 ml of 50 mM Hepes buffer, pH 7.5, containing 10 mM CaCl₂, and incubated at 25°C for 15 min before hydrolysis was initiated. Enzyme solution (2.0 ml; 0.17–0.50 μM) was added to the azocoll suspension at 25°C, and the reaction was stopped at an appropriate time by removing insoluble azocoll by filtration with Whatman paper filter. The initial concentrations of the native and nitrated matrilysins containing 4.8 nitrotyrosine residues in the reaction mixture were 0.34 and 0.33 μM respectively, and the azocoll concentration was 0–2.0 mg/ml. Hydrolysis at each azocoll concentration was performed in triplicate and monitored by measuring absorbance at 520 nm.

Site-directed mutagenesis of Tyr-150 in matrilysin

A pET-matrilysin plasmid expressing mature matrilysin was constructed in a similar way to that reported previously by Cha et al. [16]. A site-directed mutation was introduced into matrilysin using a GeneEditor™ *in vitro* site-directed mutagenesis

Figure 2 Effects of nitration of tyrosine residues on matrilysin activity at pH 7.5 and 25 °C

The reaction was performed in 50 mM Hepes buffer containing 10 mM CaCl_2 and 0.6% DMSO at pH 7.5 and 25 °C. The initial concentration of the native and nitrated matrilysins was 4.8–8.5 nM, and that of the substrate MOCac-PLGL(Dpa)AR was 1.5 μM . Matrilysin activity was not affected by nitration, and the specific constant, $k_{\text{cat}}/K_{\text{m}}$, was $(1.4\text{--}1.6) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

system (Promega, Madison, WI, U.S.A.). The oligonucleotide 5' ATGGGGACTCCGACCCATTTGATGG 3' was used for mutation of Tyr-150 to Asp. The introduced mutation was confirmed by sequencing with a SQ5500E DNA sequencer (Hitachi, Tokyo, Japan) and a Thermo Sequenase cycle sequencing kit (Amersham Life Science, Cleveland, OH, U.S.A.). The mutant matrilysin was expressed in *E. coli* BL21 (DE3) as insoluble inclusion bodies. The inclusion bodies were solubilized and refolded according to the method proposed previously [25], and the mutant enzyme was purified using the method of Cha et al. [16] with some modifications. The hydrolysis of MOCac-PLGL(Dpa)AR by the mutant enzyme was measured fluorimetrically as described above.

RESULTS

Nitration of tyrosine residues in matrilysin

The nitration of matrilysin was carried out at $[\text{TNM}]/[\text{matrilysin}]$ ratios of up to 7900:1 (mol/mol) in buffer A at 25 °C for 1 h. The number of tyrosine residues nitrated was increased dramatically up to 4.8 with increasing $[\text{TNM}]/[\text{matrilysin}]$ ratios up to 2000:1 (mol/mol), reaching a maximal level. Five residues (4.8–5.1) were nitrated at ratios from 2000:1 to 7900:1 (mol/mol) (Figure 1B). Under these conditions, a ratio of 2000:1 (mol/mol) was sufficient to achieve maximal nitration, and a maximum of five out of the eight tyrosine residues in matrilysin were nitrated. The CD spectra of the nitrated matrilysins at 195–260 nm were essentially the same as that of the native enzyme, suggesting that significant conformational change was not induced in matrilysin by nitration (results not shown). The nitrated matrilysin showed almost the same activity as the native enzyme at pH 7.5 and 25 °C, irrespective of the number of tyrosine residues nitrated (Figure 2). The nitrated residues should be ionized and negatively charged at pH 7.5, although nitration of matrilysin had no influence on its activity at pH 7.5.

Effects of nitration on the pH-dependence of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

We examined the effect of nitration on the pH-dependence of $k_{\text{cat}}/K_{\text{m}}$ in the pH range 3.6–10.4 at 25 °C (Figure 3). With native matrilysin, the pH-dependence exhibited a bell-shaped curve, with a catalytically optimum pH within the wide range 5.5–8.5

Figure 3 Effects of nitration on the pH-dependence of matrilysin activity

(A) pH-dependence of native matrilysin activity. The initial concentrations of the enzyme and MOCac-PLGL(Dpa)AR were 6.3 nM and 1.5 μM respectively. The $(k_{\text{cat}}/K_{\text{m}})_0$ value was $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ were 4.0 and 9.8 respectively. (B) pH-dependence of the activity of matrilysin containing 2.1 nitrotyrosines. The initial concentrations of the enzyme and substrate were 7.0 nM and 1.5 μM respectively. The $(k_{\text{cat}}/K_{\text{m}})_0$ value was $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ were 4.0 and 10.4 respectively. (C) pH-dependence of the activity of matrilysin containing 4.8 nitrotyrosines. The initial concentrations of the enzyme and substrate were 4.8 nM and 1.5 μM respectively. The $(k_{\text{cat}}/K_{\text{m}})_0$ and $(k_{\text{cat}}/K_{\text{m}})_0'$ values were $1.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $9.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively, and $\text{p}K_{\text{e1}}$, $\text{p}K_{\text{e2}}$ and $\text{p}K_{\text{e3}}$ were 4.2, 10.4 and 6.4 respectively.

(Figure 3A). The acidic and alkaline $\text{p}K_{\text{e}}$ values ($\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$) were determined to be 4.0 and 9.8 respectively, on the basis of Scheme 1. These values are consistent with previously reported values of 4.0 and 9.6 [14,16]. On the other hand, on nitration of 2.1 tyrosine residues, $\text{p}K_{\text{e2}}$ was shifted from 9.8 to 10.4, while $\text{p}K_{\text{e1}}$ remained constant at 4.0 (Figure 3B). $\text{p}K_{\text{e2}}$ for matrilysin containing 4.8 nitrotyrosine residues was the same as that for matrilysin containing 2.1 nitrotyrosines. However, the activity of matrilysin containing 4.8 nitrotyrosines decreased significantly at pH values below 7, and an anomalous pH-dependence profile with a shoulder around pH 5.5 was observed (Figure 3C). This pH-dependence can be analysed better using Scheme 2 rather than Scheme 1, suggesting that the activity of the matrilysin containing 4.8 nitrotyrosines is controlled not only by two ionizable groups with $\text{p}K_{\text{e1}} = 4.2$ and $\text{p}K_{\text{e2}} = 10.4$, but also additionally by a third group with a $\text{p}K_{\text{e3}}$ of 6.4. The parameters for the pH-dependence of the activity of the native and nitrated matrilysins are summarized in Table 1. The effect on $\text{p}K_{\text{e2}}$ was completed by introducing 1.3 nitrotyrosines. The bell-shaped pH-dependence remained up to 2.6 nitrotyrosines, and anomalous pH-dependence emerged when the number of nitrotyrosines was increased from 2.6 to 3.7.

Effects of amination on the pH-dependence of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

The effect of amination on the pH-dependence of $k_{\text{cat}}/K_{\text{m}}$ was examined in comparison with that of nitration (Figure 4). The pH-dependence of matrilysin containing 2.1 aminotyrosines was superimposed upon that of the native enzyme, and its $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ values were 4.1 and 9.7 respectively (Figure 4A). The $\text{p}K_{\text{e2}}$ that was shifted from 9.8 to 10.4 by nitration of 2.1 tyrosines was entirely restored to 9.7 by subsequent amination. Given that the size of an amino group is similar to that of a nitro group, the $\text{p}K_{\text{e2}}$ shift induced by nitration might be attributed to negative

Table 1 Kinetic parameters for the pH-dependence of the activity of native and nitrated matrilysins at 25 °CValues are means $\pm$ S.D.

No. of Tyr residues nitrated	$10^{-5} \times (k_{\text{cat}}/K_m)_0$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^{-5} \times (k_{\text{cat}}/K_m)_0'$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$\text{p}K_{\text{e1}}$	$\text{p}K_{\text{e2}}$	$\text{p}K_{\text{e3}}$
0	1.54 ± 0.01	3.97 ± 0.01	9.83 ± 0.01		
1.3	1.42 ± 0.01	4.03 ± 0.02	10.27 ± 0.02		
2.1	1.49 ± 0.01	4.02 ± 0.02	10.38 ± 0.03		
2.6	1.45 ± 0.01	4.15 ± 0.02	10.52 ± 0.02		
3.7	1.44 ± 0.01	1.05 ± 0.03	4.24 ± 0.03	10.56 ± 0.02	6.22 ± 0.11
4.8	1.52 ± 0.01	0.93 ± 0.03	4.24 ± 0.04	10.40 ± 0.04	6.39 ± 0.07

Figure 4 Effects of amination on the pH-dependence of matrilysin activity in comparison with those of nitration

(A) pH-dependence of the activity of matrilysin containing 2.1 aminotyrosines (●) and that of the enzyme containing 2.1 nitrotyrosines (○). The initial concentrations of the enzyme and substrate were 7.0 nM and 1.5 μM respectively. The $(k_{\text{cat}}/K_m)_0$ value for the aminated enzyme was $1.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ were 4.1 and 9.7 respectively. (B) pH-dependence of the activity of matrilysin containing 4.8 aminotyrosines (●) and that of the enzyme containing 4.8 nitrotyrosines (○). The initial concentrations of the enzyme and substrate were 4.8 nM and 1.5 μM respectively. The $(k_{\text{cat}}/K_m)_0$ value for the aminated enzyme was determined to be $8.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ were 4.4 and 9.4 respectively.

Table 2 Kinetic parameters for the pH-dependence of the activity of aminated matrilysins at 25 °CValues are means $\pm$ S.D.

No. of Tyr residues aminated	$10^{-5} \times (k_{\text{cat}}/K_m)_0$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$\text{p}K_{\text{e1}}$	$\text{p}K_{\text{e2}}$
2.1	1.43 ± 0.01	4.14 ± 0.02	9.66 ± 0.02
3.7	0.83 ± 0.02	4.23 ± 0.03	9.38 ± 0.03
4.8	0.83 ± 0.02	4.35 ± 0.02	9.37 ± 0.02

charges introduced on the tyrosine residues. It is also apparent that 1.3–2.1 aminotyrosines had no effect on maximal activity. On the other hand, the pH-dependence of matrilysin containing 4.8 aminotyrosines, unlike that of the nitrated enzyme, showed a bell-shaped curve, with $\text{p}K_{\text{e1}}$ and $\text{p}K_{\text{e2}}$ values of 4.3 and 9.3 respectively, on the basis of Scheme 1 (Figure 4B). However, the activity (k_{cat}/K_m) of this aminated matrilysin was approx. 50 % of the maximal activity of the native enzyme and that of the matrilysin containing 4.8 nitrotyrosines in the alkaline pH region. The kinetic parameters for the pH-dependence of the activity of the aminated matrilysins are summarized in Table 2. The anomalous pH-dependence profile observed on introducing 3.7–4.8 nitrotyrosine residues was reversed following their amination to the bell-shaped profile, accompanied by the decrease in maximal activity.

The introduction of 4–5 aminotyrosines decreased $(k_{\text{cat}}/K_m)_0$ significantly without changing the $\text{p}K_{\text{e}}$ values. The $(k_{\text{cat}}/K_m)_0$ value of matrilysin containing 4–5 aminotyrosines was the same as that of the enzyme containing 4–5 nitrotyrosines when the group

Figure 5 HPLC analysis of effects of nitration on the kinetic parameters k_{cat} and K_m for matrilysin activity

The reaction was performed in assay buffer (pH 5.0–10.0) plus 3.0 % (v/v) DMSO at 25 °C. The initial concentrations of the native and nitrated matrilysins were in the range 6.0–8.5 nM, and that of the substrate was 10–91 μM . (A) pH-dependence of the catalytic constant (k_{cat}) for native matrilysin (○) and matrilysins containing 2.1 (△) and 4.8 (□) nitrotyrosines. (B) pH-dependence of the Michaelis constant (K_m) for native matrilysin (○) and matrilysins containing 2.1 (△) and 4.8 (□) nitrotyrosines.

with $\text{p}K_{\text{e3}}$ is protonated at pH < 6 (Figure 4B), and the $(k_{\text{cat}}/K_m)_0$ for the aminated enzyme is similar to the $(k_{\text{cat}}/K_m)_0'$ for the nitrated enzyme. When the $\text{p}K_{\text{e3}}$ group is protonated, the introduction of 4–5 aminotyrosines or nitrotyrosines decreases $(k_{\text{cat}}/K_m)_0$ to 60–70 % of that of the native enzyme; however, when the $\text{p}K_{\text{e3}}$ group is deprotonated, the value for the matrilysin containing 4–5 nitrotyrosines recovers to that of the native enzyme (Tables 1 and 2).

Effects of nitration on k_{cat} and K_m for the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR

In the fluorophotometric assay of the matrilysin-catalysed hydrolysis of MOCac-PLGL(Dpa)AR, which was inevitably performed in the presence of 0.5 % DMSO, the kinetic parameters, k_{cat} and K_m , could not be determined separately because of the sparing solubility of the substrate. In order to circumvent this point, hydrolysis was monitored by HPLC. The substrate solubility is very low in aqueous solution, but increases up to 90 μM by adding 3 % (v/v) DMSO [13]. As this substrate concentration is much higher than the K_m value, it is possible to determine k_{cat} and K_m separately, although fluorescence detection was not available because of the inner-filter effect of the substrate. Accordingly, the products were detected by HPLC. The k_{cat} and K_m values for native matrilysin at pH 7.5 and 25 °C were determined to be $6.3 \pm 0.3 \text{ s}^{-1}$ and $62 \pm 6 \mu\text{M}$ respectively.

Figure 5 shows the pH-dependence of k_{cat} and K_m for native matrilysin and the matrilysins containing 2.1 and 4.8 nitrotyrosines. For the native enzyme, k_{cat} was almost constant at pH 5–9,

Table 3 Effects of nitration on the matrilysin-catalysed hydrolysis of azocoll

Matrilysin activity against azocoll was measured in 50 mM Hepes buffer (pH 7.5) containing 10 mM CaCl_2 at 25 °C. Values are means $\pm$ S.D.

No. of Tyr residues nitrated	K_m (mg · ml ⁻¹)	k_{cat} (mg · ml ⁻¹ · M ⁻¹ · s ⁻¹)	k_{cat}/K_m (M ⁻¹ · s ⁻¹)
0	2.57 $\pm$ 0.22	337 $\pm$ 18	134 $\pm$ 16
4.8	2.29 $\pm$ 0.32	305 $\pm$ 26	140 $\pm$ 27

and decreased slightly at pH 10 (Figure 5A), while K_m showed a concave curve with significant increase at pH 10 (Figure 5B). The k_{cat} value of matrilysin containing 2.1 nitrotyrosines showed similar pH-dependence to that of the native enzyme, but the value at any pH was approx. 80–90 % of that of the native enzyme. On the other hand, K_m for the enzyme containing 2.1 nitrotyrosines was not much affected at pH 5–10, but the value at pH 10 was lowered significantly relative to that of the native enzyme. For matrilysin containing 4.8 nitrotyrosines, k_{cat} was almost 50 % of that of the native enzyme at pH 5–10, and K_m was lowered by 50 % at pH 7–9 in comparison with the native enzyme. This agrees well with the observation that the constant k_{cat}/K_m at pH 7.5 does not depend on the degree of nitration (Figure 2, Table 1). The decreases in k_{cat} and K_m suggest that the ES complex is more stabilized and the transition state is less stabilized by increasing the degree of nitration. The ES complex stabilization observed at pH 10 might correspond to the shift in $\text{p}K_{\text{e}2}$ from 9.8 to 10.3–10.6. The anomalous pH-dependence of k_{cat}/K_m for matrilysin containing 4.8 nitrotyrosines is brought about synergistically by the complex changes in k_{cat} and K_m .

The effect of nitration on matrilysin activity against azocoll was also examined at pH 7.5. The k_{cat} and K_m values of matrilysin containing 4.8 nitrotyrosines were the same as those of the native enzyme (Table 3). This suggests that the decreased k_{cat} and K_m values observed with MOCAC-PLGL(Dpa)AR might be derived from an interaction between a tyrosine residue in the enzyme and the arginine residue in the substrate. The decreased (k_{cat}/K_m)_o of the matrilysin containing 4–5 nitrotyrosines compared with the native enzyme at pH 4–6 may be due predominantly to the decrease in k_{cat} (Figures 3C and 5).

Effects of a negative charge at position 150 on the matrilysin-catalysed hydrolysis of MOCAC-PLGL(Dpa)AR

A tyrosine residue predominantly nitrated in the matrilysin containing 1.3 nitrotyrosines was identified as Tyr-150 by automated Edman degradation. In order to clarify the effects of a negative charge raised on Tyr-150 by nitration, it was replaced with aspartic acid by site-directed mutagenesis, and the catalytic properties of this mutant enzyme (Y150D) were examined. The activity (k_{cat}/K_m) of Y150D in the hydrolysis of MOCAC-PLGL(Dpa)AR showed a broad bell-shaped pH-dependence, like that of the native enzyme (results not shown). On the basis of Scheme 1, the $\text{p}K_{\text{e}1}$ and $\text{p}K_{\text{e}2}$ values were determined to be 4.3 ± 0.1 and 10.6 ± 0.1 respectively, while (k_{cat}/K_m)_o was $(3.7 \pm 0.1) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e. only 25 % of that of the native enzyme. This indicates that introducing a negative charge at position 150 increases $\text{p}K_{\text{e}2}$ to the level observed with matrilysin containing 1.3–4.8 nitrotyrosines, without influencing $\text{p}K_{\text{e}1}$, and the shape of the pH-dependence curve is similar to that observed with the matrilysin containing 1.3–2.6 nitrotyrosines. Replacing Tyr-150 with an aspartate might induce a conformational change in the enzyme to lower the activity to 25 %, whereas replacing it with nitrotyrosine or aminotyrosine has no effect.

DISCUSSION

Use of nitrotyrosine and aminotyrosine as structural probes of matrilysin

Tyrosine residues show characteristic spectrophotometric properties that depend on the degree of ionization of phenolic hydroxy groups. On the other hand, nitrotyrosine residues ionize at neutral pH, and appear yellow. Thus tyrosines and nitrotyrosines could represent convenient probes for protein studies [23,34–36]. In the present study, the effects of the nitration and amination of tyrosine residues in matrilysin on enzyme activity were examined. A maximum of five out of eight tyrosines were nitrated (Figure 1B), suggesting that these five residues are accessible to the nitrating agent, a nitronium ion. Nitration of matrilysin had no influence on its activity (k_{cat}/K_m) at pH 7.5 (Figure 2 and Table 3). The effects of nitration of enzymes differ depending on the species of enzyme and substrate. The nitration of thermolysin markedly decreases its peptidase activity [29]. Nitration of *Bacillus subtilis* neutral metalloproteinase decreases its caseinolytic activity markedly, but increases its peptidase activity, and the peptidase activity is lost by reducing the nitrated enzyme to the aminated one [37]. The resistance of matrilysin to nitration might be meaningful from the perspective that it could retain activity even under pathological conditions when exposed to nitrating agents such as peroxynitrite.

Effects of nitration and amination of matrilysin on $\text{p}K_{\text{e}2}$

The ionizable residues controlling the activity of MMPs have not been well established [14]. The matrilysin residue Glu-198 is conserved among MMPs [21], and has been proposed to be responsible for $\text{p}K_{\text{e}1}$ as a general-base catalyst, based on structural and mechanistic similarities to thermolysin and other zinc proteinases [18,19,38,39]. However, a mutagenesis study implied that the zinc-bound water, rather than Glu-198, might be responsible for $\text{p}K_{\text{e}1}$ in matrilysin, in which Glu-198 is thought to play a role in catalysis to stabilize the transition state or to act as a general-acid catalyst after the rate-determining step [20]. On the other hand, the most plausible candidate for the residue responsible for $\text{p}K_{\text{e}2}$ is the conserved Tyr-219 [14,16]. Tyr-219, together with Tyr-193 and His-197, constitutes the shallow S1' subsite, and plays an important role in determining the preference for hydrophobic residues in the P1' position of substrates [6,40]. If we suppose that a tyrosine residue is responsible for $\text{p}K_{\text{e}2}$ (9.8), $\text{p}K_{\text{e}2}$ must be shifted down to approx. 7 when it is nitrated. However, contrary to expectations, $\text{p}K_{\text{e}2}$ was increased from 9.8 to 10.3 by nitration of 1.3 tyrosine residues, and the elevated value (10.3–10.6) was maintained even when 1.3–4.8 tyrosines were nitrated (Table 1). Subsequent amination of these residues restored the value to the level (9.4–9.7) of the native enzyme (Table 2). The most reactive tyrosine residue was identified as Tyr-150. The Y150D mutant showed activity with bell-shaped pH dependence. Thus Tyr-150 is not dispensable for activity, but a negative charge introduced at this position suppressed ionization of the group responsible for $\text{p}K_{\text{e}2}$. These results suggest that the shift in $\text{p}K_{\text{e}2}$ from 9.8 to 10.3 is brought about by nitration of Tyr-150, and that Tyr-150 is not responsible for $\text{p}K_{\text{e}2}$. However, it must be located within an electrostatically interacting distance of the group responsible for $\text{p}K_{\text{e}2}$. The accessibility to water of Tyr-150 is $54.3 \pm 4.1 \%$, which is the highest value among the eight tyrosines [6]. The spectrophotometric titration of Tyr-150 in the matrilysin containing 1.3 nitrotyrosines indicated that its $\text{p}K_{\text{a}}$ was 7.0 ± 0.1 (results not shown), suggesting that this nitrated tyrosine is normally ionizable.

A quantitative formulation of the electrostatic effects of a charged group on the acidity of a neighbouring group was given

by Bjerrum (see [41,42]). This relationship can be applied to the interaction between Tyr-150 and the probable candidate for pK_{e2} , Tyr-219 [14,16,21]. The distance between the hydroxy groups of Tyr-150 and Tyr-219 is 2.45 ± 0.03 nm [6]. Although this distance is much greater than that of a hydrogen bond, a negative charge introduced on Tyr-150 might suppress the ionization of Tyr-219 under conditions with a low dielectric constant. Using the pK_{e2} change of 0.5 caused by nitration, the effective dielectric constant D of the environment for both tyrosines was calculated to be 20 ± 1 . Although this value is markedly smaller than those ($D = 40\text{--}70$) so far evaluated for the protein surface [41,42], it might be reasonable for a long-range interaction across the inside of a protein.

The shift in pK_{e2} induced by nitrating 1–5 tyrosines implies that the residue responsible for pK_{e2} cannot be one of the five tyrosine residues nitrated. The water accessibility of Tyr-219 is $18.8 \pm 2.9\%$ [6], suggesting a possibility that it could be nitrated even with low reactivity. If it is nitrated, it is not eligible as the residue for pK_{e2} . A preliminary study on substitution of Tyr-219 by Phe or Asp indicated that the mutant enzymes exhibited almost the same activity as the native enzyme (K. Inouye and Y. Muta, unpublished work). Hence Tyr-219 is not a candidate for pK_{e2} . The thermolysin residue His-231 has been proposed to stabilize the transition state by donating a hydrogen bond to the hydrated peptide [19,43], and to be the group responsible for pK_{e2} . However, there is no analogous residue in the active sites of MMPs, and a protein-bound water molecule forms a hydrogen bond with the oxyanion of the tetrahedral intermediate [6]. This water molecule in matrilysin occupies approximately the same position as Nε of His-231 in thermolysin. Therefore ionization of the water molecule might be responsible for pK_{e2} in matrilysin.

Anomalous pH-dependence of nitrated matrilysin

Most of the MMPs, including matrilysin, show activity over a wide pH range that is controlled by two ionizable groups, while stromelysin-1 has a third pK_e around pH 6, resulting in a uniquely sharp catalytic optimum in the acidic region [31,44,45]. pK_{e3} has been attributed to His-224 in stromelysin-1, but a residue equivalent to His-224 cannot be identified in the other MMPs. The present study revealed that matrilysin involves three ionizable groups in its catalytic mechanism when four or five tyrosine residues are nitrated (Figure 3C, Table 1). The water accessibility of tyrosine residues in matrilysin is in the order Tyr-150 (54%) > Tyr-78 (33%) > Tyr-99 (24%) > Tyr-216 (23%) > Tyr-219 (19%) > Tyr-241 (12%) > Tyr-193 (9%) > Tyr-94 (7%) [6]; the order of their reactivity to TNM will be the same. A possibility is that a tyrosine residue with the fourth or fifth highest reactivity to TNM is responsible for pK_{e3} . In this case, this residue would not affect the activity of native matrilysin but, when it is nitrated, it will emerge as a significant influence on activity. Considering that the pK_{e3} value of 6.2–6.4 is close to that of nitrotyrosine, this possibility seems to be reasonable. However, another possibility has not been eliminated, namely that matrilysin activity at pH 3.6–10.4 (Figure 3) is controlled by two ionizable residues, and the third with pK_{e3} is kept either deprotonated or protonated within this pH range, i.e. the pK_{e3} is < 4 or > 11 . The pK_{e3} might be changed to 6.2–6.4 when the fourth or fifth most reactive tyrosine is nitrated to bear a negative charge. When we take this possibility into account, the residue with pK_{e3} might be Glu-198. If this is the case, the group with pK_{e1} could be a water molecule bound to the active zinc. To our knowledge, there are only few papers demonstrating anomalous pH-dependence of enzyme activity [31,44–47]. Further investigations are required in order to assign the ionizable residues controlling matri-

lysin activity and to understand the catalytic mechanism of this MMP.

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