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Tyr219 of human matrix metalloproteinase 7 is not critical for catalytic activity, but is involved in the broad pH-dependence of the activity

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Yuko Muta and Kuniyo Inouye*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

*Kuniyo Inouye, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. Tel: +81-75-753-6266, Fax: +81-75-753-6265, email: inouye@kais.kyoto-u.ac.jp

Human matrix metalloproteinase 7 (MMP-7) exhibits a broad bell-shaped pH-dependence with the acidic and alkaline pK_e (pK_{e1} and pK_{e2}) values of about 4 and 10. Its active-site tyrosyl residue, Tyr219, is conserved in all other MMPs, and thus has been thought for the ionizable group responsible for pK_{e2}. In this study, we examined the mutational effects of Tyr219 on enzyme activity. Five Tyr219 variants, Y219F (Tyr219 is replaced with Phe), Y219D, Y219A, Y219C and Y219S, were constructed by site-directed mutagenesis. In the hydrolysis of (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2, all five variants retained the activity, indicating that Tyr219 is not the ionizable group responsible for pK_{e2}. Unexpectedly, all five variants exhibited narrower pH-dependence than the wild-type MMP-7, with the pK_{e1} and pK_{e2} values in the range of 5.2–5.4 and 8.6–9.4, respectively. Such pH-dependence shifts were not observed in other active-site tyrosyl-residue variants, Y193F and Y216F. These results suggest that Tyr219 is not critical for catalytic activity, but is involved in the broad pH-dependence of the activity.

Keywords: matrix metalloproteinase/MMP-7/pH-activity profile/site-directed mutagenesis/tyrosyl residue.

Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane sulphonic acid; DMSO, dimethyl sulfoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid; K_e, proton dissociation constant; MES, 2-(*N*-morpholino)ethanesulfonic acid; 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-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2.

Human matrix metalloproteinase 7 (MMP-7, Matrilysin) [EC 3.4.24.23] is the smallest MMP, lacking a carboxyl terminal hemopexin-like domain conserved in common MMPs. It is believed to play an important role in tumour invasion and metastasis (*1*, *2*). The molecular mass of the latent pro-form is 28 kDa, and that of its mature form is 19 kDa (*3*). MMP-7 is composed of a five-stranded β-sheet and three α-helices, and contains a zinc ion essential for activity and other zinc and calcium ions that are considered necessary for stability (*4*). Like all other MMPs, it has the consensus sequence HEXXHXXGXXH, in which three histidine residues chelate a catalytic zinc ion, and a methionine-containing turn (Met-turn). Hence, it is grouped in clan MA(M) (*5*). In recent years, target molecules through which MMP-7 exerts biological functions have become apparent, such as heparin (*6*), heparan sulphate (*6*), cholesterol sulphate (*7*, *8*) and ErbB4 receptor (*9*). The inhibitions of MMP-7 activity by natural compounds (*10*, *11*), synthetic compounds (*12*) and detergents (*13*) have been reported.

Generally, ionizable groups involved in the catalytic mechanisms of enzyme are estimated from the 3D structure and pK_e values. Figure 1A shows the structure of MMP-7-hydroxymate inhibitor complex (*4*). Tyr193 [the numbering of amino acid residues of pro-MMP-7 is applied to the mature MMP-7 beginning at Tyr78 (*14*)] and Glu198 are located at the α-helix, whereas Tyr216 and Tyr219 are located at the Met-turn. Glu198 and Tyr219 are conserved in all MMPs, whereas Tyr216 is conserved in several MMPs, and Tyr193 is unique to MMP-7 (*15*). Tyr219 and Tyr216 form the S1′ subsite. MMP-7 exhibits a broad bell-shaped pH-dependence with the acidic and alkaline pK_e (pK_{e1} and pK_{e2}) values of about 4 and 10 (*12*, *16*). As a result, Glu198 and Tyr219 are believed to be the ionizable groups responsible for pK_{e1} and pK_{e2}, respectively. However, Cha *et al.* (*17*) found that the mutation of Glu198→Ala did not completely abolish catalytic activity, suggesting that the zinc-bound water might be the ionizable group responsible for pK_{e1}. We found that the MMP-7 whose tyrosyl residues were nitrated with tetranitromethane retained catalytic activity, suggesting a possibility that Tyr219 could be eliminated from the candidate for the ionizable group responsible for pK_{e2} (*18*). In this study, to address this issue we made site-directed mutagenesis

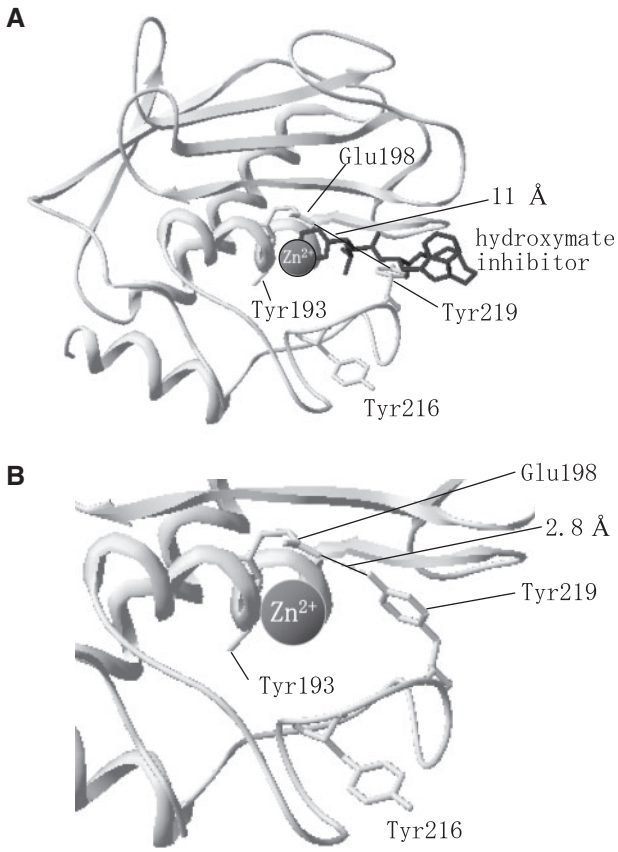

Fig. 1 Structure of MMP-7. The MMP-7-hydroxymate inhibitor complex (Protein Data Bank no. 1MMQ) (*4*) was drawn using Swiss-Pdb Viewer 4.0. Peptide chain is represented by a ribbon model. Catalytically important residue Glu198 and mutated residues, Tyr193, Tyr216 and Tyr219, are shown as ball and stick model. Zn^{2+} ion is shown as sphere. (A) Overall structure. The hydroxymate inhibitor is shown. (B) Close-up view of the active site. The hydroxymate inhibitor is deleted, and the side chain of Tyr219 is rotated to form a hydrogen bond between the hydroxyl group of Tyr219 and the carboxyl group of Glu198.

analysis. The results have demonstrated that Tyr219 is not critical for catalytic activity, but is involved in the broad pH-dependence of the activity.

Materials and Methods

Materials

(7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2, [MOCAc-PLGL(Dpa)AR] (Lot 491214, molecular mass 1093.2 Da) (*19*) and (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly (MOCAc-PLG) (Lot 510913, molecular mass 501.54 Da) were purchased from the Peptide Institute (Osaka, Japan). Their concentrations were determined by the denoted weight and the molecule weight. 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulphonic acid (AMPSO, Lot 9355C, molecular mass 227.3 Da) was from Wako Pure Chemical (Osaka, Japan). All other chemicals were from Nacalai Tesque (Kyoto, Japan).

Bacterial strains, plasmids and transformation

Escherichia coli K12 JM109 [*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB), F′(traD36, proAB$^+$ lac^{Iq}, lacZΔM15)*] and BL21(DE3) [*F*$^-$, *ompT*, *hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)] were used. The pET-MMP-7 is an expression plasmid that contains the initiator codon ATG followed by a DNA sequence encoding the mature sequence (Tyr78–Lys250) and the stop codon TAG (*19*). Site-directed mutagenesis was carried out using a GeneEditor™ *in vitro* site-directed mutagenesis kit (Promega, Madison, WI) for construction of Y219F, Y219D, Y219A, Y219C and Y219S, and Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) for construction of Y193F and Y216F. The nucleotide sequences of mutated MMP-7 genes were verified by a Shimadzu DNA sequencer DSQ-2000 (Kyoto). BL21(DE3) cells were transformed with each of the plasmids and cultured in L broth. Ampicillin was used at the concentration of 50 μg/ml.

Preparation of recombinant MMP-7

Recombinant wild-type MMP-7 and its variants were expressed in *E. coli* and purified to homogeneity, as described previously (*20*, *21*). Briefly, mature MMP-7 (Met77–Lys250) was expressed in BL21(DE3) cells in the forms of inclusion bodies, solubilized with 6 M guanidine HCl, refolded with 1 M L-arginine and purified by sequential ammonium sulphate precipitation and heparin affinity column-chromatography procedures of the refolded products. The concentration of MMP-7 was determined spectrophotometrically using the molar absorption coefficient at 280 nm, ε_{280}, of 31,800 $M^{-1}s^{-1}$ (*22*).

Fluorometric analysis of hydrolysis of MOCAc-PLGL(Dpa)AR

The MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was initiated by mixing 1,222 μl of the reaction buffer, 20 μl of the MMP-7 solution (625 nM) and 8 μl of the substrate solution (234 μM) dissolved in DMSO. The initial concentrations of enzyme, MOCAc-PLGL(Dpa)AR and DMSO were 10 nM, 1.5 μM and 0.64% (v/v), respectively. The reaction buffers were 50 mM acetate–NaOH buffer at pH 3.6–5.8, 50 mM MES–NaOH buffer at pH 5.6–7.0, 50 mM HEPES–NaOH buffer at pH 6.8–8.6 and 50 mM AMPSO–NaOH buffer at pH 8.6–10.4, each containing 10 mM $CaCl_2$. The reaction was measured by following the increase in the fluorescence intensity at 393 nm with excitation at 328 nm with a JASCO FP-777 fluorescence spectrophotometer (Tokyo, Japan). The peptide bond of Gly-L-Leu residues was cleaved by MMP-7, and the amount of the product MOCAc-PLG was estimated by the fluorescence intensity by comparison with the fluorescence intensity of an authentic MOCAc-PLG solution. The hydrolysis was carried out under pseudo-first order conditions, where the initial concentration of MOCAc-PLGL(Dpa)AR (1.5 μM) was much lower than K_m (60 μM) (*22*). The Michaelis–Menten equation is, then, expressed as $v_o = (k_{cat}/K_m)[E]_o[S]_o$, where v_o, k_{cat}, $[E]_o$ and $[S]_o$ mean the initial reaction rate, the molecular activity, the initial enzyme concentration and the initial substrate concentration, respectively. The kinetic parameters, the intrinsic k_{cat}/K_m, $(k_{cat}/K_m)_o$ and the proton dissociation constants (K_{e1} and K_{e2}) for pH-dependence of the activity were calculated from Equation 1 by a non-linear least squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT).

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o/\{1 + ([H]/K_{e1}) + (K_{e2}/[H])\} \quad (1)$$

In this equation, $(k_{cat}/K_m)_{obs}$ and [H] mean the k_{cat}/K_m value observed and the proton concentration, respectively, at the specified pH.

HPLC analysis of hydrolysis of MOCAc-PLGL(Dpa)AR

The MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was initiated by adding 30 μl of MOCAc-PLGL(Dpa)AR (0.33–3.0 mM) dissolved in DMSO to 970 μl of the MMP-7 solution dissolved in 51.5 mM HEPES buffer (pH 7.5), 10 mM $CaCl_2$. The initial concentrations of enzyme, MOCAc-PLGL(Dpa)AR and DMSO were 14.4–33.0 nM, 10–90 μM 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% trifluoroacetic acid (TFA). The mixture (100 μl) was applied to reversed-phase HPLC performed on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) × 15 cm] (Tosoh, Tokyo, Japan) equilibrated with 0.1% TFA. 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 absorption of eluate was detected at 335 nm. The substrate and its two products, MOCAc-PLG and L(Dpa)AR, were separated (*23*). 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 the nonlinear least-squares method (*24*).

Results

pH-dependences of the activities of the Tyr219 variants

To see whether Tyr219 is an ionizable group responsible for pK_{e2}, five Tyr219 variants (Y219F, Y219D, Y219A, Y219C and Y219S) were designed. Y219F was aimed to remove the hydrogen bonding potential while maintaining the hydrophobic character. Y219D was aimed to introduce the negative charge. Y219A and Y219C were aimed to remove the hydrogen bonding potential and reduce the size of hydrophobic group. Y219S was aimed to remove the hydrophobic character. The wild-type MMP-7 (WT) and the variants were prepared as described in 'Materials and Methods' section. Starting with 100 ml of *E. coli* cultures, 1–3 mg purified enzyme was recovered. Upon SDS–PAGE under reducing conditions, each yielded a single band with a molecular mass of 19 kDa (data not shown).

The pH-dependences of the k_{cat}/K_m values of WT and the five Tyr219 variants in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C were examined by fluorometric analysis (Fig. 2). All variants had the activity throughout all pH examined. Unexpectedly, they exhibited narrower bell-shaped pH-activity profiles than WT, with a catalytically optimum pH in the range of 6–8. The kinetic parameters are summarized in Table I. The pK_{e1} values of the variants were in the range of 5.2–5.4, being 1.0–1.2 pH unit higher than that of WT. The pK_{e2} values of the variants were in the range of 8.6–9.4, being 0.3–1.1 pH units lower than that of WT. The intrinsic k_{cat}/K_m, $[(k_{cat}/K_m)_o]$, values of Y219F and Y219D were similar to that of WT (99 and 90% of that of WT, respectively), whereas those of Y219A, Y219C and Y219S were reduced (50, 35 and 24% of that of WT, respectively). These results indicate that Tyr219 is not critical for catalytic activity, but is involved in the broad pH-dependence of the activity.

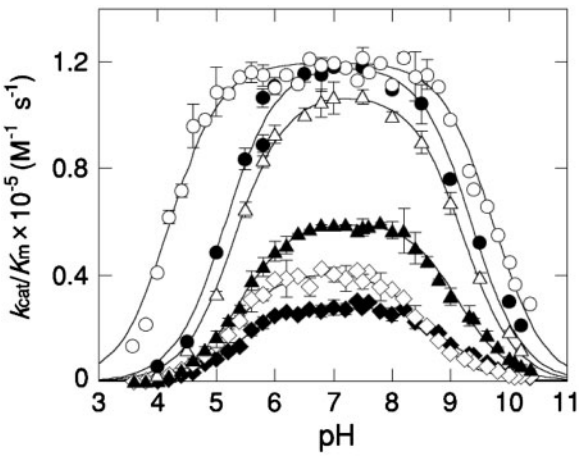

Fig. 2 Effect of pH on the activities of the wild-type MMP-7 and the Tyr219 variants in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C. The initial enzyme and substrate concentrations were 0.2 nM and 1.5 μM, respectively. Error bars indicate SD values of triplicate determination. Symbols: WT, open circle; Y219F, solid circle; Y219D, open triangle; Y219A, solid triangle; Y219C, open diamond and Y219S, solid diamond.

Kinetic parameters of the Tyr219 variants

Substrate-dependence of the initial reaction rates of the five Tyr219 variants in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C were examined by HPLC analysis. The kinetic parameters are summarized in Table II. The k_{cat}/K_m value of Y219F and Y219D were similar to that of WT (97 and 85% of that of WT, respectively), whereas those of Y219A, Y219C and Y219S were substantially reduced (52, 37 and 30% of that of WT, respectively). The decreases in k_{cat}/K_m of Y219A and Y219C were ascribed to the decreases in k_{cat}, whereas that of Y219S was ascribed to the increase in K_m. These results again indicate that Tyr219 is not critical for catalytic activity.

NaCl-induced activation of Y219F

MMP-7 activity is enhanced with increasing concentration of NaCl (*23*, *25*). We examined the degree of the activation of Y219F by NaCl in the hydrolysis of MOCAc-PLGL(Dpa)AR at pH 7.5, at 25°C (Fig. 3). The activities of WT and Y219F increased with increasing concentration of NaCl, but the increase

Table I. pK_e values and intrinsic k_{cat}/K_m $[(k_{cat}/K_m)_o]$ of the wild-type MMP-7 and its variants in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C by fluorometric analysis.

MMP-7	pK_{e1}	pK_{e2}	$(k_{cat}/K_m)_o \times 10^{-5}$ (M^{-1} s^{-1})
wild type	4.2 ± 0.0	9.7 ± 0.0	1.20 ± 0.01 (1.0)
Y219F	5.2 ± 0.0 (+1.0)	9.4 ± 0.0 (−0.3)	1.19 ± 0.01 (0.99)
Y219D	5.3 ± 0.0 (+1.1)	9.2 ± 0.0 (−0.5)	1.08 ± 0.01 (0.90)
Y219A	5.4 ± 0.0 (+1.2)	9.1 ± 0.0 (−0.6)	0.60 ± 0.01 (0.50)
Y219C	5.3 ± 0.0 (+1.1)	8.6 ± 0.0 (−1.1)	0.42 ± 0.01 (0.35)
Y219S	5.3 ± 0.0 (+1.1)	9.0 ± 0.0 (−0.7)	0.29 ± 0.00 (0.24)
Y193F	4.4 ± 0.0 (+0.2)	9.8 ± 0.0 (+0.1)	0.53 ± 0.01 (0.42)
Y216F	4.3 ± 0.0 (+0.1)	9.8 ± 0.0 (+0.1)	1.04 ± 0.01 (0.87)

The average of triplicate determination with SD values is shown. Numbers in parentheses indicate the ΔpK_e compared with the wild-type MMP-7 and the $(k_{cat}/K_m)_o$ relative to the wild-type MMP-7.

Table II. Kinetic parameters of the wild-type MMP-7 and its variants in the hydrolysis of MOCAc-PLGL(Dpa)AR at pH 7.5, at 25°C by HPLC analysis.

MMP-7	K_m (μM)	k_{cat} (s^{-1})	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1})
wild type	73.6 ± 9.0 (1.0)	6.43 ± 0.38 (1.0)	0.87 ± 0.08 (1.0)
Y219F	73.2 ± 10.4 (0.99)	6.18 ± 0.40 (0.96)	0.84 ± 0.09 (0.97)
Y219D	86.5 ± 9.4 (1.18)	6.35 ± 0.35 (0.99)	0.74 ± 0.06 (0.85)
Y219A	78.8 ± 8.8 (1.07)	3.53 ± 0.22 (0.55)	0.45 ± 0.04 (0.52)
Y219C	51.1 ± 4.4 (0.69)	1.61 ± 0.06 (0.25)	0.32 ± 0.02 (0.37)
Y219S	211 ± 48 (2.87)	5.41 ± 0.93 (0.84)	0.26 ± 0.06 (0.30)

The average of triplicate determination with SD value is shown. Numbers in parentheses indicate values relative to the wild-type MMP-7.

was more remarkable in Y219F: the activity of WT at 4 M NaCl was 550% of that at 0 M NaCl, whereas that of Y219F at 4 M NaCl was 850% of that at 0 M NaCl.

pH-dependences of the activities of Y193F and Y216F

To explore the catalytic roles of other active-site tyrosyl-residues, Tyr193 and Tyr216, the Y193F and Y216F variants were designed and prepared. The pH-dependences of k_{cat}/K_m of Y193F and Y216F in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C were examined by fluorometric analysis (Fig. 4). Y193F and Y216F had the activity throughout all pH examined. Unlike Y219F, they exhibited similar pH-dependences as WT, with a catalytically optimum pH in the range of 5–9. The kinetic parameters are summarized in Table I. The pK_{e1} and pK_{e2} values of Y193F were 4.4 and 9.8, respectively, and those of Y216F were 4.3 and 9.8, respectively, both of which were the same as those of WT (4.2 and 9.7, respectively). These results indicate that Tyr193 and Tyr216 are neither critical for catalytic activity nor involved in the broad pH-dependence of the activity.

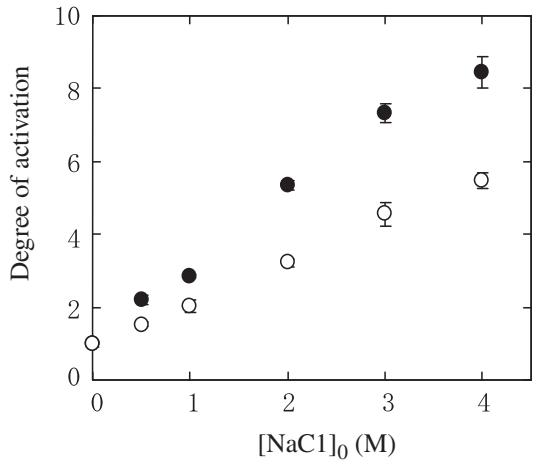

Fig. 3 Effect of NaCl on the activities of the wild-type MMP-7 and Y219F in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C. The reaction was carried out in 50 mM HEPES buffer at pH 7.5 containing 10 mM $CaCl_2$ and 0–4 M NaCl. The initial concentrations of WT (open circle) and Y219F (solid circle) were 11.4 and 13.6 nM, respectively, and that of the substrate was 400 nM. Error bars indicate SD values of triplicate determination.

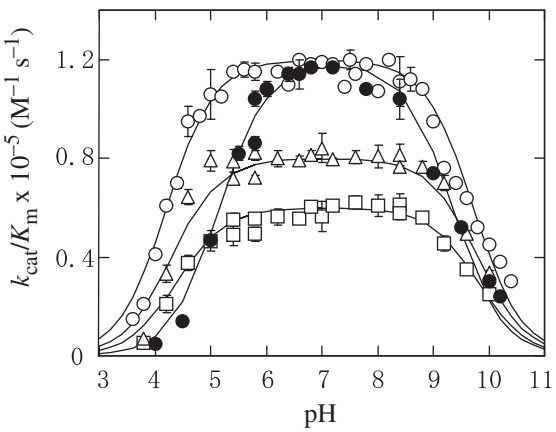

Fig. 4 Effect of pH on the activities of the wild-type MMP-7, Y193F, Y216F and Y219F in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C. The initial enzyme and substrate concentrations were 0.2 nM and 1.5 μM, respectively. Error bars indicate SD value of triplicate determination. Symbols: WT, open circle; Y193F, open square; Y216F, open triangle and Y219F, solid circle.

Discussion

Elimination of Tyr219 from the candidate for the ionizable group responsible for pK_{e2}

Although Tyr219 of MMP-7 has been thought for the ionizable group responsible for pK_{e2} (*12*, *16*), all five Tyr219 variants retained catalytic activity (Fig. 1, Tables I and II). Hence it can be concluded that Tyr219 is not critical for enzyme activity and cannot be the ionizable group responsible for pK_{e2}.

There are no lysine and arginine residues in the active site of MMP-7. What is the ionizable group of MMP-7 responsible for pK_{e2}? We previously raised a possibility that protein-bound water might be a candidate (*18*). We here again propose that protein-bound water might be the ionizable group of MMP-7 responsible for pK_{e2}, with the estimation that when it is in the protonated state, it stabilizes the tetrahedral intermediate. Similar estimations were reported in other zinc metalloproteinases (*26*, *27*). The ionizable group responsible for pK_{e2} in MMP-3 was assigned to the protein-bound water (*26*), and that in carboxypeptidase A was to the zinc-bound water (*27*). These water molecules are thought to stabilize the tetrahedral intermediate (*26*, *27*).

Insight into the catalytic role of the hydroxyl group of Tyr219

The wild-type MMP-7 exhibited a bell-shaped pH-dependence with the pK_{e1} and pK_{e2} values of 4.2 and 9.7, wherase all five Tyr219 variants exhibited narrower bell-shaped pH-activity profiles than WT (Table I). Glu198 and the zinc-bound water have been the candidates for the ionizable group for pK_{e1} (*12*, *16*, *17*). In the MMP-7-hydroxymate inhibitor complex, the distance from the hydroxyl group of Tyr219 to the carboxyl group of Glu198 is 11 Å (Fig. 1A), which is too long for the hydrogen bond. Interestingly, when the hydroxymate inhibitor was deleted, the side chain of Tyr219 could be rotated to form a hydrogen bond with the side chain of Glu198 without affecting any other residues (Fig. 1B). This preliminary finding raises a possibility that in free MMP-7, the hydroxyl group of Tyr219 promotes the deprotonation of Glu198, suggesting that Glu198 is the ionizable group responsible for pK_{e1}.

Insight into the catalytic role of the phenyl ring of Tyr219

We have proposed that neutral salts are suitable probes for exploring the active-site geometry (*25*, *28*). The activity of Y219F was almost the same as that of WT at 0 M NaCl, whereas Y219F was more activated by 4 M NaCl than WT (Fig. 3). The removal of the hydroxyl group of Tyr219 should render the active site more hydrophobic. We speculate that the increase in

degree of NaCl-induced activation of Y219F might be due to the increase in dielectric constant of its active site by the mutation.

Y219A, Y219C and Y219S had lower activities than WT, whereas Y219F had similar activity to WT (Table I). Y219S had the increased K_m values whereas Y219A and Y219C had the decreased k_{cat} values compared with WT (Table II). These results suggest that the phenyl ring of Tyr219 promotes the formation of enzyme–substrate complex and stabilizes the transition state.

Insight into the catalytic role of the active-site tyrosyl residues

Aminopeptidases have the conserved active-site tyrosyl residues thought to be involved in the stabilization of the tetrahedral intermediate. Mutational effects of such tyrosyl residues on catalytic activity were reported (*29*, *30*). In aminopeptidase A [EC 3.4.11.7], the mutation of Tyr471→Phe decreased the activity by three orders of magnitude, suggesting that Tyr471 is involved in the stabilization of the transition state (*29*). In leukotriene A4 hydrolase [EC 3.3.2.6], the mutation of Tyr456→Phe gave minimal effect, suggesting that not only Tyr456 but also several other residues are involved in the stabilization of the transition state (*30*). The mutation of Tyr219 did not much decrease MMP-7 activity, suggesting that the transition state of MMP-7 is stabilized by Tyr219 and several other residues.

In conclusion, Tyr219 is not critical for catalytic activity, but plays various catalytic roles including the broad pH-dependence of the activity. To identify the ionizable group of MMP-7 responsible for pK_{e2}, thermodynamic analysis of its proton dissociation constants is currently underway.

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Conflict of interest

None declared.

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