

**Biochemical Analysis on the Interaction of Human Matrix
Metalloproteinase 7 and Thermolysin with 8-Anilidonaphthalene
1-Sulfonate, Heparin, and Cholesterol Sulfate**

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Abbreviations

AMPSO	3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid
ANS	8-anilinonaphthalene 1-sulfonate
CD	circular dichroism
CS	cholesterol sulfate
<i>D</i>	dielectric constant
DMSO	dimethyl sulfoxide
Dnp-RPLALWRS	dinitrophenyl-L-Arg-L-Pro-L-Leu-L-Ala-L-Leu-L-Trp-L-Arg-L-Ser
FAGLA	<i>N</i> -[3-(2-furyl)acryloyl]-Gly-Leucine Amide
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
K_a	association constant
k_{cat}	molecular activity
k_{cat}/K_m	specificity constant
K_d	dissociation constant
K_m	Michaelis constant
k_{obs}	first-order rate constant for thermal inactivation
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MMP-7	human matrix metalloproteinase 7, matrilysin
MOCAC-PLG	(7-methoxycoumarin-4-yl)acetyl-L-Pro-Leu-Gly
MOCAC-PLGL(Dpa)AR	(7-methoxycoumarin-4-yl)acetyl-L-Pro-Leu-L-Gly-L-Leu-[N ³ -2,4-dinitrophenyl-L-2,3-diaminopropionyl]-Ala-L-Arg-NH ₂
pK_e	proton dissociation constant
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAPS	<i>N</i> -tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TLN	thermolysin
Tris	tris(hydroxymethyl)aminomethane

Introduction

Zinc containing neutral metalloproteases are a homologous group of structurally related proteases that have an absolute requirement for zinc ions for catalytic activity (1). The Zinc containing metalloproteases, human matrix metalloproteinase 7 (MMP-7) [EC 3.4.24.23], the smallest matrix metalloprotease (MMP) (2, 3), and thermolysin (TLN) [EC 3.4.24.27], the principal archetypal metalloprotease, (4) are the prime focus of this research.

Human matrix metalloproteinase 7 (MMP-7) lacks a carboxyl terminal haemopoxin-like domain that is conserved in most MMPs. The molecular mass of the latent pro-form is 28 kDa and that of the mature form is 19 kDa (5). MMP-7 is composed of a five-stranded β -sheet and three α -helices, and one zinc ion, essential for catalytic activity and an additional zinc and two calcium ions that are considered necessary for stability (6).

MMP-7 is frequently overexpressed in human cancer tissues (7-12) and degrades extracellular material components (2, 3, 5), suggesting that MMP-7 plays an important role in cancer metastasis/progression. From this view point, development of MMP-7 inhibitors is considered to be of therapeutic benefit. In recent years, target molecules through which MMP-7 exerts biological functions have become apparent, including heparin (13), heparan sulfate (13), cholesterol sulfate (14-16), and ErbB4 receptor (17).

Heparin is a highly negatively charged glycosaminoglycan. Glycosaminoglycans play critical roles in the conversion of promatrilysin into the active form (18). Extraction experiments and confocal microscopy studies have established that glycosaminoglycans on or around the epithelial cells and in the underlying basement membranes act as anchors or docking materials for MMP-7 (13). As anchors or docking materials, glycosaminoglycans, particularly heparin sulfate, hold the enzyme thereby preventing it from diffusing in the body fluids. The anchored protein is thus kept in a fixed position that allows it to carry out its proteolytic functions (13).

Cholesterol sulfate (CS) is a highly amphipathic molecule containing a sulfate group, a sterol ring, and hydrophobic side chains. It is widely distributed in various body fluids

and in tissues and cells, including erythrocytes, platelets, skin, hair, adrenals, lung, and the brain. It has been reported that under physiological conditions, MMP-7 binds to the CS present in the cellular membranes of colon cancer cells and causes proteolysis (14, 19, 20). These reports on heparin and CS have revealed that they are involved in the biological functions of MMP-7.

8-Anilinonaphthalene 1-sulfonate (ANS) is a fluorescent probe widely used for the analysis of proteins (21, 22). It has hydrophobic and hydrophilic groups. It emits large fluorescence energy when the anilinonaphthalene group binds with proteins through hydrophobic interaction, however it does not fluoresce when the sulfonic group binds with proteins through electrostatic interaction (23). ANS is used in the analysis of proteins to characterize folding intermediates because of its high affinity for partially molten-like globule states which are absent in native or denatured protein (24-27), to detect environmental or ligand-induced conformational changes (28-29), to measure surface hydrophobicity (30), to characterize the binding sites of ligands on proteins, and to detect aggregation or fibrillation (31-33).

To explore the mechanism of the binding of MMP-7 with naturally occurring sulfated glycosaminoglycans and CS, ANS is the probe of choice due to the following reasons (i) it contains a sulfonic group similar to the sulfated groups in glycosaminoglycans and a hydrophobic group just like CS, thus raising the possibility that the binding sites of MMP-7 for ANS are similar to those for the sulfated glycosaminoglycans or CS and (ii) the binding of MMP-7 with ANS is more easily characterized than that for sulfated glycosaminoglycans.

Thermolysin (TLN) is a thermostable neutral metalloprotease (34, 35). It consists of 316 amino acid residues (36), one zinc ion for activity, and four calcium ions required for structural stability (37-39). X-ray crystallographic analysis has revealed that it consists of a β -rich N-terminal domain and an α -helical C-terminal domain (40, 41). As previously highlighted, TLN is the principal archetypal metalloprotease and for years now it has served as a model system to study the inhibition and activation mechanisms of other metalloproteinases (4, 42). To date much of the data used to derive the currently held views of the chemical mechanisms of the metalloproteases is derived from detailed

structural and kinetic analysis of this protease (4). Thiorphan (44, 45) and silanediol based compounds, (43) that were initially discovered as inhibitors of TLN activity, have proved to be equally potent when used against other fellow metalloproteases. TLN, like MMP-7, is also activated and stabilized by neutral salts (46-48). Both enzymes also exhibit a bell shaped pH profile with pH optimal around 6-7 (49-53). Due to the similarity in the interactions between TLN and other metalloproteases with various compounds, we decided to also study the interactions of TLN with the above named containing groups.

The objective of this study was to explore the interaction of MMP-7 and TLN with ANS, heparin, and CS as a function of their catalytic activity, thermal, and conformational stability, with the hope of understanding and comparing the speculated and known *invivo* and *invitro* interactions of sulfated glycosaminoglycans and cholesterol sulfate that lead MMP-7 to play a critical role in cancer progression. In Chapter 1, we examined the effect of ANS on MMP-7 activity. In chapter 2, we examined the effects of heparin and CS on MMP-7's activity and stability. In chapter 3, we explored the effects of salts on TLN using ANS.

Chapter 1

Interaction of 8-Anilidonaphthalene 1-Sulfonate (ANS) and Human Matrix Metalloproteinase 7 (MMP-7) as Examined by MMP-7 Activity and ANS Fluorescence

Introduction

MMP-7 has been detected in lesions of prostate (7), colon (8), brain (9), stomach (10), lung (11), and breast (12), and degrades extracellular material components, including gelatins of types I, III, IV, and V, type IV basement membrane collagen, fibronectin, vitronectin, proteoglycan, laminin, and elastin (2, 3, 5). This suggests that MMP-7 plays important roles in tumour invasion and metastasis. In recent years, target molecules through which MMP-7 exerts biological functions have become apparent, including heparin (13), heparan sulfate (13), CS (14-16), and ErbB4 receptor (17). From this viewpoint, understanding the exact nature of MMP-7's interactions with the above biological molecules for the development of MMP-7 inhibitors is considered to be of therapeutic benefit.

8-Anilidonaphthalene 1-sulfonate (ANS) (Fig. 1) is a fluorescent probe widely used for the analysis of proteins (21, 22). It has hydrophobic and hydrophilic groups. It emits large fluorescence energy when the anilidonaphthalene group binds with proteins through hydrophobic interaction, however it does not emit fluorescence when the sulfonic group binds with proteins through electrostatic interaction (23). ANS was used to explore the mechanism of the binding of MMP-7 with naturally occurring sulfated glycosaminoglycans, such as heparin, heparan sulfate, and CS, due to the following reasons: (i) ANS contains a sulfonic group similar to the sulfate groups in glycosaminoglycans and CS and also a hydrophobic group just like CS, thus raising the possibility that the binding sites of MMP-7 for ANS are similar to those for the sulfated glycosaminoglycans or CS and (ii) the binding of MMP-7 with ANS is more easily characterized than that for sulfated glycosaminoglycans. In this study, we

describe the interaction of ANS and MMP-7 by examining the effects of ANS on MMP-7 activity and the effects of MMP-7 on ANS fluorescence.

Materials and Methods

Materials - MOCAc-PLGL(Dpa)AR (lot no. 491214, 1093.2 Da) (Fig 2) (54) MOCAc-PLG (lot no. 510913, 501.54 Da) were purchased from Peptide Institute (Osaka, Japan). Their concentrations were determined by the denoted molecular weight. ANS (lot no. CM10-118, 299.34 Da) was from AnaSpec Inc. (San Jose, CA, USA). Its concentration was determined spectrophotometrically using the molar absorption coefficient at 350 nm, ϵ_{350} , of $5,000 \text{ M}^{-1} \text{ cm}^{-1}$ (55, 56). 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid (AMPSO) was from Wako Pure Chemical (Osaka, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Expression and purification of MMP-7 - Expression in *Escherichia coli* and purification of recombinant MMP-7 were carried out, as described previously (57, 58). Briefly, mature MMP-7 (Tyr78–Lys250) was expressed in BL21(DE3) cells in the form of inclusion bodies, solubilized with 6 M guanidine HCl, refolded with 1 M L-arginine and purified by sequential ammonium sulfate 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 $31,800 \text{ M}^{-1} \text{ cm}^{-1}$ (57, 58).

Fluorometric analysis of the MMP-7-catalysed hydrolysis of MOCAc-PLGL(Dpa)AR - The reaction buffers were 50 mM acetate–NaOH buffer at pH 3.5 and 4.5, 50 mM MES–NaOH buffer at pH 5.5 and 6.5, 50 mM HEPES–NaOH buffer at pH 7.5, and 50 mM AMPSO–NaOH buffer at pH 8.5 and 9.5, each containing 10 mM CaCl_2 . Pre-incubation (2,484 μl) was done by mixing 40 μl of the MMP-7 solution (2 μM in the reaction buffer), 0–250 μl of the ANS solution (5 mM

in the reaction buffer), and 2,194–2,444 μl of the reaction buffer. After pre-incubation at 25°C for 10 min, the reaction was initiated by adding 16 μl of the substrate solution (234 μM) dissolved in DMSO. The initial concentrations of enzyme, MOCAC-PLGL(Dpa)AR, and DMSO were 32 nM, 1.5 μM and 0.64% v/v, respectively. The reaction was measured by following the increase in fluorescence intensity at 393 nm with excitation at 328 nm using a Shimadzu RF-5300 fluorescence spectrophotometer (Kyoto, Japan) for 1 min at 25°C. The temperature was maintained in a range of $\pm 0.2^\circ\text{C}$. The peptide bond of the Gly–L-Leu residues was cleaved by MMP-7, and the amount of the product, MOCAC-PLG, was estimated by fluorescence intensity by comparison with that of the MOCAC-PLG standard solution.

The increase in the fluorescence intensity at 393 nm due to the production of MOCAC-PLG (ΔFI_{393}) of the reaction mixture is affected by the presence of ANS. Hence, FI_{393} was measured with various concentrations of ANS in the absence and in the presence of 0.46 μM MOCAC-PLG. Based on the results, the ΔFI_{393} observed at x μM ANS was corrected as follows:

$$\frac{[(FI_{393} \text{ of } 0.46 \mu\text{M MOCAC-PLG}) - (FI_{393} \text{ of buffer})]}{\{(FI_{393} \text{ of } 0.46 \mu\text{M MOCAC-PLG plus } x \mu\text{M ANS}) - (FI_{393} \text{ of } x \mu\text{M ANS})\}}$$

The MMP-7-catalysed hydrolysis of the MOCAC-PLGL(Dpa)AR substrate was carried out under pseudo-first order conditions, where the initial concentration (1.5 μM) of the substrate was much lower than K_m (60 μM) (59). The Michaelis–Menten equation is then expressed as:

$$\text{Eq. 1: } v_o = (k_{\text{cat}} / K_m) [E]_o [S]_o$$

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.

HPLC analysis of the MMP-7-catalysed hydrolysis of MOCAC-PLGL(Dpa)AR - Pre-incubation (950 μl) was initiated by mixing 16 μl of the MMP-7 solution (2 μM in

the reaction buffer), 0, 25, 50, 100, 150, or 200 μl of the ANS solution (1 mM in 50 mM HEPES–NaOH buffer at pH 7.5) and 934, 909, 884, 834, 784, or 734 μl of the reaction buffer. After pre-incubation at 25°C for 10 min, the reaction was initiated by adding 50 μl of the MOCac-PLGL(Dpa)AR (0–2.8 mM) dissolved in DMSO (the total volume of 1,000 μl) at 25°C. The initial concentrations of MMP-7, MOCac-PLGL(Dpa)AR and DMSO were 32 nM, 0–140 μM and 5%, respectively. The reaction was stopped at an appropriate time by mixing 100 μl of the reaction solution with 400 μl of 1% trifluoroacetic acid (TFA). The mixture (100 μl) was then applied to reversed-phase HPLC on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) $\times$ 150 mm] (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 20 min at a flow-rate of 1.0 ml/min. The absorption of elutes was detected at 335 nm. The substrate and its two products, MOCac-PLG and L(Dpa)AR, were separated (48). They were evaluated by the respective peak areas. The v_o was determined from the time course of the production of MOCac-PLG. The kinetic parameters, k_{cat} and K_m , were determined based on the Michaelis–Menten equation using the nonlinear least-squares methods. The HPLC apparatus consisted of a solvent delivery system CCPM, a UV monitoring system UV-8010, a computer control system PX-8010 and an integrator Chromatocorder 21.

Fluorometric analysis of ANS - Pre-incubation (1,000 μl) was carried out by mixing 100 μl of the ANS solution (250 μM in the reaction buffer), 400 μl of the reaction buffer and 500 μl of the MMP-7 solution (1,000 nM in the reaction buffer) at 25°C for 10 min. After the pre-incubation, the fluorescence spectra were measured with excitation at 380 nm and emission at 400–600 nm with a Shimadzu RF-5300PC fluorescence spectrophotometer at 25°C.

Circular dichroism (CD) measurement - Pre-incubation (500 μl) was carried out by mixing 164 μl of the reaction buffer and 336 μl of the MMP-7 solution (10.4 μM in the reaction buffer) at 25°C for 10 min. After the pre-incubation, the CD spectra were

measured using 2-mm cell with Jasco J-820 (Tokyo, Japan) spectropolarimeter equipped with a Peltier system of cell temperature control. Ellipticity was reported as mean residue molar ellipticity $[\theta]$ ($\text{deg cm}^2 \text{ dmol}^{-1}$). The spectrometer conditions were: spectral range 200–270 nm; 100 mdeg sensitivity; 0.2 nm resolutions; 0.2 s response time; 50 nm min^{-1} scan rate; and 5 accumulations. The control baseline was obtained with solvent and all the components without the proteins.

Results

Inhibition of MMP-7 activity by ANS - Figure 3A shows the effects of increasing concentrations of ANS on MMP-7 activity in the hydrolysis of MOCac-PLGL(Dpa)AR. MMP-7 (32 nM) and ANS (0–500 μM) were pre-incubated at pH 3.5–9.5 at 25°C for 10 min followed by the reaction at the same pH at 25°C. At pH 3.5, MMP-7 did not exhibit activity. At pH 6.5–8.5, it exhibited the highest activity. It is known that MMP-7 has a bell-shaped pH-dependence activity with the pK_{e1} and pK_{e2} values of 4.2–4.6 and 9.7–9.8 (52, 53, 60). The pH dependence of activity observed in the absence of ANS was in good agreement with the previous results. At pH 4.5–9.5, the initial reaction rates decreased with increasing ANS concentration, indicating that ANS inhibits MMP-7 activity. The ANS concentrations giving 50% inhibition (IC_{50} values) at pH 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 were 120, 64, 90, 96, 92, and 135 μM , respectively. Sodium sulfate (0–500 μM) did not inhibit MMP-7 activity (data not shown), suggesting that anilidonaphthalene group of ANS is required for the inhibition of MMP-7 activity. Figure 3B shows the effects of pre-incubation time on MMP-7 activity. MMP-7 (32 nM) and ANS (50 μM) were pre-incubated at pH 7.5 at 25°C for 0–35 min followed by the reaction at pH 7.5 at 25°C. The initial reaction rates decreased with increasing pre-incubation time and reached the minimum at 10 min, indicating that ANS exhibits slow-binding inhibition of MMP-7.

Inhibitory manner of ANS against MMP-7 activity - To determine k_{cat} and K_{m} of MMP-7 in the hydrolysis of MOCac-PLGL(Dpa)AR separately, the initial reaction

rates in the absence and in the presence of ANS were measured. It was necessary to increase the concentration of MOCAC-PLGL(Dpa)AR up to 140 μM that is higher than the K_m value (60 μM) (59), and fluorescence detection was not available because of the internal-filter effect of the substrate. Consequently, the products were detected by HPLC on a TSK-gel ODS column (48). All the plots showed saturated profiles (Fig. 4A). The plot of $[S]_0/v_0$ versus $[S]_0$ (Hanes–Wolf plot) in the absence and in the presence of ANS showed non-parallel lines intersecting at the X-axis, suggesting that the K_m value was 52 μM and that the inhibition is non-competitive (Fig. 4B). The k_{cat} values with 0, 25, 50, 100, 150, and 200 μM were determined to be 1.6 ± 0.1 , 1.2 ± 0.1 , 1.1 ± 0.1 , 1.0 ± 0.1 , 0.7 ± 0.1 , and $0.5 \pm 0.1 \text{ s}^{-1}$, respectively. Based on this, the reaction rate can be described as,

$$\text{Eq. 2: } v_0 = \frac{k_{\text{cat}}[E]_0[S]_0}{(K_m + [S]_0)\left(1 + \frac{[I]_0}{K_i}\right)}$$

where $[I]_0$ is the initial inhibitor concentration, and K_i is the inhibitor constant. The K_i value of ANS at pH 7.5 was calculated to be $110 \pm 20 \mu\text{M}$ from Eq. 2, which was similar to the IC_{50} at pH 7.5 (96 μM) as described above.

Effects of MMP-7 on the fluorescence of ANS - Fluorescence spectra of ANS in the absence and in the presence of MMP-7 were measured (Fig. 5). The shape of the fluorescence spectra of ANS measured at pH 3.5–9.5 in the absence of MMP-7 were almost the same, although the intensity of those at pH 3.5–4.5 was slightly higher than that of those at pH 5.0–9.5 (Fig. 5A), but the spectrum observed at pH 3.5 in the presence of 500 nM MMP-7 was drastically changed in comparison to that in its absence (Fig. 5B): the fluorescence intensity at 490 nm (FI_{490}) with 500 nM MMP-7 was 200% of that without it (Fig. 5C), and the wavelength giving the maximum fluorescence ($\lambda_{FI_{\text{max}}}$) with 500 nM MMP-7 was shorter by 20 nm than that without it. On the other hand, there were no differences in FI_{490} (Fig. 5C) and $\lambda_{FI_{\text{max}}}$ (Fig. 5D) among the spectra without MMP-7 at pH 3.5–9.5 and those with 500 nM MMP-7 at pH 4.0–9.5. These results suggest that the anilinonaphthalene group of ANS does not

bind with hydrophobic regions of MMP-7 at pH 4.0–9.5, but binds at pH 3.5.

Effects of pH on the CD spectra of MMP-7 - CD spectra of MMP-7 in the absence of ANS were measured at pH 3.5–9.5 (Fig. 6). Each spectrum was characterized by negative ellipticities at around 206–230 nm with the peaks around 208 and 225 nm, indicating that there is little difference in secondary structure. The ellipticities at 206–230 nm at pH 3.5 were slightly smaller than those at pH 4.5–9.5. The contents of α -helix, β -sheet, calculated based on the reference spectra (61), at pH 3.5 were 31, 31, and 38%, respectively, and those at pH 4.5–9.5 in average were 31, 34, and 35%, respectively. This suggests that MMP-7 did not receive a drastic structural change at pH 3.5.

Effects of alcohols on the fluorescence of ANS - Fluorescence spectra of ANS in the presence of ethanol and 2-propanol were measured (Fig. 7). The fluorescence intensity increased with increasing concentrations of ethanol (Fig. 7A) and 2-propanol (Fig. 7B). FI_{490} of ANS increased with increasing alcohol concentrations (Fig. 7C). $\lambda_{F_{\text{max}}}$ of ANS decreased with increasing alcohol concentrations and reached 487 nm at 80% ethanol and 482 nm at 80% 2-propanol (Fig. 7D). Relative FI_{490} of the ANS solution in the presence of ethanol was defined as the relative value of FI_{490} to that without ethanol. (Fig. 7E) shows relative FI_{490} of the ANS solution in the presence of ethanol against dielectric constant (D) of the solution, calculated as described previously (37). The relative FI_{490} of 2.0 of ANS, which was observed in the presence of 500 nM MMP-7 at pH 3.5 corresponds to the D value of 72.

Discussion

Mechanism of inhibition of MMP-7 activity by ANS - We have analysed the inhibition of MMP-7 activity by various compounds (45, 59, 62–65). Alcohols inhibit MMP-7 activity competitively with K_i values of 0.66–4.80 μM (56). It is suggested that

MMP-7 has the inhibitor-binding site for alcohol with the size large enough to accommodate the length of four-carbon chain and the bulk of tertiary alcohols (45). Thiorphan and R-94318, which were originally designed as inhibitors of enkephalinase and gelatinase, respectively, inhibit MMP-7 activity competitively with the K_i values of 11.2 and 7.65 μM , respectively (57). Lignans also inhibit MMP-7 activity competitively with the IC_{50} or K_i values of 50–280 μM (59). It is suggested that non-polar character of the dibenzylbutyrolactone structure is important in the inhibition (59). Green tea catechins with the galloyl group inhibit MMP-7 activity non-competitively, with the K_i values of 0.47–1.65 μM (63). In this study, it is demonstrated that ANS inhibits MMP-7 activity in a non-competitive manner with the K_i value of 110 ± 20 μM (Fig. 4). The degree of inhibition depends on the time for pre-incubation of ANS and MMP-7 (Fig. 3B). ANS fluorescence is not changed by the addition of MMP-7 at pH 4.0–9.5 (Fig. 5), suggesting that the sulfonic, but not anilinonaphthalene, group of ANS binds with MMP-7 through hydrophilic interaction. From these results, there is an interesting observation that hydrophobic inhibitors inhibit MMP-7 activity competitively, while hydrophilic inhibitors inhibit it non-competitively.

Considering the cleavage site of MOCac-PLGL(Dpa)AR by MMP-7 and the structure of thiorphan (45), the binding of substrate and inhibitors to the S1' pocket of the active site is the most important. It is thought that the S1' pocket is hydrophobic and prefers hydrophobic residues, such as Leu, Phe and Trp, but is not large enough to accommodate the anilinonaphthalene group of ANS.

Binding of MMP-7 with ANS -The pI value of MMP-7 is 5.9 (5). The pK_a value of the sulfonic group of ANS is less than 2 (65, 66). Considering that (i) the sulfonic group is negatively charged at pH 4.0–9.5, (ii) the fluorescence of ANS is not increased by the addition of MMP-7 at pH 4.0–9.5 and (iii) MMP-7 binds with heparin, heparan sulfate and cholesterol sulfate (13-16). It is suggested that ANS binds with the positively charged surface regions of MMP-7. MMP-7 has 11 Lys residues and 8 Arg residues. Figure 8 shows the overall structure of MMP-7. When MMP-7 is viewed with the active site on the centre, only three Lys residues (Lys235, Lys239, and Lys243) and two Arg

residues (Arg143 and Arg180) are on the surface, while the reverse side shows Lys and Arg residues all over the surface (6, 13) (Fig. 8), suggesting that ANS could bind with the regions far from the active site of MMP-7. Regarding this, it was reported that the binding site of MMP-7 with cholesterol sulfate is located on the other side of the active site (14), and that the amino acid residues of MMP-7 involved in the binding to cholesterol sulfate are Ile106, Arg110, Arg128, Trp132, Arg248, Lys249, and Lys250 (Fig. 8B) (15). In this article, we speculate that several CS molecules bind with one MMP-7 molecule because five basic residues (Arg110, Arg128, Arg248, Lys249, and Lys250) contribute to the binding (15). Although the binding sites of MMP-7 for ANS have not been identified, we speculate that several ANS molecules bind with one MMP-7 molecule. It was also reported that MMP-7 that binds with negatively charged liposomes retains the activity, whereas MMP-7 that binds with positively charged liposomes loses it (43). Therefore, in a physiological condition, MMP-7 might bind with sulfated glycosaminoglycans on the surface of tumour cells through the basic regions far from the active site.

FI_{490} of ANS with 500 nM MMP-7 and without alcohol at pH 3.5 was 200% of that without MMP-7 and alcohol at pH 3.5 (Fig. 5C). FI_{490} of ANS with 15% ethanol without MMP-7 at pH 7.5 was 200% of that without alcohol and MMP-7 at pH 7.5 (Fig. 7C). This increase in FI_{490} of ANS corresponds to the dielectric constant (D) value of 72 (Fig. 7E), suggesting that the environment at the binding sites of MMP-7 for ANS at pH 3.5 is more hydrophobic than water ($D = 78$).

ANS has been used to explore conformational changes of proteins as a hydrophobic probe (25-28, 68): in the complex of ANS with arginine kinase (68) or bromelain (69). ANS fluorescence first increased and then decreased with increasing concentration of guanidine hydrochloride, suggesting the presence of a partially unfolded intermediate between the native and the fully unfolded states. In the complex of ANS with citrate synthase, ANS fluorescence was increased by the addition acetyl-CoA or ATP, which is ascribed to the ligand-induced conformational change (28). In the complex of ANS with phospholipase A2, ANS fluorescence was increased by the addition of Ca^{2+} , which is ascribed to the binding of Ca^{2+} with the active-site aspartate residue (26). In the complex

of ANS with interleukin-6 (IL-6), ANS fluorescence was increased by disrupting the intramolecular disulphide by dithiothreitol (27). In this study, MMP-7 did not exhibit activity at pH 3.5 after pre-incubation without ANS for 10 min at pH 3.5 (Fig. 3A). The fluorescence of ANS was increased by the addition of MMP-7 at pH 3.5, but not at pH 4.0–9.5 (Fig. 5). This can be explained by that at pH 3.5, MMP-7 undergoes a structural change and the anilinonaphthalene group of ANS binds with hydrophobic regions of MMP-7 although the degree of structural change of MMP-7 at pH 3.5 is thought to be small according to the results of CD spectra of MMP-7 (Fig. 6).

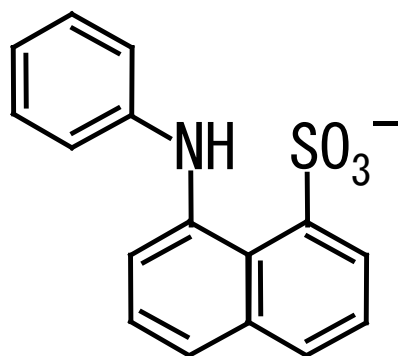


Fig. 1. Molecular structure of ANS. ANS is a fluorescent probe used in protein analysis. It consists of a hydrophobic anilinonaphthalene ring and a negatively charged sulfonate group. ANS fluorescence is enhanced when bound to hydrophobic regions of proteins.

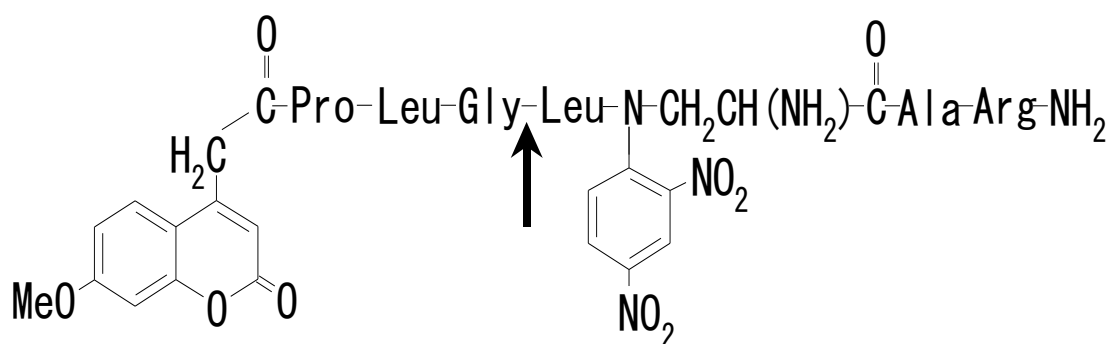


Fig. 2. Molecular structure of MOCac-PLGL(Dpa)AR. MOCac-PLGL(Dpa)AR is a FRET peptide. The arrow above indicates the point of cleavage by MMP-7 or TLN.

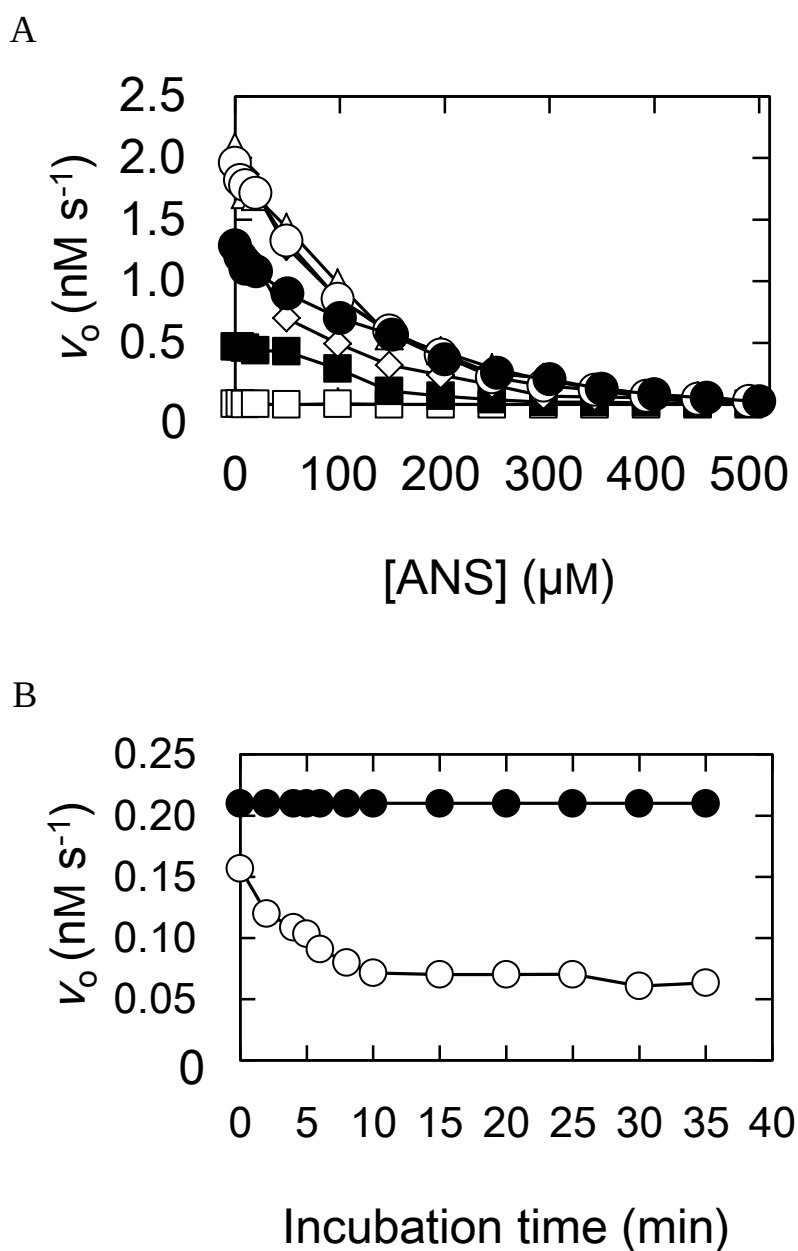


Fig. 3. Fluorometric analysis of inhibition of the MMP-7-catalysed hydrolysis of MOCac-PLGL(Dpa)AR by ANS. (A) Effect of pH and ANS concentration on the initial reaction rate, v_o . Pre-incubation of ANS and MMP-7 was carried out at 25°C for 10 min. The reaction was carried out with 32 nM MMP-7, 1.5 μM MOCac-PLGL(Dpa)AR, 0-500 μM ANS, and 0.64% DMSO at pH 3.5, □; 4.5, ■; 5.5, ◇; 6.5, ◆; 7.5, Δ; 8.5, ○; and 9.5, ● at 25°C. (B) Effect of pre-incubation time on v_o . Pre-incubation was carried out at 25°C for 0-35 min. The reaction was carried out with 50 μM ANS (○) or without it (●) at pH 7.5 at 25°C.

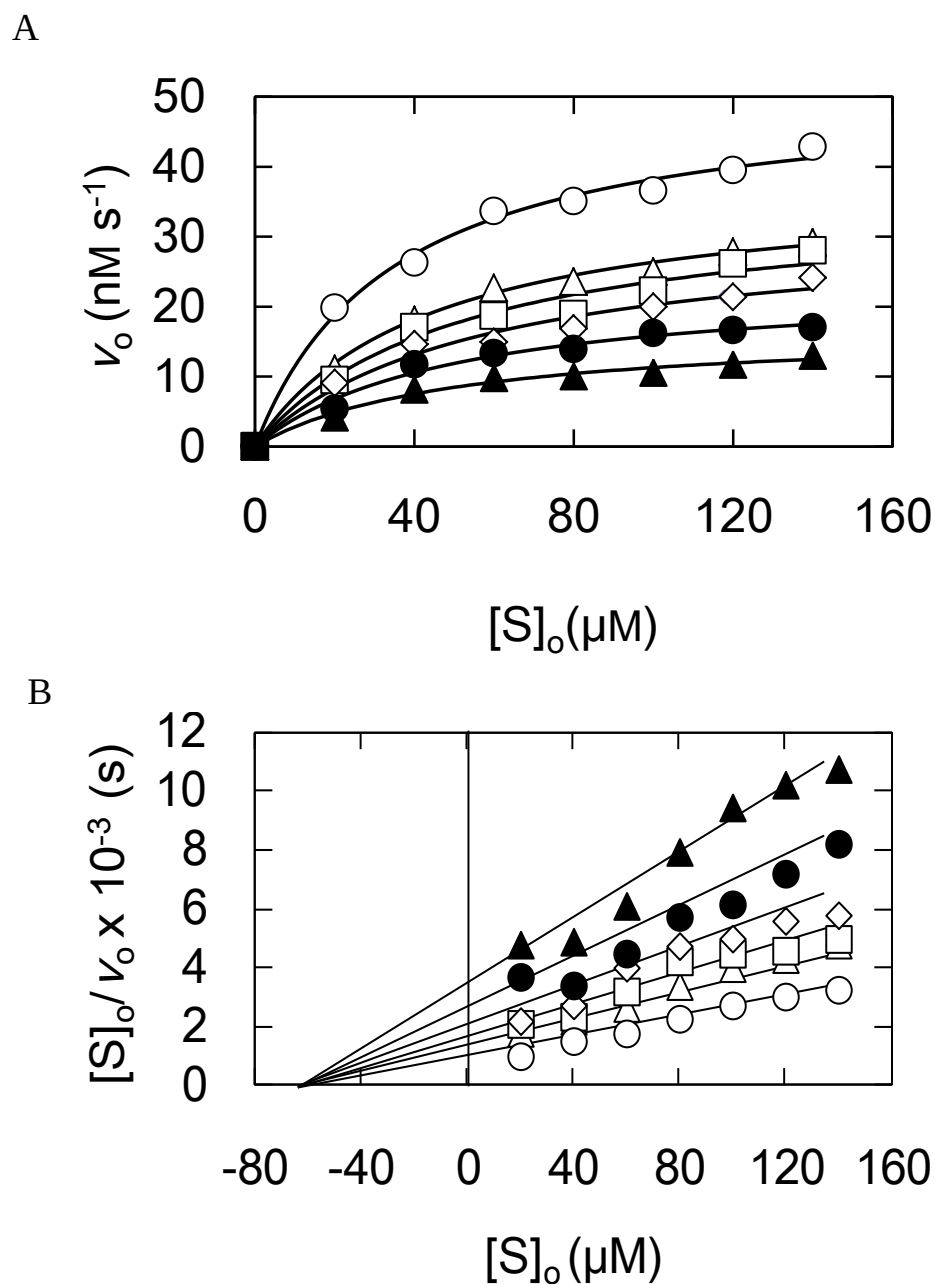


Fig. 4. HPLC analysis of inhibition of the MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR by ANS. The reaction was carried out with 32 nM MMP-7, 0-140 μM MOCAc-PLGL(Dpa)AR, 0, $\circ$; 25, Δ ; 50, $\square$; 100, $\bullet$; 150, $\blacktriangle$; or 200, $\blacksquare$ μM ANS, and 5 % DMSO at 25°C, and stopped at an appropriate time. (A) Effect of the initial substrate concentrations, $[S]_0$ on v_0 . Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. (B) Hanes-Woolf plot.

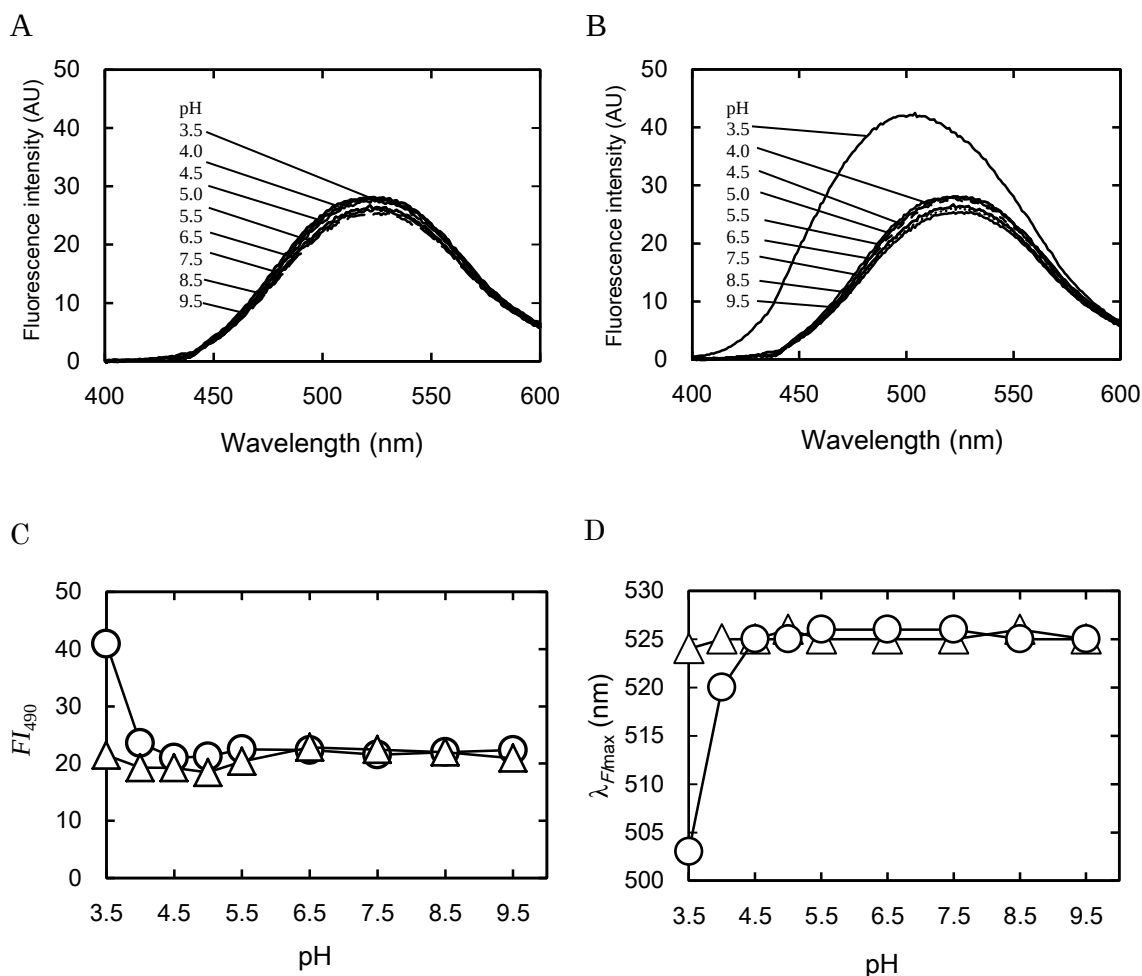


Fig. 5. Effect of pH and MMP-7 on the fluorescence of ANS. Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 5 μ M ANS in 50 mM acetate-NaOH buffer at pH 3.5, 4.0, and 4.5; 50 mM MES-NaOH buffer at pH 5.0, 5.5 and 6.5; 50 mM HEPES-NaOH buffer at pH 7.5; and 50 mM AMPPO-NaOH buffer at pH 8.5 and 9.5, each containing 10 mM CaCl_2 at 25°C. (A) Fluorescence spectra without MMP-7. (B) Fluorescence spectra with 500 nM MMP-7. (C) Fluorescence intensity at 490 nm (FI_{490}). (D) Wavelength giving the maximum fluorescence (λ_{FImax}). FI_{490} (C) and λ_{FImax} (D) of ANS with of 500 nM MMP-7, ($\circ$) and without MMP-7, (Δ) were plotted against pH.

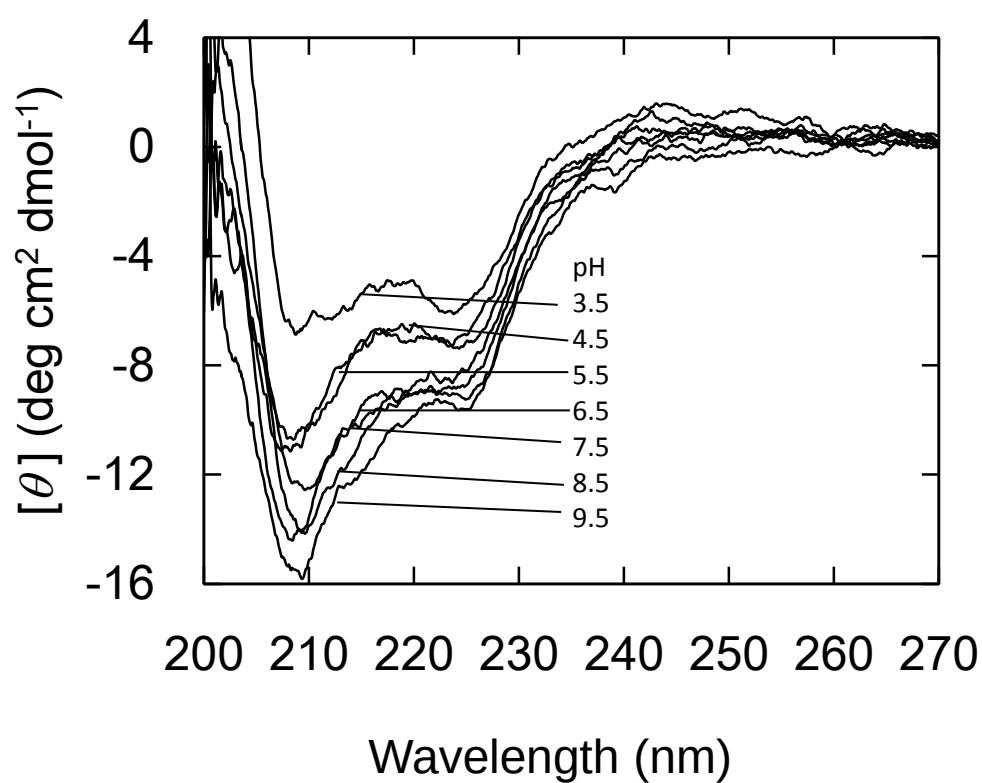


Fig. 6. Effect of pH on the CD spectra of MMP-7. CD spectra were measured for 7.0 μ M MMP-7 in 50 mM acetate-NaOH buffer at pH 3.5 and 4.5, 50 mM MES-NaOH buffer at pH 5.5 and 6.5, 50 mM Tris-HCl buffer at pH 7.5, and 50 mM AMP-SO-NaOH buffer at pH 8.5 and 9.5, each containing 10 mM CaCl₂ at 25°C.

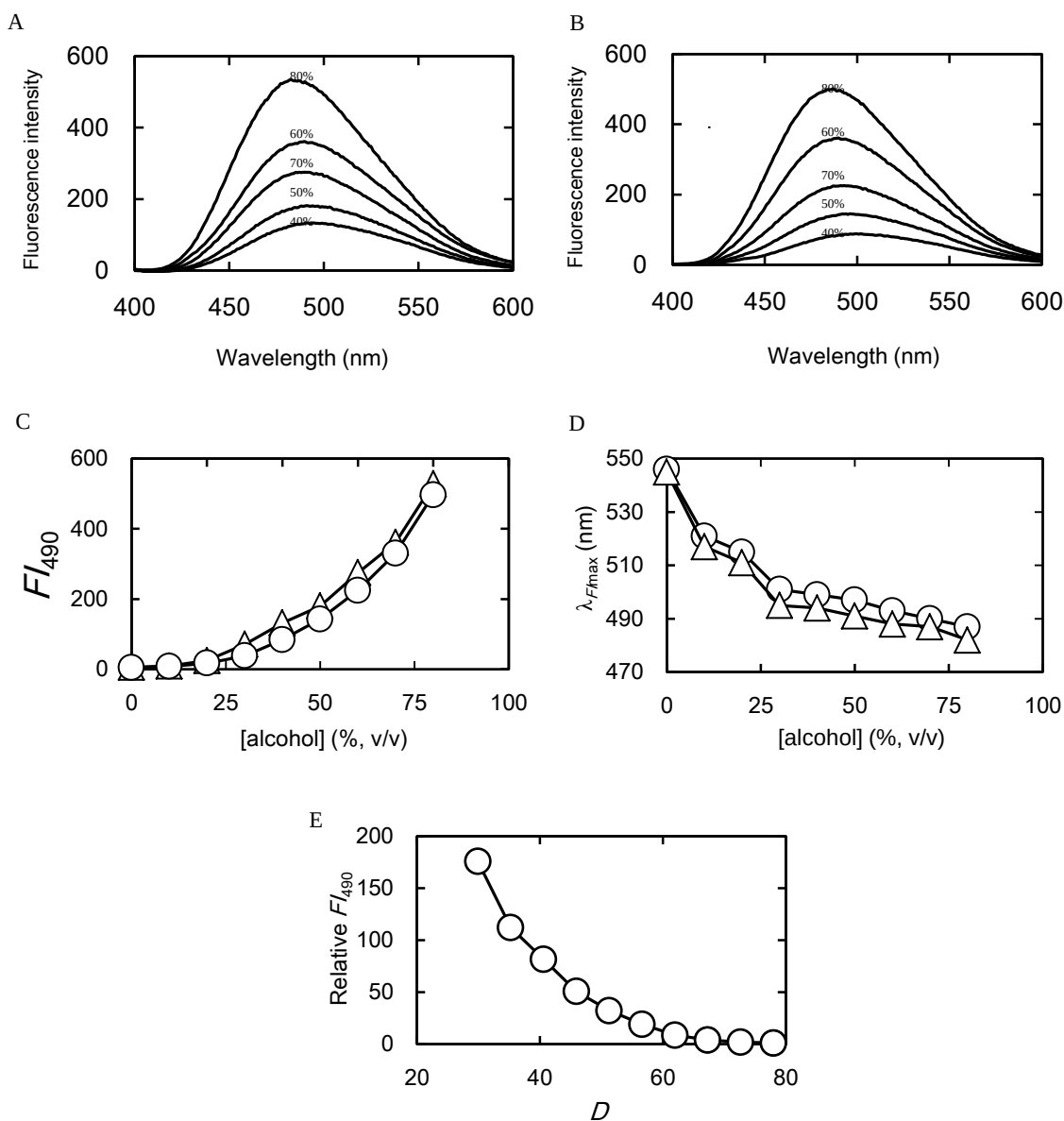


Fig. 7. Effect of alcohols on the fluorescence of ANS. Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 5 μM ANS in 50 mM HEPES-NaOH, 10 mM of CaCl_2 , pH 7.5 with various concentrations of alcohols at 25°C. (A) Fluorescence spectra with 40, 50, 60, 70, and 80% of ethanol. (B) Fluorescence spectra with 40, 50, 60, 70, and 80% 2-propanol. (C) Fluorescence intensity at 490 nm (FI_{490}). (D) Wavelength giving the maximum fluorescence ($\lambda_{F\text{max}}$). Symbols for alcohols (C, D): ethanol, $\circ$; 2-propanol, Δ . (E) Relative FI_{490} . Relative FI_{490} was defined as the relative value of FI_{490} in the presence of ethanol to the value in its absence. Dielectric constant, D , is calculated according to the previous report (64).

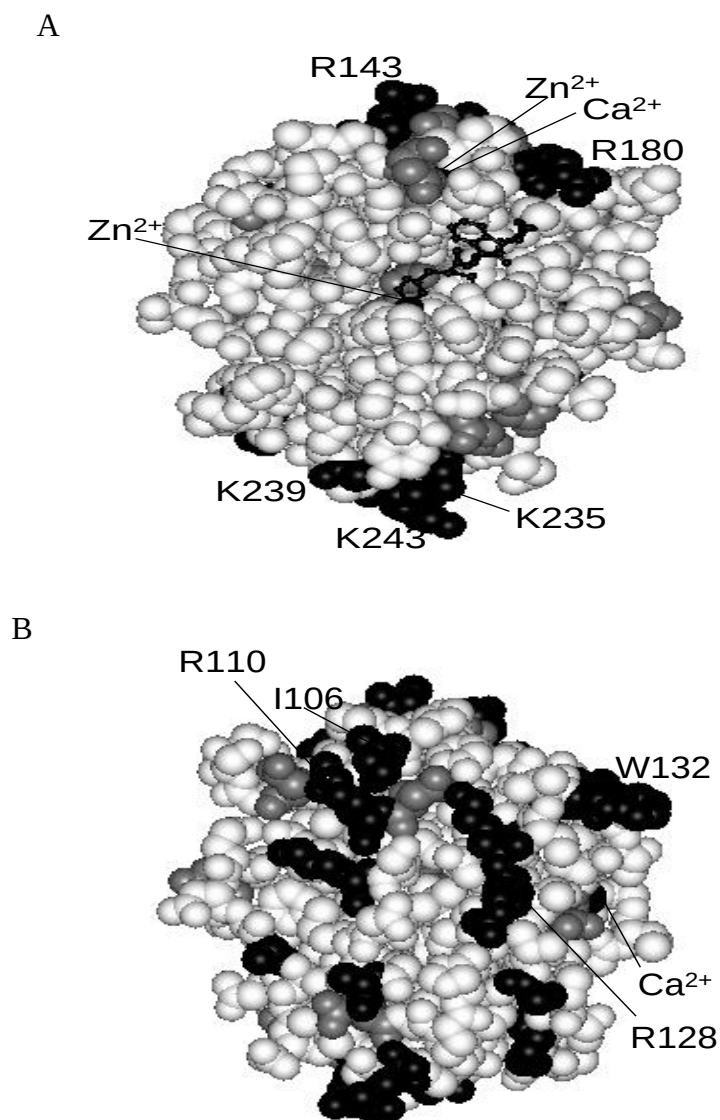


Fig. 8. Crystallographic structure of MMP-7. The MMP-7-hydroxymate inhibitor complex is based on the Protein Data Bank no. 1MMQ (6). MMP-7 residues (Tyr78-Lys243) are represented by a CPK model. Lys residues (dark gray), Arg residues (light gray), Ile106, and Trp132 are indicated. Arg244, Ser245, Asn246, Ser247, Arg248, Lys249, and Lys250 are not shown because they are not contained in 1MMQ. The hydroxymate inhibitor is represented by a ball and stick model. (A) MMP-7 viewed with the active site at the center. (B) MMP-7 viewed from the opposite side.

Chapter 2

Effects of Heparin and Cholesterol Sulfate on the Activity and Stability of Human Matrix Metalloproteinase 7

Introduction

Sulfated glycosaminoglycans and sulfated lipids are involved in the biological functions of human matrix metalloproteinase 7 (MMP-7). Heparin (Fig. 1) is a highly negatively charged sulfated glycosaminoglycan, it contains glucosamine and glucuronic acid or iduronic acid as disaccharide unit. Heparin is localized on the surface of various cells and is involved in the activation or inhibition of a number of proteinases and protein proteinase inhibitors an example of this being the activation of promatrilysin into its active form (18). Extraction experiments and confocal microscopy studies have established that glycosaminoglycans on or around the epithelial cells and in the underlying basement membranes act as anchors/docking material for MMP-7 (13) that hold the enzyme in position preventing it from diffusing in the body fluids, thus allowing it to carry out its proteolytic functions (13).

CS (Fig. 2) is a highly amphipathic molecule containing a sulfate group, a sterol ring, and hydrophobic side chains. It is widely distributed in various body fluids and in tissues and cells, including erythrocytes, platelets, skin, hair, adrenals, lung, and the brain. It has been reported that under physiological conditions, MMP-7 binds to the CS present in the cellular membranes of colon cancer cells and causes proteolysis (14, 19, 20). These reports on heparin and CS have revealed that they are involved in the biological functions of MMP-7. This chapter describes the effects of heparin and CS on the activity and stability of MMP-7 in the hydrolysis of synthetic peptides. The results indicate that heparin increases the activity and stability of MMP-7 while CS decreases its activity.

Materials and Methods

Materials - MOCAC-PLGL(Dpa)AR (molecular mass 1093.2 Da) (54) and MOCAC-PLG (501.54 Da) were purchased from Peptide Institute (Osaka, Japan). (Dnp-RPLALWRS) was from Bachem Holding AG (Budendorf, Switzerland). Their concentrations were determined by the denoted molecular weight. Porcine intestinal heparin (an average molecular mass of 5,000 Da) was from Nacalai Tesque (Kyoto, Japan). CS (466.72 Da) was from Sigma (St. Louis, MO). All other chemicals were from Nacalai Tesque.

Expression and purification of MMP-7 - Expression in *Escherichia coli* and purification of recombinant MMP-7 were carried out as described in Chapter 1.

Fluorometric analysis of the MMP-7-catalyzed hydrolysis of MOCAC-PLGL(Dpa)AR - In the experiment with heparin, pre-incubation (270 μ l) was initiated by mixing 20 μ l of the MMP-7 solution (4.0 μ M in 50 mM HEPES-NaOH buffer, 10 mM CaCl_2 at pH 7.5 (buffer A), 0-250 μ l of the heparin solution (5.0 mM in buffer A), and 0-250 μ l of buffer A. In the experiment with CS, pre-incubation (270 μ l) was initiated by mixing 20 μ l of the MMP-7 solution (4.0 μ M in buffer A), 0-250 μ l of the CS solution (5.0 mM in methanol), and 0-250 μ l of methanol. After the pre-incubation at 25°C for 0–60 min, the reaction was initiated by adding 2,214 μ l of buffer A and 16 μ l of the MOCAC-PLGL(Dpa)AR solution (234 μ M) dissolved in DMSO (the total volume of 2,500 μ l). The initial concentrations of enzyme, substrate, and DMSO were 32 nM, 1.5 μ M and 0.64% v/v, respectively. The reaction was measured by following the increase in fluorescence intensity at 393 nm with excitation at 328 nm with a Shimadzu RF-5300 fluorescence spectrophotometer (Kyoto, Japan) for 1 min at 25°C. The peptide bond of the Gly-I-Leu residues was cleaved by MMP-7, and the amount of the product, MOCAC-PLG, was estimated by fluorescence intensity by comparison with that of the MOCAC-PLG solution.

The reaction was carried out under pseudo-first order conditions, where the initial concentration (1.5 μM) of the substrate was much lower than K_m (59 μM) (14). The Michaelis-Menten equation is, then, expressed as:

$$\text{Eq. 1: } v_o = (k_{\text{cat}}/K_m) [E]_o [S]_o$$

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.

HPLC analysis of the MMP-7-catalyzed hydrolysis of MOCac-PLGL(Dpa)AR
HPLC - In the experiment with heparin, pre-incubation (238 μl) was initiated by mixing 4 μl of the MMP-7 solution (2.0 μM in buffer A) 0, 2.5, 5.0, 10, 15, or 20 μl of the heparin solution (2.5 mM in buffer A) and 234, 231.5, 229, 224, 219, or 214 μl of buffer A. In the experiment with CS, pre-incubation (238 μl) was initiated by mixing 4.0 μl of the MMP-7 solution (2.0 μM in buffer A) 0, 1.0, 2.5, 5.0, or 10 μl of the CS solution (2.5 mM in methanol) 10, 9.0, 7.5, 5.0, or 0 μl of methanol, and 224 μl of buffer A. After the pre-incubation at 25°C for 10 min, the reaction was initiated by adding 12 μl of the substrate solution (0–2.8 mM) dissolved in DMSO (the total volume of 250 μl) at 25°C. The initial concentrations of enzyme, substrate, and DMSO were 32 nM, 0–140 μM , and 5%, respectively. The reaction was stopped at an appropriate time by mixing 100 μl of the reaction solution with 400 μl of 1% trifluoroacetic acid (TFA). The mixture (100 μl) was then applied to reversed-phase HPLC performed on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) x 150 mm] (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 20 min at a flow-rate of 1.0 ml/min. The absorption of elutes was detected at 335 nm. The substrate and its two products, MOCac-PLG and L(Dpa)AR, were separated. They were evaluated by the respective peak areas. The v_o was determined from the time course of the production of MOCac-PLG. The kinetic parameters, k_{cat} and K_m , were determined based on the Michaelis-Menten equation using the nonlinear

least-squares methods. The HPLC apparatus consisting of a solvent delivery system CCPM, a UV monitoring system UV-8010, a computer control system PX-8010, and an integrator Chromatocoder 21, were from Tosoh.

HPLC analysis of the MMP-7-catalyzed hydrolysis of Dnp-RPLALWRS - Reaction (105 μ l) was initiated by mixing 3.4 μ l of the MMP-7 solution (1.0 μ M in buffer A), 0 or 2.1 μ l of the CS solution (0.5 mM in methanol), 2.1 or 0 μ l of methanol, 94.9 μ l of buffer A, and 4.6 μ l of the substrate solution (0–11.5 mM) dissolved in DMSO at 25°C. The initial concentrations of enzyme, substrate, and DMSO were 32 nM, 0–500 μ M and 5%, respectively. The reaction was stopped at an appropriate time by mixing 100 μ l of the reaction solution with 400 μ l of 1% TFA and applied to reversed-phase HPLC as described above. The absorption of elutes was detected at 335 nm. The substrate and its two products, Dnp-RPLA and LWRS, were separated. The v_o was determined from the time course of the decrease of Dnp-RPLALWRS.

Thermal inactivation of MMP-7 - In the experiment with heparin, thermal incubation (1,250 μ l) was initiated by mixing 20 μ l of the MMP-7 solution (4.0 μ M in buffer A), 0, 12.5, 25, or 50 μ l of the heparin solution (5.0 mM in buffer A) and 1230, 1,217.5, 1,205, or 1,180 μ l of buffer A. In the experiment with CS, thermal incubation (1,250 μ l) was initiated by mixing 20 μ l of the MMP-7 solution (4.0 μ M in buffer A), 0, 12.5, 25, or 50 μ l of the CS solution (5.0 mM in methanol), 125, 112.5, 100, or 75 μ l of methanol, and 1,105 μ l of buffer A. After the incubation at 25, 50, 60, or 70°C for 10 min, the reaction was initiated by adding 1,234 μ l of buffer A and 16 μ l of the substrate solution.

Results

Activation of MMP-7 activity by heparin - Figure 3A shows the effects of increasing concentrations of heparin on MMP-7 activity in the hydrolysis of MOCAC-PLGL(Dpa)AR. MMP-7 and heparin were pre-incubated at pH 7.5 at 25°C for 30 min followed by the reaction at pH 7.5 at 25°C. The initial reaction rate without heparin was 1.2 nM s^{-1} . It increased with increasing concentrations of heparin and reached almost the maximum (3.0 nM s^{-1}) at $50 \text{ }\mu\text{M}$. Figure 3B shows the effects of pre-incubation time on MMP-7 activity. MMP-7 was pre-incubated without or with heparin for 0–60 min followed by the reaction. The initial reaction rates without and with $100 \text{ }\mu\text{M}$ heparin were in the range $1.1\text{--}1.2$ and $2.5\text{--}3.1 \text{ nM s}^{-1}$, respectively, indicating that the duration of pre-incubation does not affect the activity of MMP-7.

Inhibition of MMP-7 activity by CS - Figure 4A shows the effects of increasing concentrations of CS on MMP-7 activity. MMP-7 and CS were pre-incubated at pH 7.5 at 25°C for 30 min followed by the reaction at pH 7.5 at 25°C. The initial reaction rate decreased with increasing concentrations of CS and diminished almost completely at $450 \text{ }\mu\text{M}$. The CS concentration giving 50% inhibition (IC_{50} value) was $1.6 \text{ }\mu\text{M}$. Figure 4B shows the effects of pre-incubation time on MMP-7 activity. MMP-7 was pre-incubated without or with CS for 0–60 min followed by the reaction. The initial reaction rates without and with $25 \text{ }\mu\text{M}$ CS were in the range $1.1\text{--}1.2$ and $0.4\text{--}0.6 \text{ nM s}^{-1}$, respectively, indicating that the duration of pre-incubation does not affect the activity of MMP-7.

Activation of MMP-7 by heparin and inhibition of MMP-7 by CS - Figure 5A shows the effects of increasing concentrations of heparin on MMP-7 activity in the hydrolysis of MOCAC-PLGL(Dpa)AR in the presence of $0\text{--}500 \text{ }\mu\text{M}$ CS. MMP-7, heparin, and CS were pre-incubated at pH 7.5 at 25°C for 30 min followed by the reaction at pH 7.5 at 25°C. The initial reaction rate with $1, 10, \text{ and } 100 \text{ }\mu\text{M}$ CS and 0

μM heparin was 0.94, 0.52, and 0.26 nM s^{-1} , respectively, which were 78, 43, and 22%, respectively, of that with 0 μM CS and 0 μM heparin (1.2 nM s^{-1}). Like the case without CS (Fig. 3A), the initial reaction rates increased with increasing concentrations of heparin and reached almost the maximum (2.7, 1.4, and 0.4 nM s^{-1} , respectively) at 50 μM , which were 90, 47, and 8% of that without CS (3.0 nM s^{-1}). Figure 5B shows the effects of increasing concentrations of CS on MMP-7 activity in the hydrolysis of MOCAC-PLGL(Dpa)AR in the presence of 0–500 μM heparin. The initial reaction rate with 10 and 500 μM heparin and 0 μM CS was 1.9 and 2.9 nM s^{-1} , respectively, which were 160 and 240%, respectively, of that with 0 μM CS and 0 μM heparin (1.2 nM s^{-1}). Like the case without heparin (Fig. 4A), the initial reaction rates decreased with increasing concentrations of CS and reached 0.72 and 1.2 nM s^{-1} , respectively, at 5 μM , which were 150 and 250% of that without heparin (0.48 nM s^{-1}). These results indicated that the dose-response curves of the activation of MMP-7 by heparin in the presence of various concentrations of CS are similar and that those of the inhibition of MMP-7 by CS in the presence of various concentrations of heparin are similar. Hence, it is thought that the binding of heparin and that of CS to MMP-7 are non-competitive.

Activation manner of heparin against MMP-7 activity - To determine the k_{cat} and K_{m} of MMP-7 in the hydrolysis of MOCAC-PLGL(Dpa)AR separately, the initial reaction rates in the absence or presence of heparin were measured. It was necessary to increase the concentration of MOCAC-PLGL(Dpa)AR up to 140 μM that is higher than the K_{m} value (59 μM), (14) and fluorescence detection was not available because of the internal-filter effect of the substrate. Accordingly, the products were detected by reversed-phase HPLC (48)

The effects of heparin and CS on the solubility of MOCAC-PLGL(Dpa)AR were examined. MOCAC-PLGL(Dpa)AR (0–140 μM) was incubated with heparin (200 μM) or CS (100 μM) without MMP-7. After the centrifugation, the supernatant was applied to reversed-phase HPLC (Fig. 8). The peak area of MOCAC-PLGL(Dpa)AR increased

proportionally with increasing MOCAC-PLGL(Dpa)AR concentration in the presence of heparin or CS and in their absence, indicating that neither 200 μM heparin nor 100 μM CS affected the solubility of MOCAC-PLGL(Dpa)AR.

The initial reaction rates in the absence or presence of various concentrations of heparin were measured. All plots showed saturated profiles (Fig. 5A), and the k_{cat} and K_{m} values of MMP-7 were determined separately (Fig. 4B). The K_{m} values at 25–200 μM heparin were in the range 17–21 μM , which was in the range 30–37% of that without heparin ($57 \pm 8 \mu\text{M}$). The k_{cat} values at 25–200 μM heparin were 1.2 s^{-1} , which was 75% of that without heparin ($1.6 \pm 0.1 \text{ s}^{-1}$). Consequently, the $k_{\text{cat}}/K_{\text{m}}$ values at 25–200 μM heparin were 200–250% of that without heparin. This indicates that heparin increased the activity by decreasing K_{m} .

Inhibition manner of CS against MMP-7 activity - The initial reaction rates in the absence or presence of various concentrations of CS were measured. All the plots showed saturated profiles (Fig. 7A). The plot of $1/v_o$ vs. $1/[S]_o$ (Lineweaver-Burk plot) in the absence and presence of CS showed non-parallel lines intersecting at the X-axis, indicating that the K_{m} value was $60 \pm 7 \mu\text{M}$ and that the inhibition is non-competitive (Fig. 7B). The k_{cat} values with 0, 10, 25, 50, and 100 μM CS were determined to be 1.6 ± 0.2 , 0.71 ± 0.09 , 0.57 ± 0.07 , 0.26 ± 0.03 , and $0.18 \pm 0.02 \text{ s}^{-1}$, respectively. Based on this, the reaction rate can be described as

$$\text{Eq. 2:} \quad v_o = \frac{k_{\text{cat}}[E]_o[S]_o}{(K_{\text{m}} + [S]_o) \left(1 + \frac{[I]_o}{K_i} \right)}$$

where $[I]_o$ and K_i are the initial inhibitor concentration and the inhibitor constant, respectively. The K_i value of CS at pH 7.5 was calculated to be $11 \pm 3 \mu\text{M}$ from eq. 2.

The initial reaction rates in the absence or presence of 10 μM CS were measured. All the plots showed saturated profiles (Fig. 9A). The Lineweaver-Burk plot in the

absence and presence of 10 μM CS showed non-parallel lines intersecting at the Y-axis, indicating that the k_{cat} value was $47 \pm 1 \text{ s}^{-1}$, and that the inhibition is competitive (Fig. 9B). The K_{m} values with 0 and 10 μM CS were determined to be 0.26 ± 0.02 and $0.92 \pm 0.04 \text{ mM}$, respectively. The results coincided with the previously reported one that CS inhibits MMP-7 activity competitively in the hydrolysis of Dnp-RPLALWRS with the K_{m} values of 0.26 mM for 0 μM CS and 1.2 mM for 10 μM CS (25).

Stabilization of MMP-7 by heparin and destabilization of MMP-7 by CS - Figure 7 shows the remaining activities of MMP-7 to hydrolyze MOCac-PLGL(Dpa)AR after thermal treatment at 50–70°C for 10 min. The relative activities, which were defined as the ratio of the initial reaction rate with incubation at the indicated temperature for 10 min to that without it, of MMP-7 without heparin or CS decreased with increasing temperature. At all temperatures examined, the relative activities of MMP-7 increased with increasing heparin concentration (Fig. 10A) while they decreased with increasing CS concentration (Fig. 10B). This indicates that heparin increased MMP-7 stability while CS decreased it.

Discussion

Mechanism of activation of MMP-7 by heparin - Heparin binds numerous proteins including enzymes and inhibitors involved in the blood clotting cascade (68). In this study, we found that heparin increased the activity (Figs. 3 and 6) and stability (Fig. 10) of MMP-7. Activation was brought about by a decrease in K_{m} . MMP-7 has 11 Lys and 8 Arg residues. According to the X-ray structure (Protein Data Bank accession number, all Lys and Arg residues except for Arg143 and Arg180 are located on the reverse side when MMP-7 is viewed with the active site at the center (6, 13) (Fig. 8). The pI value of MMP-7 is 5.9 (7), suggesting that MMP-7 is positively charged at neutral and alkaline pH. Although the amino acid residues of MMP-7 involved in binding to heparin are unknown, we speculate that MMP-7 binds to heparin through its basic region far from

the active site.

It has been reported that the heparin-binding site of protein kinase CK2 consists the four consecutive residues (Lys74-Lys75-Lys76-Lys77) and three residues (Arg191, Arg195, and Lys198) (69) and that of phospholipase A2 is Lys49 (69). They are located in the vicinity of their active sites, and heparin inhibits their activities competitively (69, 70). MMP-7, which binds with negatively charged liposomes, retains activity whereas MMP-7, which binds with positively charged ones, loses activity (67) Hence it can be said that heparin does not decrease enzyme activities except when the heparin-binding sites are located near the active sites.

Mechanism of stabilization of MMP-7 by heparin - Heparin stabilizes basic fibroblast growth factor (bFGF) (71), tissue transglutaminase (72), and tripeptidyl-peptidase I (TPP I) (73), and destabilizes cytochrome c (74). The mechanism of stabilization of MMP-7 by heparin is unknown. Considering that not only heparin but also other sulfated glycosaminoglycans such as heparan sulfate and chondroitin sulfate increase enzyme stability (73), we speculate that the negative charge density of heparin protects MMP-7 against inactivation by heating. Heparin protects bFGF against inactivation by heating it more strongly at high temperatures (71) and protects tissue transglutaminase against inactivation by heating and also degradation by other proteases (72). This evidence suggests that under physiological conditions, heparin is involved in the stabilization of MMP-7.

Mechanism of inhibition of MMP-7 by CS - CS is present in cellular membranes and also circulates in the plasma and gastric juices (75). In cellular membranes, CS is oriented parallel to the fatty acid chains of phospholipids with the sulfate group on the surface. In plasma and gastric juices, it is associated with other proteins (75). It regulates the activity of a variety of enzymes (76, 78). It activates factor XII, triggering the intrinsic blood coagulation system.

In this study, we found that CS inhibited the activity of MMP-7 in hydrolyzing MOCAc-PLGL(Dpa)AR (Figs. 4A and 5B) and Dnp-RPLALWRS (Fig. 9), and

decreased the stability (Fig. 10b) of MMP-7. The manner of inhibition was non-competitive for MOCAC-PLGL(Dpa)AR (Fig. 7B), and competitive for Dnp-RPLALWRS (Fig. 9). It has been reported that CS (10 μ M) competitively inhibited the activity of MMP-7 in hydrolyzing Dnp-RPLALWRS, suggesting that CS reduces the affinity between Dnp-RPLALWRS and MMP-7 (15). The results suggest that CS inhibits the activity in hydrolyzing MOCAC-PLGL(Dpa)AR without reducing the affinity between MOCAC-PLGL(Dpa)AR and MMP-7. The reason for this discrepancy is unknown, and calls for further study, but we speculate that the binding of CS imparts a long-range interaction to the active site and affects substrate binding. Regarding the difference in the manner of inhibition of MMP-7 by CS depending on the substrate, the difference in the interaction modes of CS with substrates must be considered.

MMP-7 binds to the surface CS of colon cancer cells and this binding is essential for MMP-7-catalyzed proteolysis (14–16). We speculate that the difference in the effects on MMP-7 activity as between free CS and membrane-bound CS can be explained by the fact that both the sulfate group and the hydrophobic side chain of free CS are involved in the inhibition of MMP-7, in view of the following evidence: (i) ANS, but not sodium sulfate, inhibits MMP-7 activity (77), and (ii) in the crystal structure of the complex of retinoic acid-related orphan receptor α (BOR α) and CS, both the sulfate group and the hydrophobic side chain of CS are completely surrounded by amino acid residues of BOR α (78). The amino acid residues of MMP-7 involved in binding to the surface CS of cancer cells have been identified. They are Ile106, Arg110, Arg128, Trp132, Arg248, Lys249, and Lys250, located on the side opposite to the active site (Fig. 11) (15).

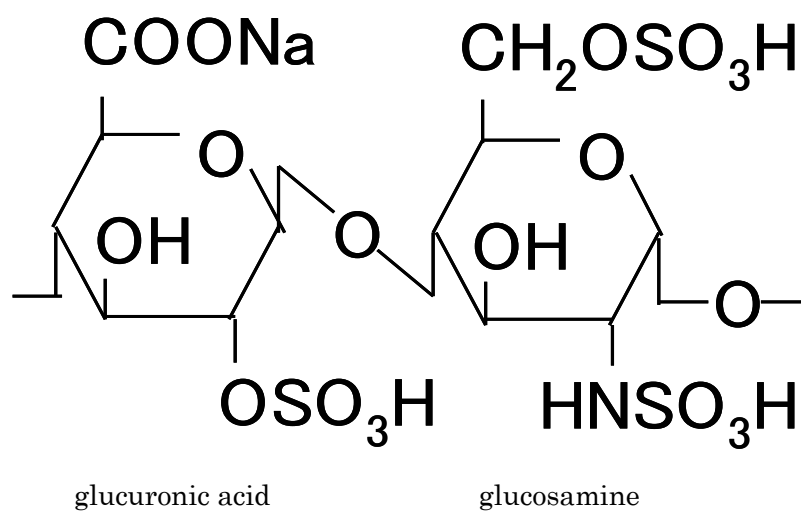


Fig. 1. Molecular structure of heparin. A naturally occurring sulfated glycosaminoglycan, composed of glucosamine and either glucuronic acid or iduronic acid as a disaccharide unit.

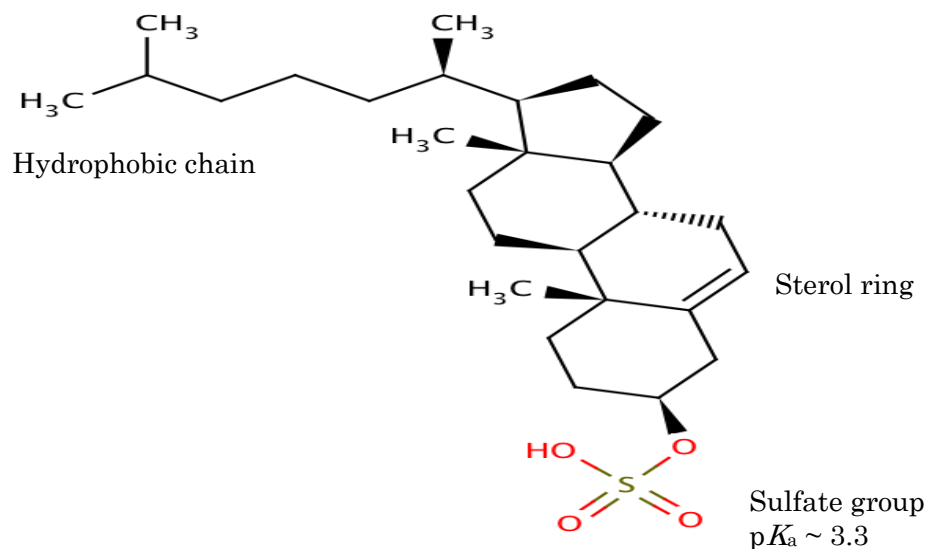


Fig. 2. Molecular structure of cholesterol sulfate. An amphipathic molecule, composed of a sulfate group, a sterol ring and a hydrophobic chain.

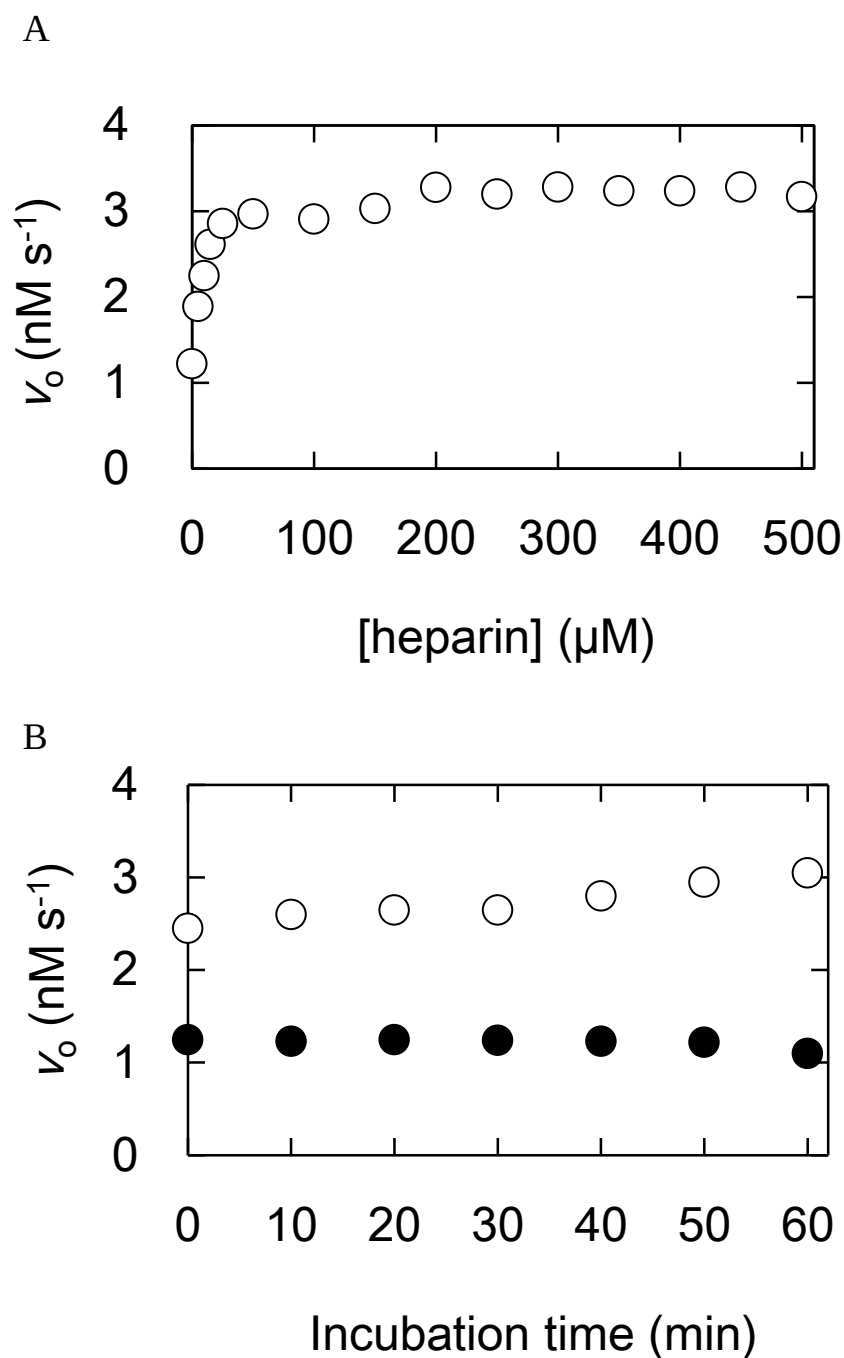


Fig. 3. Activation of the MMP-7-catalyzed hydrolysis of MOCac-PLGL(Dpa)AR by heparin. Pre-incubation of MMP-7 was carried out in the presence or absence of heparin at pH 7.5 at 25°C, and the reaction was carried out with 32 nM MMP-7 and 1.5 μM MOCac-PLGL(Dpa)AR at pH 7.5 at 25°C. (A) Effect of heparin concentration on the initial reaction rate, v_o . Pre-incubation was carried out for 30 min, and the reaction was carried out with 0–500 μM heparin. (B) Effect of pre-incubation time on v_o . Pre-incubation was carried out for 0–60 min, and the reaction was carried out with 0, (●) or 100, (○) μM heparin.

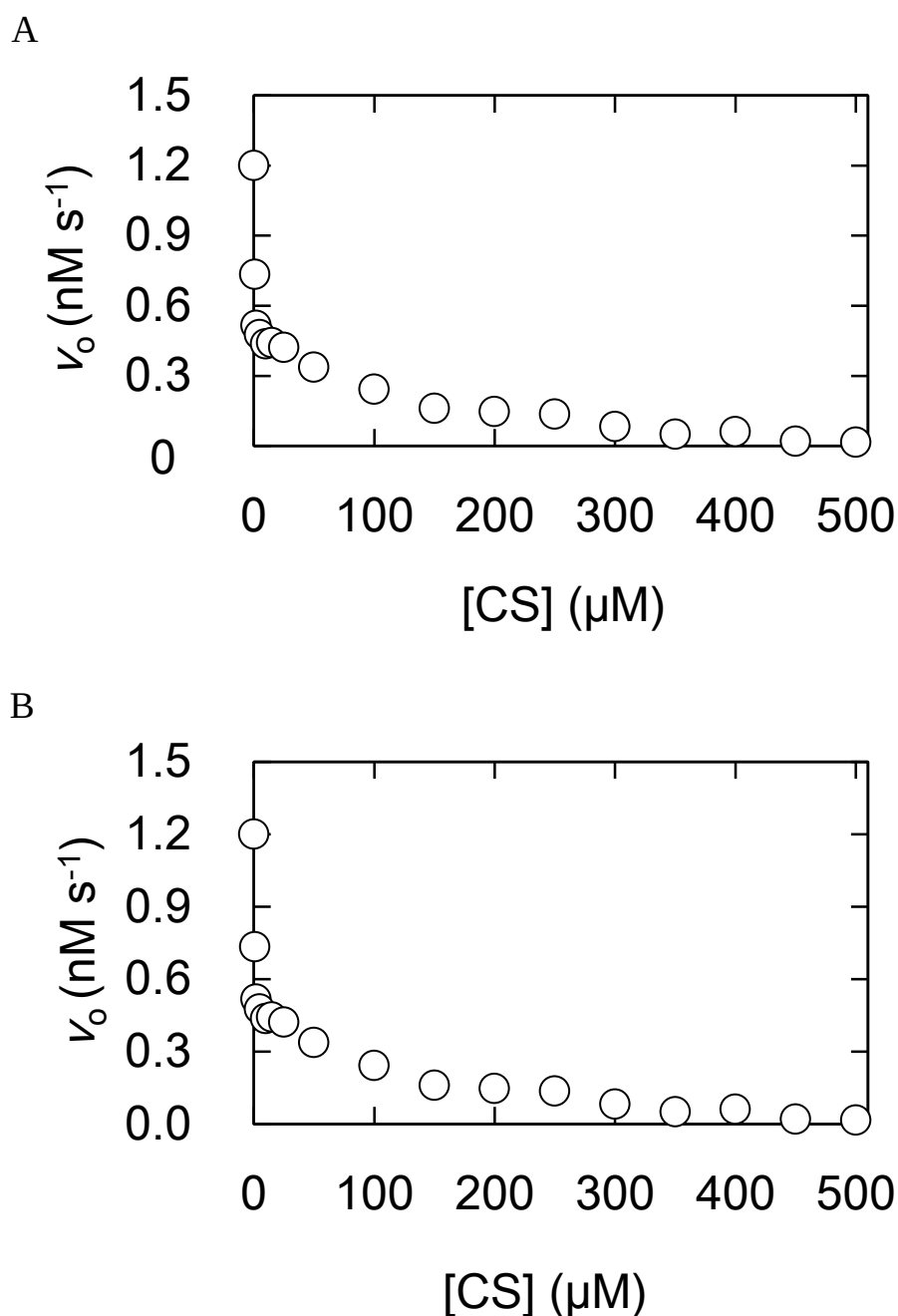


Fig. 4. Inhibition of the MMP-7-catalyzed hydrolysis of MOCac-PLGL(Dpa)AR by CS. Pre-incubation of MMP-7 was carried out in the presence or absence of CS at pH 7.5 at 25°C, and the reaction was carried out with 32 nM MMP-7 and 1.5 μM MOCac-PLGL(Dpa)AR at pH 7.5 at 25°C. (A) Effect of CS concentration on the initial reaction rate, v_o . Pre-incubation was carried out for 30 min, and the reaction was carried out with 0–500 μM CS. (B) Effect of pre-incubation time on v_o . Pre-incubation was carried out for 0–60 min, and the reaction was carried out with 0, (●) or 25, (○) μM CS.

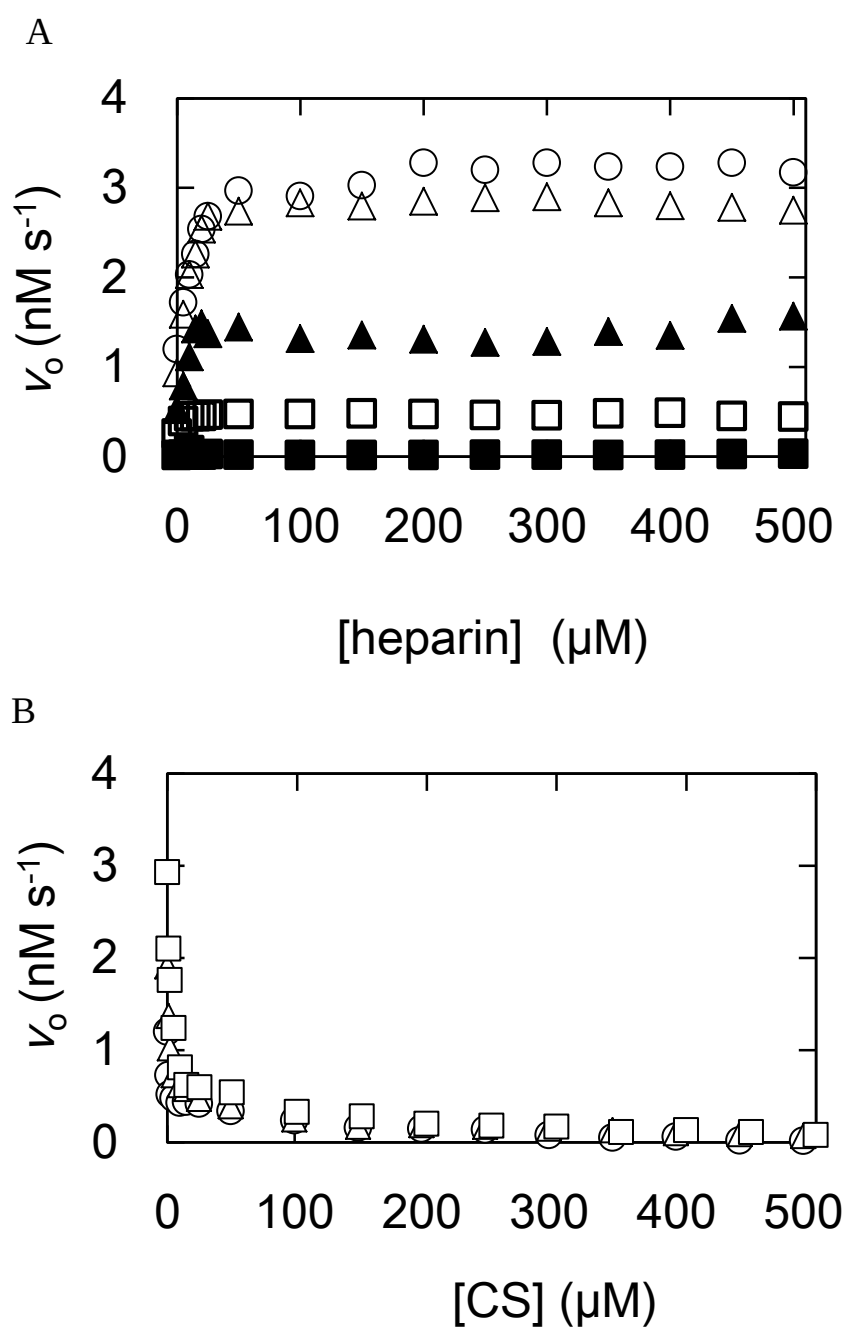


Fig. 5. Effects of heparin and CS on the MMP-7-catalyzed hydrolysis of MOCac-PLGL(Dpa)AR. Pre-incubation of MMP-7 was carried out in the presence or absence of heparin and CS at pH 7.5 at 25°C for 30 min, and the reaction was carried out with 32 nM MMP-7, 1.5 μM MOCac-PLGL(Dpa)AR, 0–500 μM heparin, and 0–500 μM CS at pH 7.5 at 25°C. (A) Activation of the MMP-7 activity by heparin in the presence of 0, ○; 1, △; 10, ▲; 100, □; and 200, ■ μM CS. (B) Inhibition of the MMP-7 activity by CS in the presence of 0, ○; 10, △; and 500, □ μM CS.

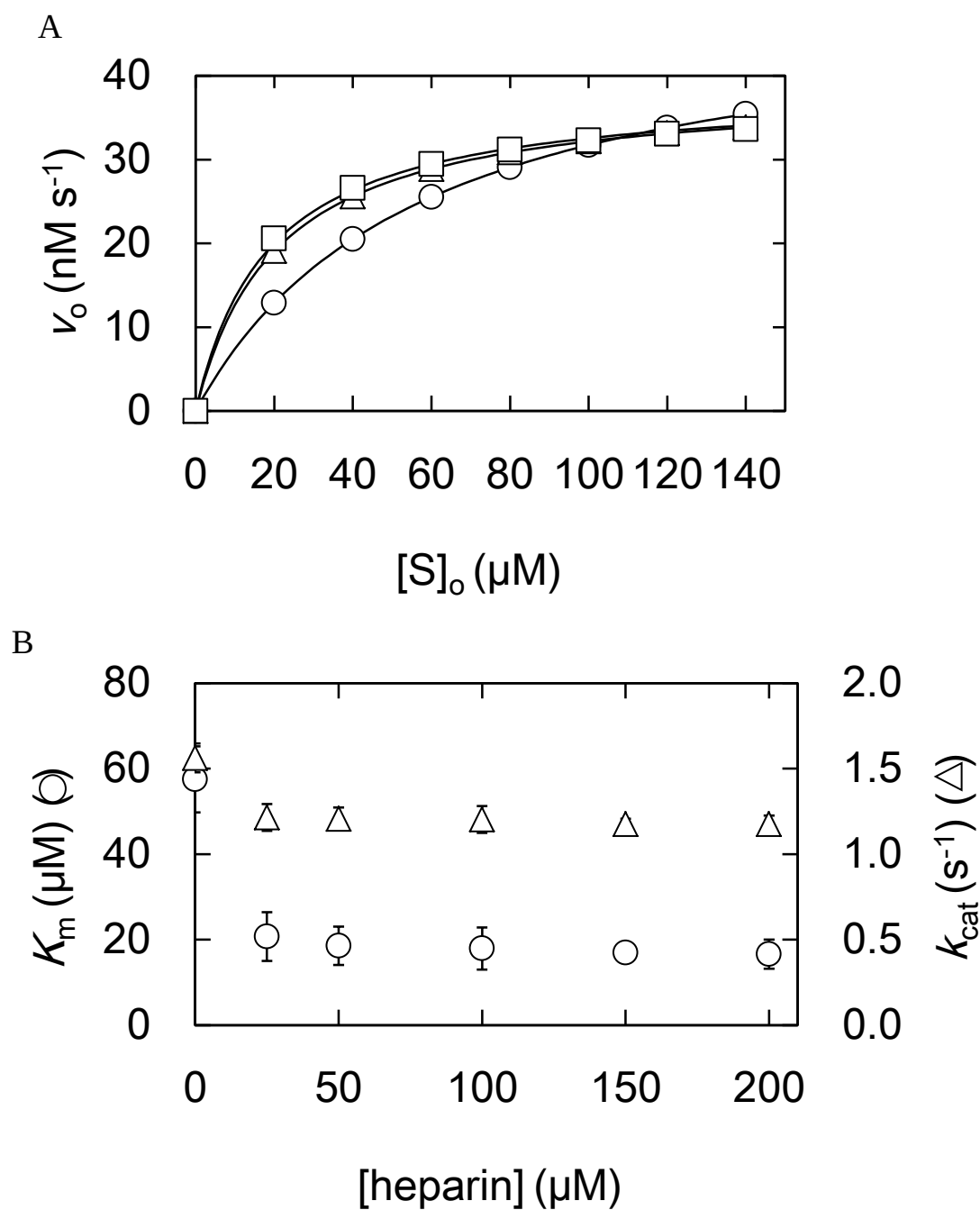


Fig. 6. HPLC analysis of activation of the MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR by heparin. (A) MMP-7 was pre-incubated in the presence or absence of heparin at pH 7.5 at 25°C for 10 min. The reaction was carried out with 32 nM MMP-7, 0-140 μM MOCAc-PLGL(Dpa)AR, and 0, ○; 25, Δ; and 200, □ μM. (B) Effect of the heparin concentration on K_m , (○); and k_{cat} , (Δ).

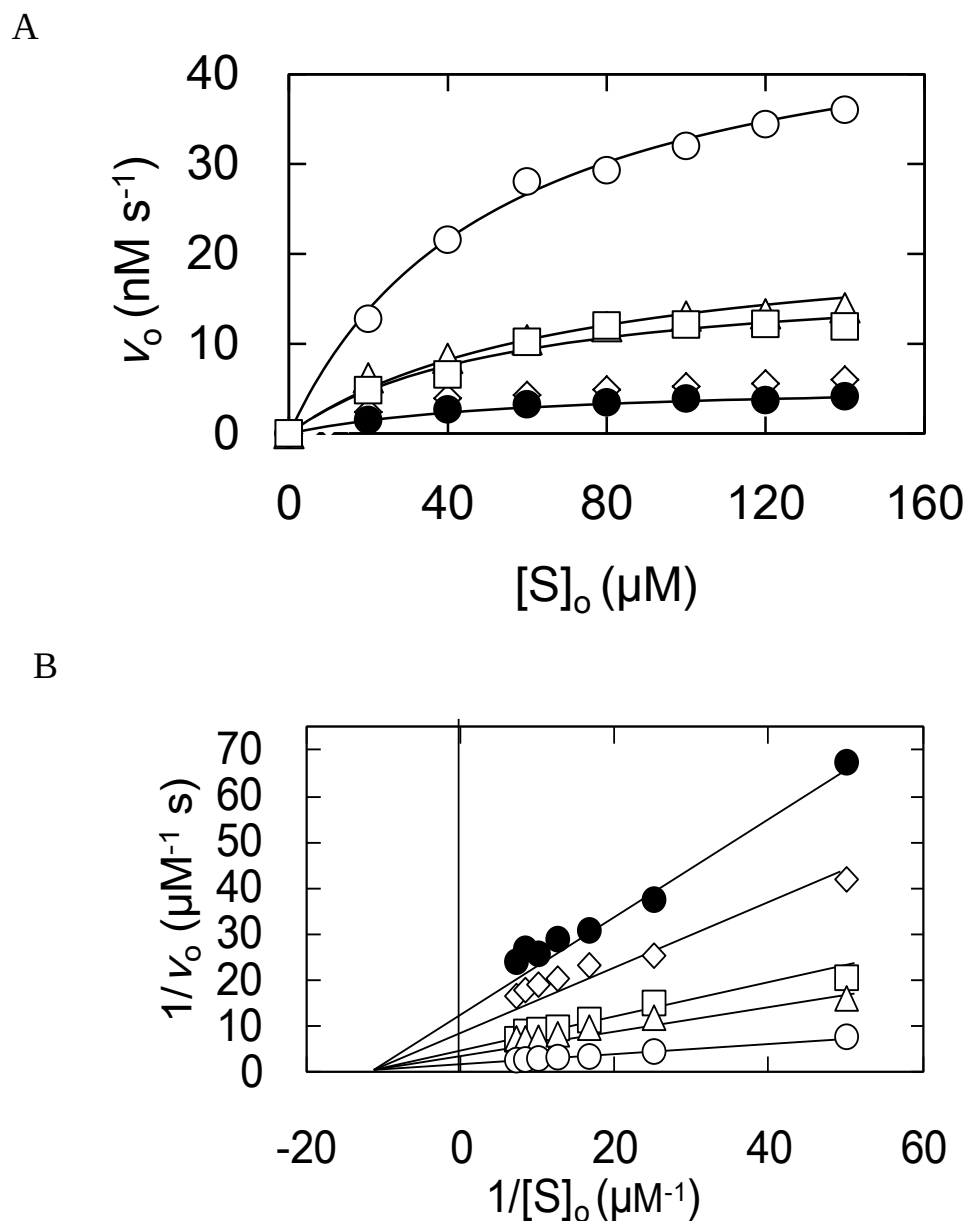


Fig. 7. HPLC analysis of inhibition of the MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR by CS. (A) MMP-7 was pre-incubated in the presence or absence of CS at pH 7.5 at 25°C for 10 min. The reaction was carried out with 32 nM MMP-7, 0-140 μM MOCAc-PLGL(Dpa)AR, and 0, $\circ$; 10, Δ ; 25, $\square$; 50, $\diamond$; or 100, $\bullet$ μM CS at pH 7.5 at 25°C, and stopped at an appropriate time. (B) Lineweaver-Burk plot.

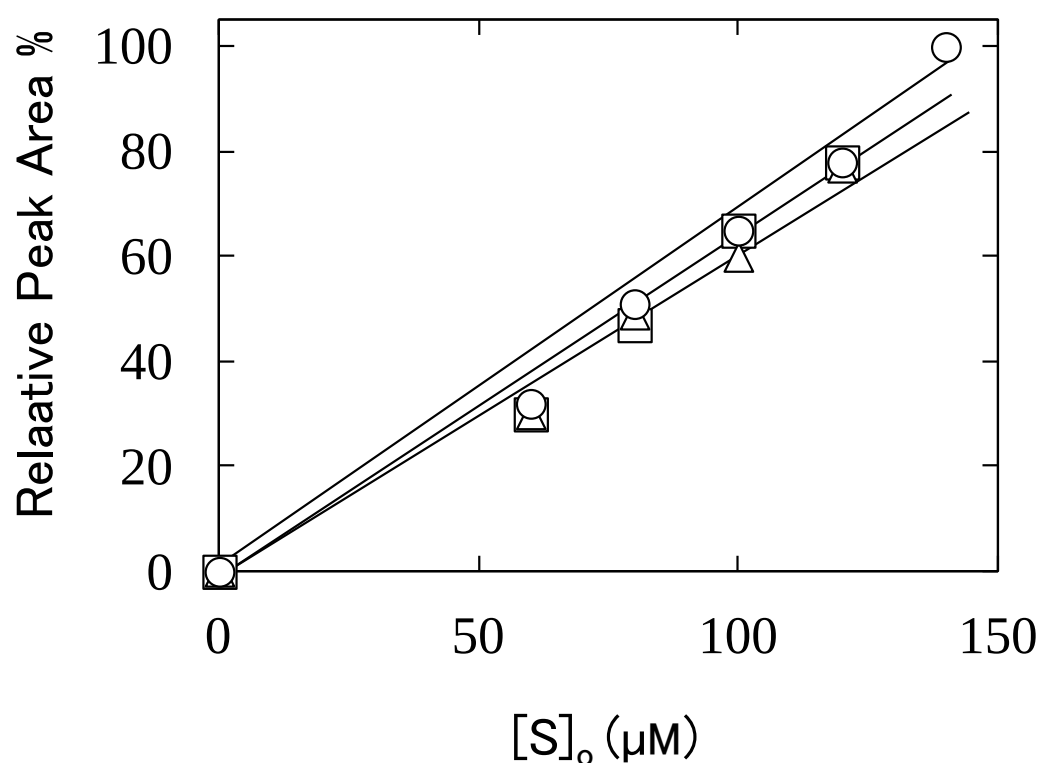


Fig. 8. HPLC analysis of MOCAC-PLGL(Dpa)AR. Incubation was initiated by mixing 237.5 μ l of buffer A, $\circ$; 217.5 μ l of buffer A and 20 μ l of heparin solution (2.5 mM in buffer A), Δ ; or 227.5 μ l of buffer A and 10 μ l of CS solution (2.5 mM in methanol) (open square) and 12 μ l of the substrate solution (0–2.8 mM) (Total volume of 250 μ l). After five min, 100 μ l of each solution was mixed with 400 μ l of 1% TFA. The mixture was then centrifuged for one min at 10,000 $\times$ g. The supernatant (100 μ l) was applied to reversed-phase HPLC column equilibrated with 0.1% TFA. The relative peak area (%) is defined as the peak area when the substrate at the indicated concentration was incubated without heparin or CS, $\circ$; 200 μ M heparin, Δ ; or 100 μ M CS, $\square$; to that when 140 μ M substrate was incubated without heparin or CS.

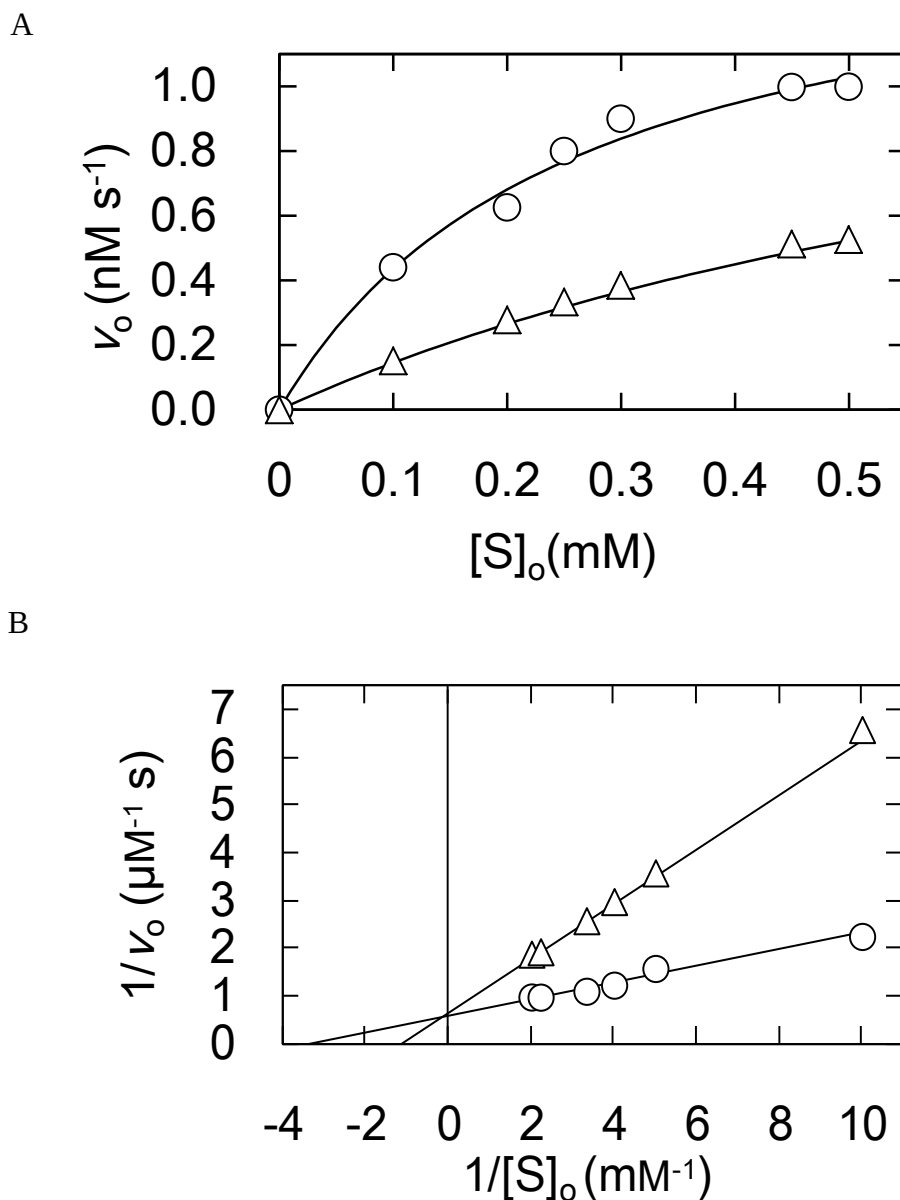
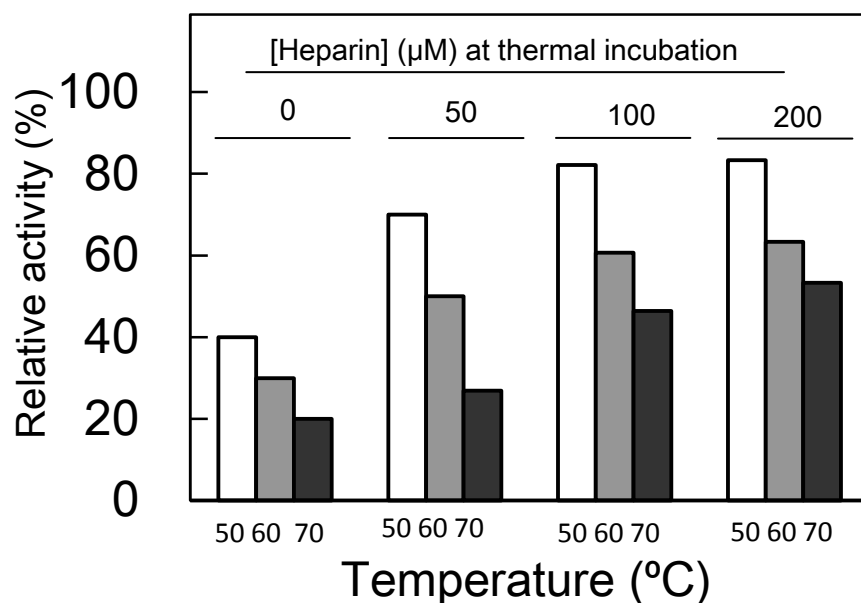


Fig. 9. HPLC analysis of inhibition of the MMP-7-catalyzed hydrolysis of Dnp-RPLALWRS by CS. (A) MMP-7 was pre-incubated in the presence or absence of CS at pH 7.5 at 25°C for 10 min. The reaction was carried out with 32 nM MMP-7, 0-0.5 mM Dnp-RPLALWRS, and 0, $\circ$; or 10, Δ μ M CS at pH 7.5 at 25°C, and stopped at an appropriate time. (A) Effect of the initial substrate concentrations, $[S]_0$, on v_0 . Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. (B) Lineweaver-Burk plot.

A



B

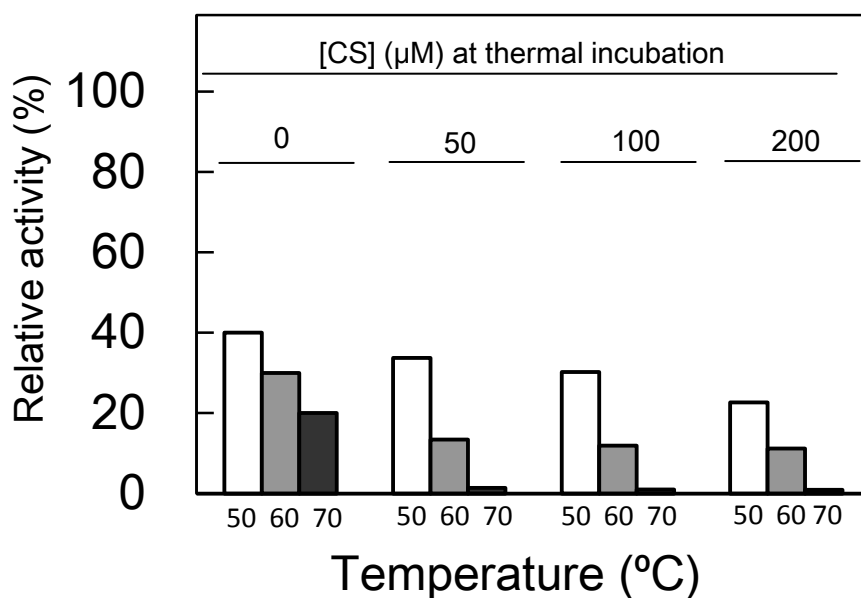


Fig. 10. Irreversible thermal inactivation of MMP-7. MMP-7 was incubated at pH 7.5 at 25, 50, 60, or 70°C with 0, 50, 100, or 200 μM heparin or CS for 10 min, and the hydrolyzing reaction was carried out with 0, 25, 50, or 100 μM heparin or CS, respectively, at pH 7.5 at 25°C. The relative activity of MMP-7 was defined as the ratio of the initial reaction rate with thermal incubation for 10 min at 50, 60, or 70°C to that at 25°C (1.0 nM s⁻¹ for thermal incubation without heparin or CS; 2.6, 2.8, and 3.0 nM s⁻¹ for those with 50, 100, and 200 μM heparin; and 0.36, 0.15, and 0.07 nM s⁻¹ for those with 50, 100, and 200 μM CS, respectively).

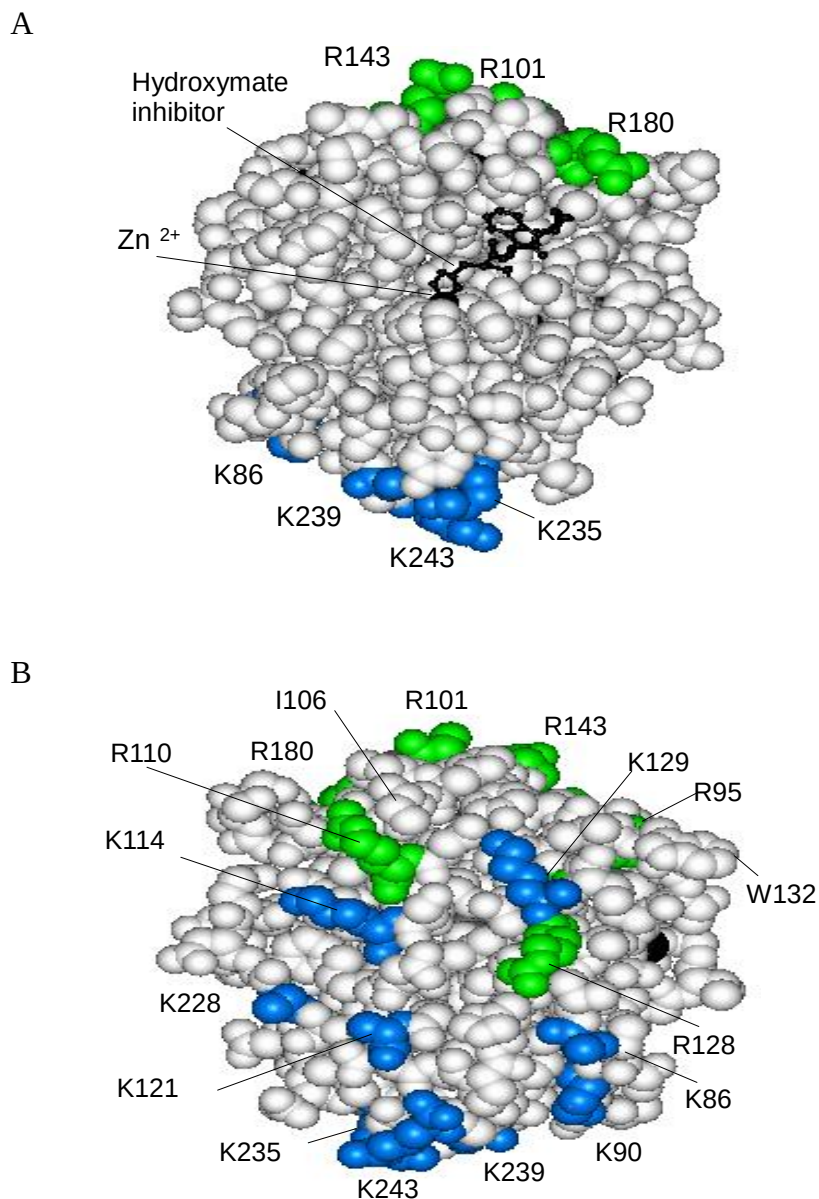


Fig. 11. Overall structure of MMP-7. The MMP-7-hydroxymate inhibitor complex is based on the Protein Data Bank no. 1MMQ.(5). MMP-7 residues (Tyr78-Lys243) are represented by a CPK model. Lys residues (dark gray), Arg residues (light gray), Ile106, and Trp132 are indicated. Arg244, Ser245, Asn246, Ser247, Arg248, Lys249, and Lys250 are not shown because they are not contained in 1MMQ. The hydroxymate inhibitor is represented by a ball and stick model. (A) MMP-7 viewed with the active site at the center. (B) MMP-7 viewed from the opposite side.

Chapter 3

Interaction of 8-Anilinonaphthalene 1-Sulfonate (ANS) and Thermolysin as Examined by ANS Fluorescence

Introduction

Thermolysin (TLN) is the principal archetypal metalloprotease and it has served for years as a model system to study the inhibition and activation mechanisms of other metalloproteinases (4, 42) such that to date much of the data used to derive the currently held views of the chemical mechanisms of the metalloproteases is derived from detailed structural and kinetic analysis of this protease (4). The activities of thermolysin and human matrix metalloprotease are known to increase in an exponential fashion with increasing concentrations of neutral salts. The degree of activation has been shown to be different from that of the hofmeister series, with sodium ions being more effective in enhancing the activity of thermolysin. However the exact mechanism of salt-induced activation of thermolysin is still unknown.

ANS is a fluorescent probe widely used for the analysis of proteins (21, 22), to characterize folding intermediates because of its high affinity for partially molten like globule states which are absent in native or denatured protein (24-27), to detect environmental or ligand induced conformational changes (28-29), to measure surface hydrophobicity (30), to characterize the binding sites of ligands on proteins, and to detect aggregation or fibrillation (31-33). In this chapter, we explore the mechanism of salt induced activation of TLN by NaCl using ANS.

Materials and Methods

Materials. ANS (Lot CM10-118, 299.34 Da) was purchased from AnaSpec Inc. (San Jose, CA). The concentration of ANS was determined spectrophotometrically

using the molar absorption coefficient at 350 nm, ϵ_{350} , of $5,000 \text{ M}^{-1} \text{ cm}^{-1}$ (55, 56). A three-times-crystallized and lyophilized preparation of TLN (Lot TIDC391, 34.6 kDa) was purchased from Daiwa Kasei (Osaka, Japan). The preparation was used without further purification. The TLN solution was filtered through a Millipore membrane filter, Type HA (pore size, $0.45 \text{ }\mu\text{M}$), before use. The concentration of TLN was determined using ϵ_{277} of $63,000 \text{ M}^{-1} \text{ cm}^{-1}$ (46). FAGLA (Lot 111K1764) was purchased from Sigma (St. Louis, MO). The concentration of FAGLA was determined using ϵ_{345} of $766 \text{ M}^{-1} \text{ cm}^{-1}$ (55, 56). MOCAc-PLGL(Dpa)AR (1,093.2 Da) (19) and MOCAc-PLG (501.54 Da) were purchased from Peptide Institute (Osaka, Japan). Their concentrations were determined by the denoted molecular weights. All other chemicals were from Nacalai Tesque (Kyoto, Japan).

Fluorometric analysis of ANS - Pre-incubation ($1,000 \text{ }\mu\text{l}$) was initiated by mixing $10 \text{ }\mu\text{l}$ of the ANS solution ($2,500 \text{ }\mu\text{M}$ in 40 mM HEPES buffer at 10 mM CaCl_2 , $0\text{--}4.0 \text{ M}$ NaCl at pH 7.5 (buffer A), $0\text{--}490 \text{ }\mu\text{l}$ of buffer A, and $0\text{--}500 \text{ }\mu\text{l}$ of the TLN solution ($4.0 \text{ }\mu\text{M}$ in buffer A) at 25°C for 10 min. After the pre-incubation, the fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm with a Shimadzu RF-5300PC fluorescence spectrophotometer at 25°C .

HPLC analysis of the TLN-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. MOCAc-PLGL(Dpa)AR - hydrolyzing activity was determined by methods described previously (48). Briefly, pre-incubation ($240 \text{ }\mu\text{l}$) was initiated by mixing $0\text{--}50 \text{ }\mu\text{l}$ of the ANS solution ($2,500 \text{ }\mu\text{M}$ in buffer A), $0\text{--}236 \text{ }\mu\text{l}$ of buffer A, and $4 \text{ }\mu\text{l}$ of the TLN solution ($2.0 \text{ }\mu\text{M}$ in buffer A) at 25°C for 10 min. The reaction was initiated by adding $10 \text{ }\mu\text{l}$ of the substrate solution (0.5 mM) dissolved in DMSO to $240 \text{ }\mu\text{l}$ of the pre-incubated solution (total volume $250 \text{ }\mu\text{l}$) at 25°C . The initial concentrations of TLN, substrate, ANS, and DMSO were 32 nM , $20 \text{ }\mu\text{M}$, $0\text{--}500 \text{ }\mu\text{M}$, and $4\% \text{ v/v}$, respectively. The reaction was stopped at appropriate times, by mixing $100 \text{ }\mu\text{l}$ of the reaction solution with $400 \text{ }\mu\text{l}$ of 1% trifluoroacetic acid (TFA). This mixture ($100 \text{ }\mu\text{l}$) was then applied to

reversed-phase HPLC done on a TSKgel ODS-80Ts column (4.6 mm inner diameter x 150 mm) (Tosoh, Tokyo) equilibrated with 0.1% TFA, 20% v/v acetonitrile. A linear gradient was generated from 20 to 70% acetonitrile at a retention time of 5 min over 20 min at a flow-rate of 1.0 ml/min. The absorption of elutes was detected at 335 nm. The substrate and its two products, MOCac-PLG and L(Dpa)AR, were separated, and they were evaluated by the respective peak areas. Reaction rate was determined from the time course of the production of MOCac-PLG.

Thermal inactivation of TLN - The thermal inactivation of TLN was examined by methods described previously (79). Briefly, 100 μ l of a solution containing 1.0 μ M thermolysin, in 50 mM acetate-NaOH buffer at pH 3.5, 4.0, and 4.5, 50 mM MES-NaOH buffer at pH 5.0, 5.5 and 6.5, 50 mM HEPES-NaOH buffer at pH 7.5 and 8.0 and 50 mM AMPSO-NaOH buffer at pH 8.5 and 9.0, each containing 10 mM CaCl_2 was incubated at 70°C for specified durations (30, 60, 90, 120, 150, or 180 min). Then it was incubated at 25°C for 5 min. Relative activity for FAGLA hydrolysis was determined as described above. Under the assumption that the thermal inactivation of TLN is irreversible and consists of only one step, the first-order rate constant (k_{obs}) of thermal inactivation was evaluated by plotting the logarithm of the activity ($k_{\text{cat}}/K_{\text{m}}$) against the duration of thermal treatment.

Spectrophotometric analysis of the TLN-catalyzed hydrolysis of FAGLA - FAGLA-hydrolyzing activity was determined by methods described previously (46). Briefly, the reaction was initiated by adding 50 μ l of the TLN solution to 950 μ l of the substrate solution in buffer at various pH above described (total volume 1,000 μ l) at 25°C. The initial concentrations of TLN and substrate were 0.1 μ M and 400 μ M, respectively. A_{345} of the reaction solution was measured continuously. The amount of FAGLA hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\epsilon_{345} = -310 \text{ M}^{-1} \text{ cm}^{-1}$, at 25°C (46, 54). Reaction rate was determined from the time course of the decrease of FAGLA.

Results

Effects of TLN on ANS fluorescence - Fluorescence spectra of ANS with varying concentrations of thermolysin in 40 mM HEPES, 10 mM CaCl₂, 0 M NaCl at pH 7.5 were measured (Fig. 1). The shapes of the fluorescence spectra of ANS measured with 0–2.0 μ M of TLN were almost the same, while the fluorescence intensity increased, and the wavelength giving the maximum fluorescence ($\lambda_{F\text{I}\text{max}}$) decreased with increasing concentrations of TLN (Fig. 1A). The fluorescence intensity at 490 nm (FI_{490}) at 2.0 μ M TLN was 133% of FI_{490} at 0 μ M TLN (Fig. 1B). $\lambda_{F\text{I}\text{max}}$ at 2.0 μ M TLN was shorter by 14 nm than $\lambda_{F\text{I}\text{max}}$ at 0 μ M thermolysin (Fig. 1C). These results indicate that the anilinonaphthalene group of ANS binds with TLN through hydrophobic interaction.

Effects of ANS on TLN activity - FAGLA has widely been used as a substrate for TLN (45-48). However, A_{345} detection was not available because of the effect of ANS. Accordingly, MOCAC-PLGL(Dpa)AR was used,(48) and the products were detected by reversed-phase HPLC (Fig. 2). TLN and ANS were pre-incubated at 25°C for 10 min, followed by the reaction at 25°C. The reaction rates were unchanged with increasing concentration of ANS. This indicates that ANS neither activates nor inhibits TLN activity.

Effects of salts on ANS fluorescence in the presence of TLN - Fluorescence spectra of ANS in the presence of 1.0 μ M TLN in buffer A with varying concentrations of NaCl, NaBr, LiCl, or KCl were measured. The change of FI_{490} , ΔFI_{490} , defined as FI_{490} in the presence of 1.0 μ M TLN minus FI_{490} in the absence of TLN, increased with increasing salt concentrations from zero to 4.0 M (Fig. 3A). ΔFI_{490} at 4.0 M NaCl, NaBr, LiCl, and KCl were 249, 204, 168, and 128%, respectively, of those at 0 M. In the presence of 1.0 μ M TLN, $\lambda_{F\text{I}\text{max}}$ at 4.0 M NaCl, NaBr, LiCl, and KCl were shorter by 17, 15, 11, and 8 nm, respectively, than $\lambda_{F\text{I}\text{max}}$ at

0 M NaCl (Fig. 3B). In the absence of TLN, $\lambda_{FI_{max}}$ was unchanged (data not shown). Thus, $\Delta\lambda_{FI_{max}}$, defined as $\lambda_{FI_{max}}$ in the presence of 1.0 μ M TLN minus $\lambda_{FI_{max}}$ in the absence of TLN, decreased from -6 to -17 nm with increasing NaCl concentration ([NaCl]), -6 to -15 nm with increasing [NaBr], -6 to -8 nm with increasing [LiCl], and -5 to -8 nm with increasing [KCl] from zero to 4.0 M. ΔFI_{490} and $\Delta\lambda_{FI_{max}}$ at 0–4 M NaCl are summarized in Table 1. These results indicate that the magnitude of the salt-induced changes in ANS fluorescence by 1.0 μ M TLN at pH 7.5 was in the order of NaCl, NaBr, LiCl, and KCl, suggesting that Na^+ ion potently and Li^+ and K^+ ions slightly affect the ANS fluorescence in the presence of TLN, while Cl^- and Br^- ions hardly affect it.

Binding of ANS with TLN at various NaCl concentrations - To measure the dissociation constants, K_d , of ANS with TLN, fluorescence area of varying concentrations of ANS was measured in the presence of 1.0 μ M TLN at 0, 1.0, 2.0, 3.0, and 4.0 M NaCl, pH 7.5. The plot of [ANS]/ ΔFI_{area} vs. [ANS] showed non-parallel lines intersecting near at the Y-axis (Fig. 4A). Under the assumption that ANS binds with TLN at a single site, the K_d values at pH 7.5 at 0, 1.0, 2.0, 3.0, and 4.0 M NaCl were calculated to be 33 ± 2 , 29 ± 2 , 23 ± 4 , 15 ± 4 , and 9 ± 3 μ M, respectively. Relative K_d was defined as the ratio of the K_d value at x M NaCl to that at 0 M NaCl (33 ± 2 μ M). The plot of the reciprocal of relative K_d vs. [NaCl] indicated that the reciprocal of relative K_d value increased with increasing [NaCl] (Fig. 4B). The increase in logarithmic value of the reciprocal of relative K_d value was not proportional to [NaCl] (Fig. 4C).

Binding of ANS with TLN at various salt concentrations - To measure K_d of ANS with TLN, fluorescence area of varying concentrations of ANS was measured in the presence of 1.0 μ M TLN at 4.0 M NaCl, NaBr, LiCl, or KCl at pH 7.5. The plot of [ANS]/ ΔFI_{area} vs. [ANS] showed non-parallel lines which did not intersect (Fig. 5). The K_d values at pH 7.5 at 4.0 M NaCl, NaBr, LiCl, and KCl were calculated to be $9 \pm$

3, 10 ± 2 , 12 ± 3 , and 15 ± 2 μM , which were 27–45% of that of K_d without salts (33 ± 2 μM), indicating that the K_d values hardly depend on salt species at 4 M.

Effects of pH on ANS fluorescence in the presence of TLN - Fluorescence spectra of ANS in the presence of 1.0 μM thermolysin at 0 M NaCl with varying pH were measured. ΔFI_{490} , defined as FI_{490} in the presence of 1.0 μM TLN minus FI_{490} in the absence of TLN, increased with increasing pH from 4.0 to 9.0, and ΔFI_{490} at pH 9.0 was 500% of that at pH 4.0 and 170% of that at pH 7.5 (Fig. 6A). In the presence of 1.0 μM TLN, $\lambda_{FI_{\text{max}}}$ at pH 9.0 was shorter by 17 nm than that at pH 4.0 and by 14 nm than that at pH 7.5, while in the absence of TLN, it was stable (Fig. 6B). Thus, $\Delta\lambda_{FI_{\text{max}}}$ decreased from -3 to -16 nm with increasing pH (7.0–9.0) of NaCl. These results indicate that the magnitude of the change in ANS fluorescence by 1.0 μM TLN at 0 M NaCl increased with increasing pH from 7.0 to 9.0.

Binding of ANS with TLN at various pH - TLN exhibits bell-shaped pH-activity profile with the maximum at pH 6.5 and 7.0 (54) Thermal stabilities of TLN at 70°C at pH 4.5–8.5 were measured (Fig. 7A). Relative stability was defined as the ratio of k_{obs} at pH 4.5 to that at given pH. The result indicated that the relative stability was highest at pH 6.5. To measure the dissociation constants, K_d , of ANS with TLN, fluorescence areas of varying concentrations of ANS were measured in the presence of 1.0 μM TLN at 0 M NaCl, pH 5.5, 6.5, 7.5, and 8.5. The plot of $[\text{ANS}]/\Delta FI_{\text{area}}$ vs. $[\text{ANS}]$ showed non-parallel lines intersecting at the X-axis (Fig. 7B). Under the assumption that ANS binds with TLN at a single site, the K_d values at 0 M NaCl at pH 5.5, 6.5, 7.5, and 8.5 were 34 ± 3 , 31 ± 2 , 33 ± 2 , and 32 ± 2 μM , respectively (Fig. 7B), indicating that the K_d values were unchanged with varying pH from 5.5 to 8.5.

Discussion

It has been observed that the effectiveness of monovalent cations of neutral salts to activate thermolysin is in the order of $\text{Na}^+ > \text{K}^+ > \text{Li}^+$, an order different to that of the Hofmeister series ($\text{Li}^+ > \text{Na}^+ > \text{K}^+$) (80–82) or the ionic series of the monovalent cations ($\text{K}^+ > \text{Na}^+ > \text{Li}^+$). Based on this evidence, the activation of thermolysin by neutral salts is not derived from changes in water structure or in the hydration of the enzyme or substrate. Hence it has been speculated that the salt-induced activation of TLN might be as a result of conformational changes brought about by the direct interactions of the ions with charged residues on the enzyme (80–82).

In this study, we showed that ANS binds with TLN (Fig. 1), ANS does not inhibit TLN activity (Fig. 2), and NaCl and other salts increase the affinity of TLN for ANS (Figs 3–5). The degree of the salt-induced changes in ANS fluorescence by TLN was in the order of NaCl, NaBr, LiCl, and KCl (Fig. 3), suggesting that Na^+ affects ANS fluorescence more potently than Cl^- ion. It also suggests that the effectiveness of monovalent cations of neutral salts on the salt-induced changes in ANS fluorescence by thermolysin is $\text{Na}^+ > \text{Li}^+ > \text{K}^+$, being different from that of activation of thermolysin ($\text{Na}^+ > \text{K}^+ > \text{Li}^+$). The K_d value of ANS and TLN at 4.0 M salt was in the order of KCl, LiCl, NaBr, and NaCl (Fig. 5), suggesting that the degree of the effects of salts on the interaction of ANS and TLN depends on salt species. From the result that the K_d values of ANS and TLN did not change with increasing pH from 5.5–8.5 (Fig. 7), we speculate that amino acid residues with side chains with $\text{p}K_a$ values of around 5.5–8.5 might not be located in the ANS-binding site. However, from the results presented in this study, it is difficult to precisely speculate the mechanism of the effects of salts on the interaction of ANS and TLN.

In contrast to that little is known about the mechanism of salt-induced activation of thermolysin, effects of ions on enzyme activity have been well studied in human immunodeficiency virus type-1 (HIV-1) protease. HIV-1 protease is a homodimeric aspartic protease, each containing 99 amino acid residues. Like TLN, HIV-1 is highly activated and stabilized by neutral salts (1–2 M NaCl) (83–85). Recent molecular

dynamics simulations and conductivity measurement analysis of HIV-1 protease have shown that Na^+ binds at least twice as strongly to the surface of HIV-1 protease than K^+ does (86-88).

In conclusion, this study suggests that Na^+ and /or Cl^- ions bind with thermolysin and affect its binding with ANS. We also think that the activation and stabilization of TLN by neutral salts might be due to the binding of ions with TLN.

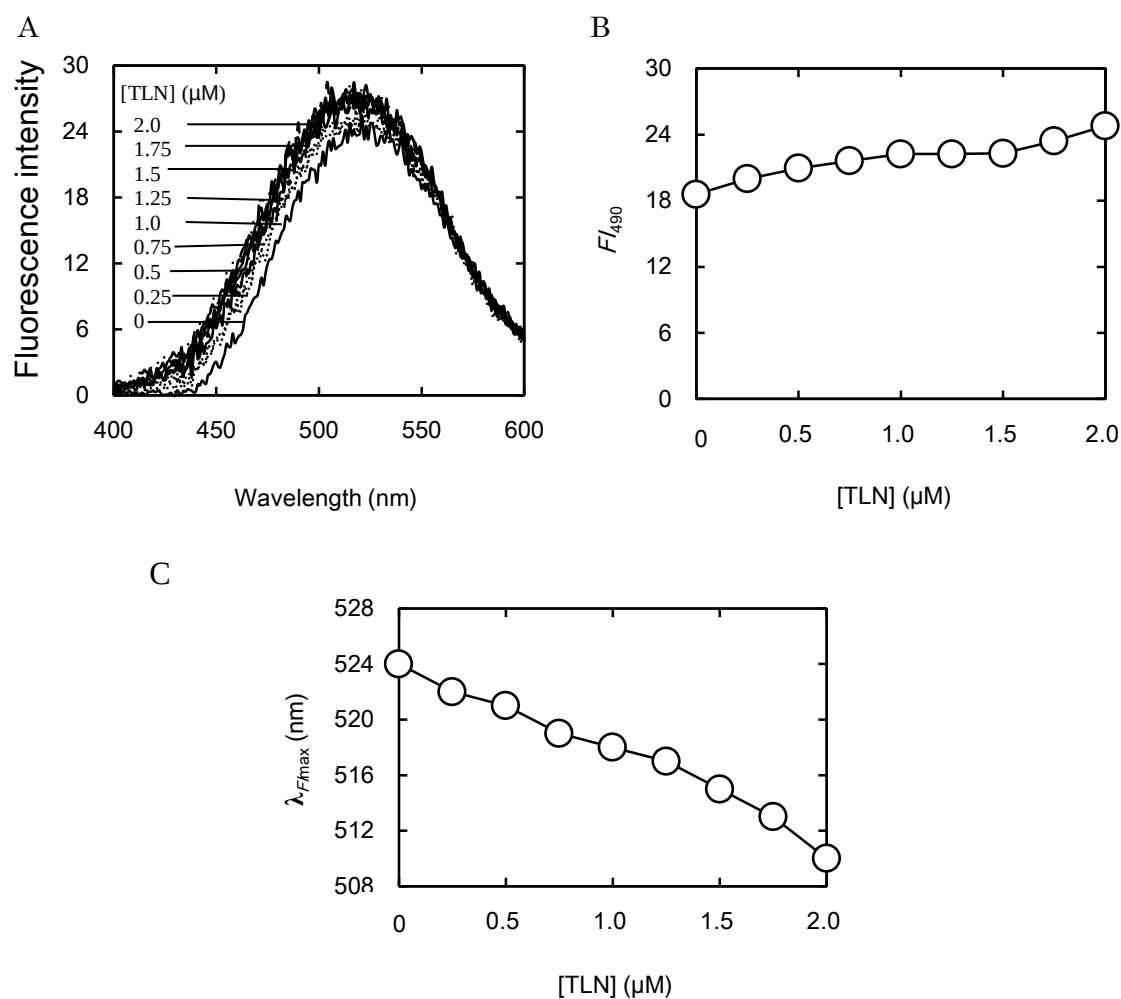


Fig. 1. Effect of TLN on the fluorescence of ANS. Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μ M ANS in 40 mM HEPES buffer, 10 mM CaCl_2 , pH 7.5 with various concentrations of TLN at 25°C. (A) Fluorescence spectra with 0–2.0 μ M TLN. (B) FI_{490} with 0–2.0 μ M TLN. (C) Wavelength giving the maximum fluorescence (λ_{FImax}).

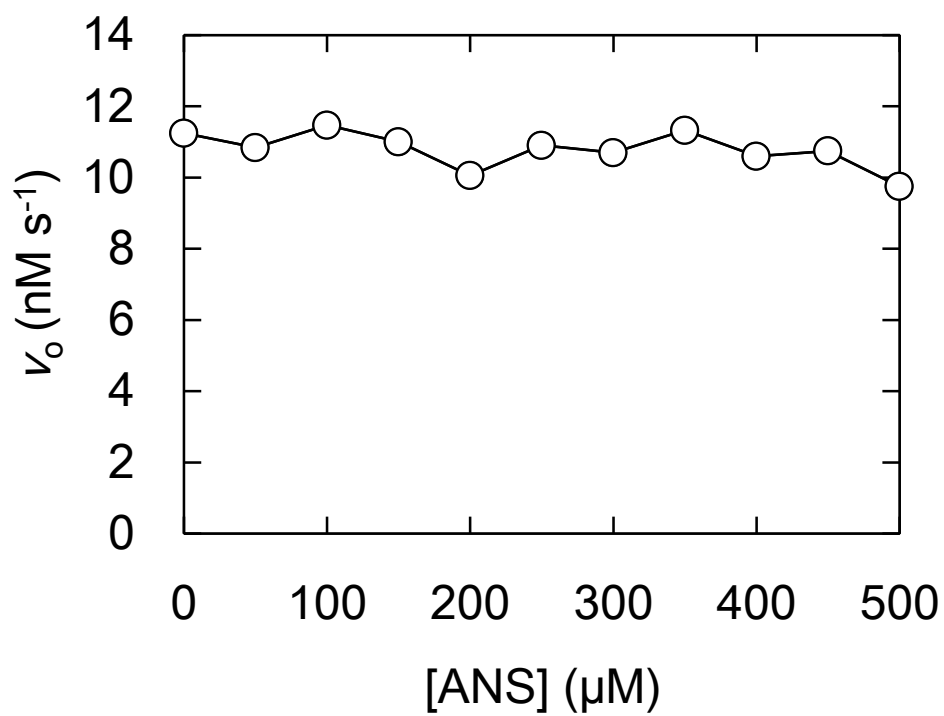


Fig. 2. Effect of ANS on the activity of TLN. Pre-incubation of TLN was carried out for 10 min in the presence and the absence of ANS at 25°C at pH 7.5, and the reaction was carried out with 32 nM TLN and 20 μM MOCac-PLGL(Dpa)AR at 25°C at pH 7.5.

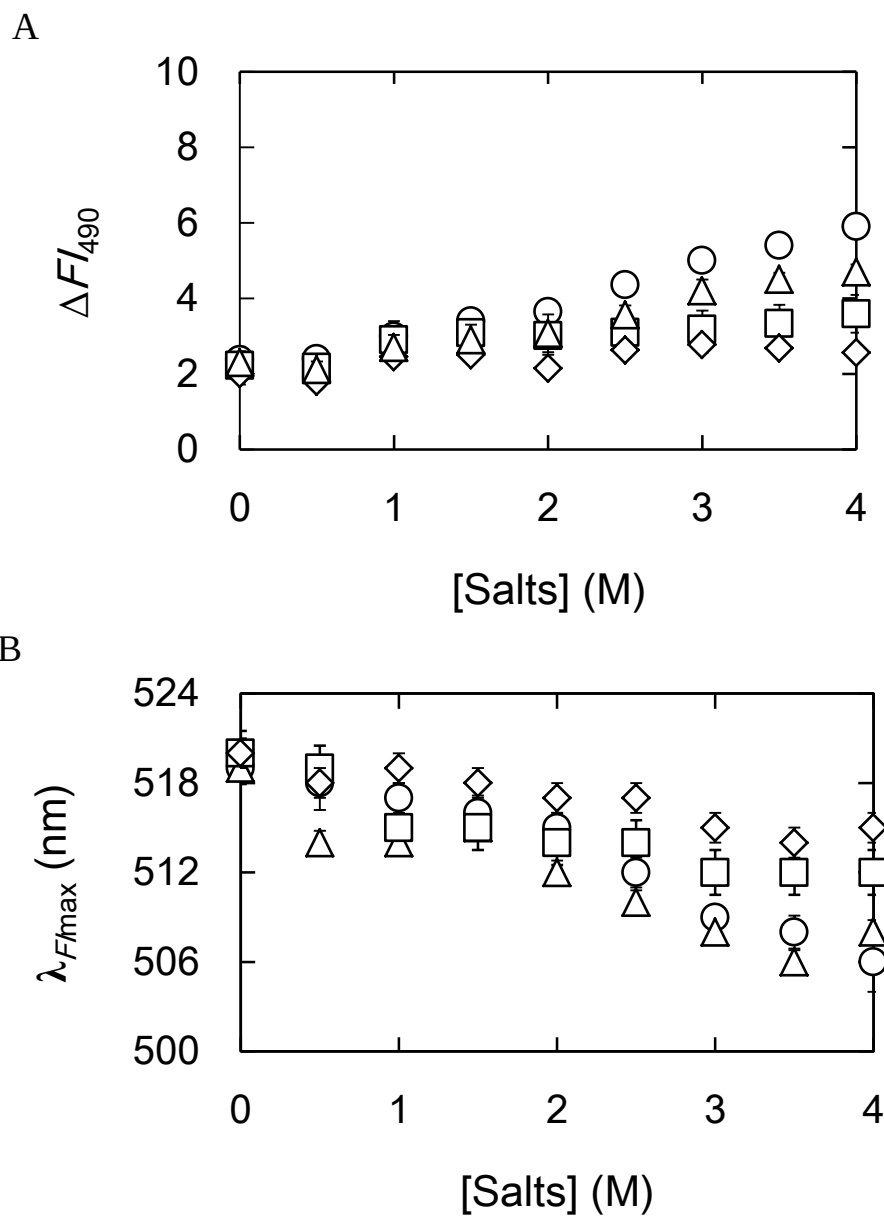


Fig. 3. Effect of salts on the fluorescence of ANS. Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μ M ANS in buffer A, 0–4.0 M NaCl, $\circ$; NaBr, Δ ; LiCl, $\square$; or KCl, $\diamond$; in the presence and absence of 1.0 μ M thermolysin at 25°C. (A) Change in fluorescence intensity at 490 nm (ΔFI_{490}). ΔFI_{490} was defined as the difference in FI_{490} between the values in the presence and the absence of 1.0 μ M TLN. ΔFI_{490} was plotted against salt concentration. (B) The wavelength giving the maximum fluorescence (λ_{Fmax}). λ_{Fmax} in the presence of 1.0 μ M TLN was plotted against salt concentration.

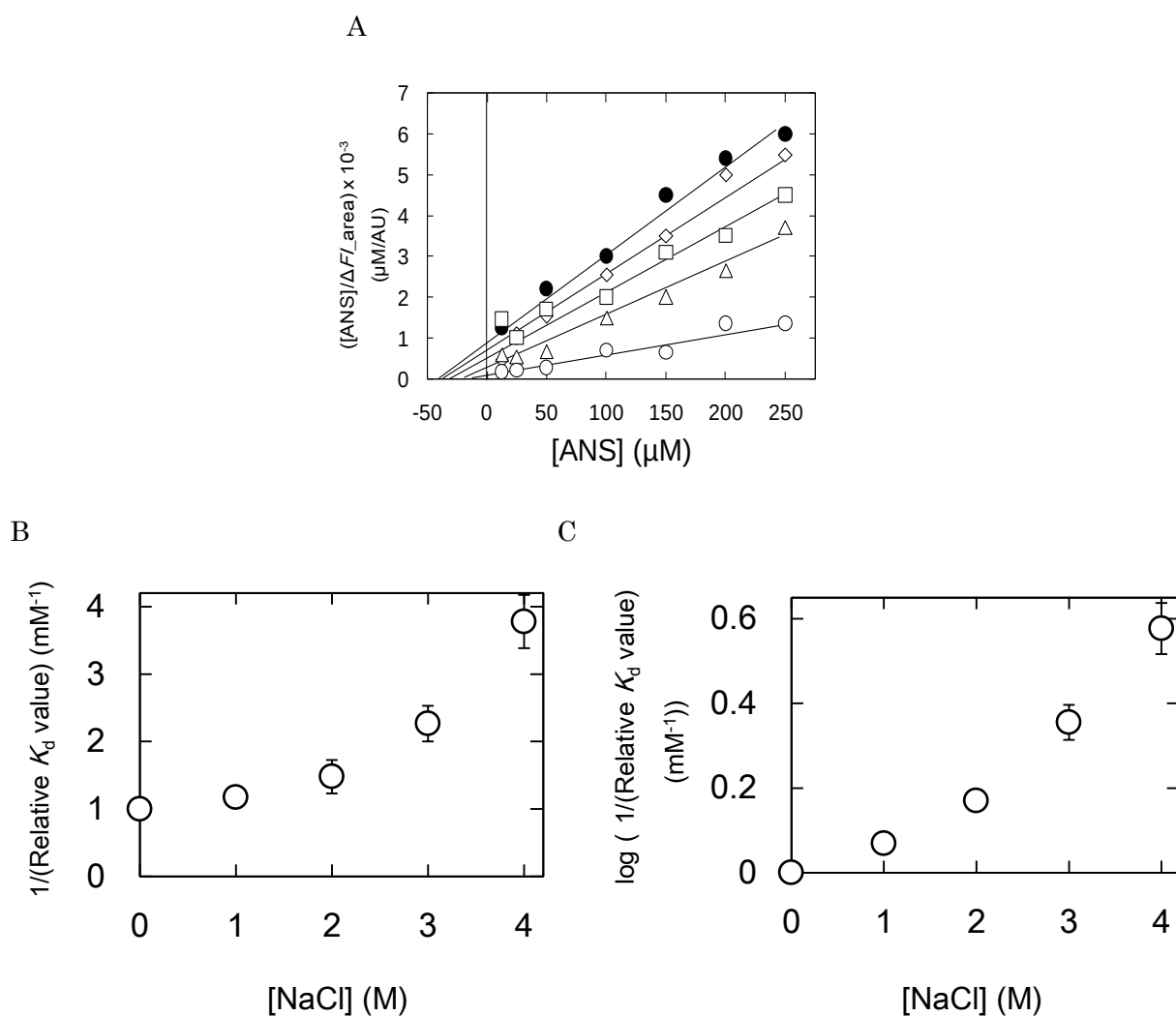


Fig. 4. Effect of NaCl on the binding of ANS with TLN. ΔFI_{area} was defined as the difference in FI_{490} between the values in the presence and the absence of $1.0 \mu\text{M}$ TLN. ΔFI_{area} were measured with $10\text{--}250 \mu\text{M}$ ANS in the presence of $0, \bullet$; $1.0, \diamond$; $2.0, \square$; $3.0, \Delta$; or $4.0 \text{ M}, \circ$ NaCl. $[\text{ANS}]/\Delta FI_{\text{area}}$ vs. $[\text{ANS}]$ plot is shown. (B) Effect of NaCl on the reciprocal of the dissociation constants (K_d). Relative K_d was defined as the ratio of the K_d value at $x \text{ M}$ NaCl to that at 0 M NaCl ($30 \pm 2 \text{ mM}^{-1}$). (C) Logarithmic relationship of the reciprocal of K_d with $[\text{NaCl}]$. Error bars indicate SD values of triplicate measurements.

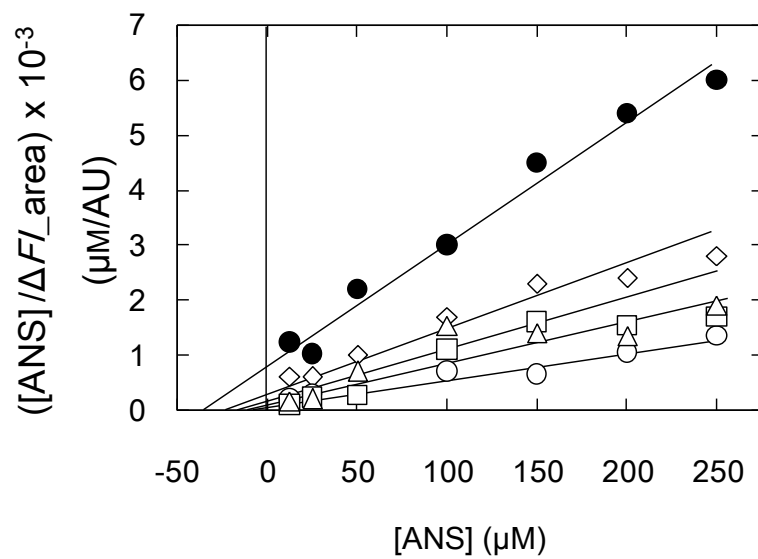


Fig. 5. Effect of salts on the binding of ANS with TLN. ΔFI_{area} were measured with 10–250 μM ANS in the presence of 4 M NaCl, $\circ$; NaBr, Δ ; LiCl, $\square$; or KCl, $\diamond$; and in the absence of salts, $\bullet$. $[\text{ANS}]/\Delta FI_{\text{area}}$ vs. $[\text{ANS}]$ plot is shown. One of the representative data is shown.

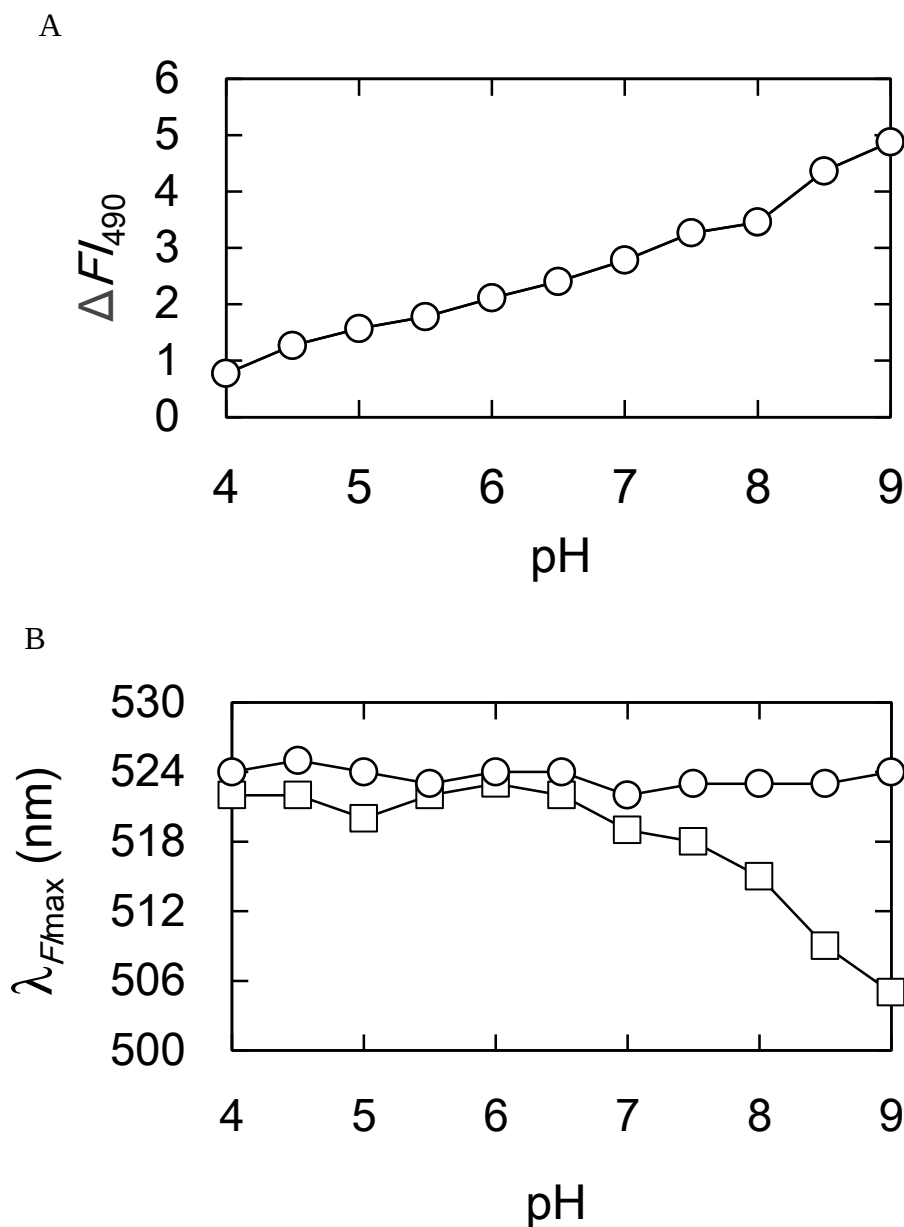


Fig. 6. Effect of pH on the fluorescence of ANS. Fluorescence spectra were measured with excitation at 380 nm and emission at 400-600 nm for 25 μ M ANS in 50 mM acetate-NaOH buffer at pH 3.5, 4.0, and 4.5, 50 mM MES-NaOH buffer at pH 5.0, 5.5 and 6.5, 50 mM HEPES-NaOH buffer at pH 7.5 and 8.0 and 50 mM AMPSO-NaOH buffer at pH 8.5 and 9.0, each containing 10 mM CaCl_2 at 25°C. (A) Change in fluorescence intensity at 490 nm (ΔFI_{490}). ΔFI_{490} was defined as the difference in FI_{490} between the values in the presence and the absence of 1.0 μ M TLN. ΔFI_{490} was plotted against pH. (B) Wavelength giving the maximum fluorescence (λ_{Fmax}). λ_{Fmax} in the presence, $\square$; and the absence, $\circ$; of 1.0 μ M TLN was plotted against pH.

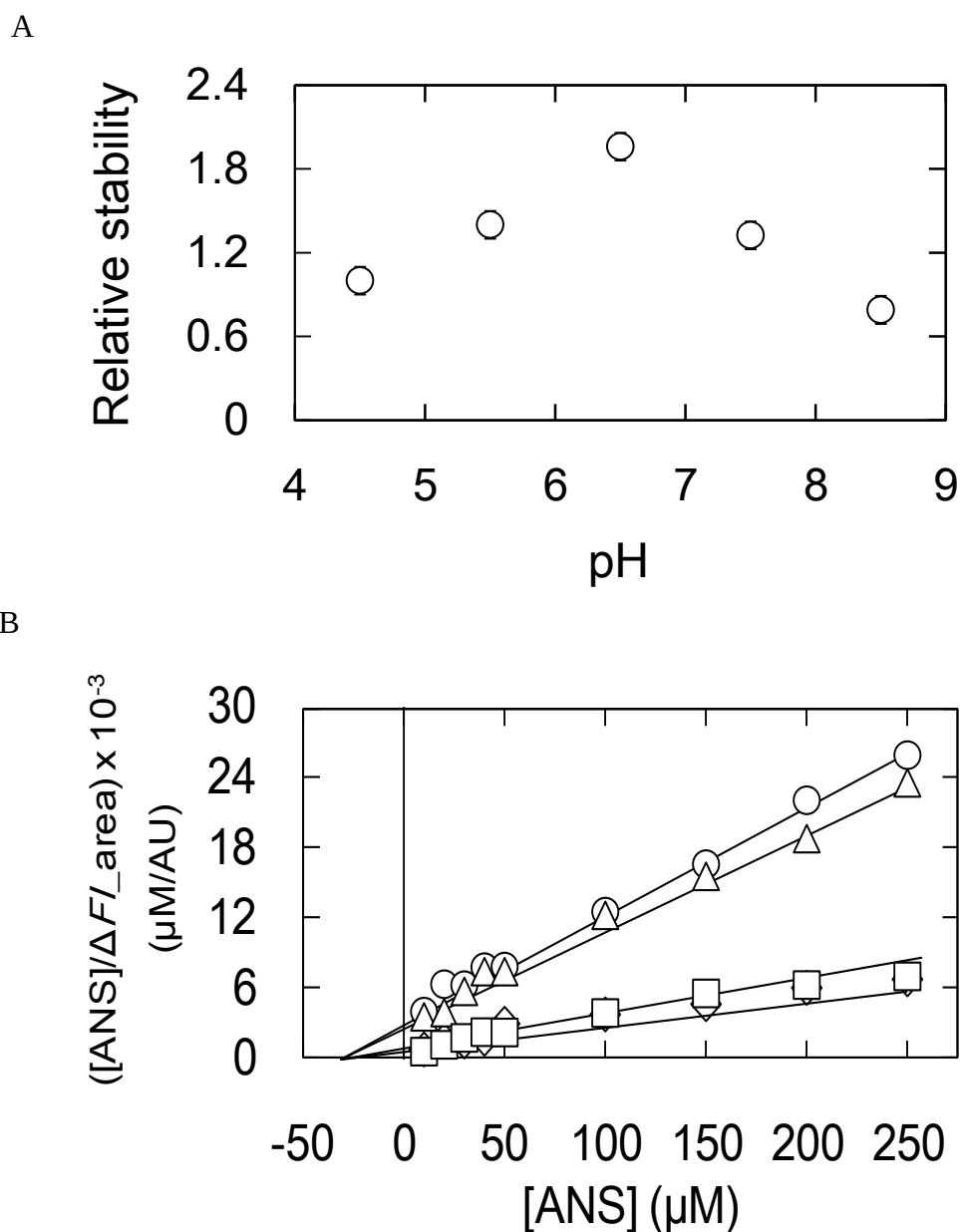


Fig. 7. Effect of pH on the binding of ANS with TLN. (A) pH-stability profile. TLN (1.0 μM) in 40 mM HEPES-NaOH, 10 mM CaCl₂, and 0–4.0 M NaCl at pH 7.5 was incubated at 70°C for specified durations. The experimental conditions for FAGLA hydrolysis were as described in the Materials and Methods section. Relative stability of TLN variants was defined as the ratio of the first-order rate constant, k_{obs} , of the thermal inactivation at 0 M NaCl ($1.2 \pm 0.1 \times 10^{-4} \text{ s}^{-1}$) to that at x M NaCl. (B) [ANS]/ΔFI_area vs. [ANS] plot. Symbols for pH: 5.5, ○; 6.5, Δ; 7.5, □; and 8.5, ◇. Error bars indicate SD values of triplicate measurements.

Summary

Chapter 1

Human matrix metalloproteinase 7 (MMP-7) is the smallest matrix metalloproteinase. It plays important roles in tumor invasion and metastasis. 8-Anilinonaphthalene 1-sulfonate (ANS) is a fluorescent probe widely used for the analysis of proteins. It emits a large fluorescence energy when its anilinonaphthalene group binds with hydrophobic regions of protein. In this study, we analyzed the interaction of ANS and MMP-7. At pH 4.5–9.5, ANS inhibited MMP-7 activity in the hydrolysis of MOCac-PLGL(Dpa)AR. The inhibition was a non-competitive manner and depended on the time for pre-incubation of ANS and MMP-7. At pH 4.5–9.5, the fluorescence of ANS was not changed by the addition of MMP-7. At pH 3.5, MMP-7 lacked activity, and the fluorescence of ANS was increased by the addition of MMP-7. These results suggest that at pH 4.5–9.5, the sulfonic group of ANS binds with MMP-7 through electrostatic interaction, while at pH 3.5, the anilinonaphthalene group of ANS binds with MMP-7 through hydrophobic interaction.

Chapter 2

Sulfated glycosaminoglycans and sulfated lipids are involved in the biological functions of human matrix metalloproteinase 7 (MMP-7). In this study, the effects of heparin and cholesterol sulfate (CS) on the activity and stability of MMP-7 in the hydrolysis of a synthetic substrate, MOCac-PLGL(Dpa)AR, were examined. Heparin increased activity by decreasing K_m , and the K_m values for 0 and 50 μM heparin were 57 ± 8 and 19 ± 5 μM , respectively. CS decreased activity in a non-competitive inhibitory manner with a K_i value of 11 ± 3 μM . In thermal incubation at 50–70°C, heparin increased relative activity (the ratio of k_{cat}/K_m of MMP-7 with incubation to that without it), while CS decreased relative activity. These results indicate that heparin increases the

activity and stability of MMP-7, while CS decreases them.

Chapter 3

Neutral salts activate and stabilize thermolysin (TLN). In this study, to explore the mechanism, we analyzed the interaction of 8-anilinonaphthalene 1-sulphonate (ANS) and thermolysin by ANS fluorescence. At pH 7.5, the fluorescence of ANS increased and blue-shifted with increasing concentrations (0–2.0 μM) of TLN, indicating that the anilinonaphthalene group of ANS binds with TLN through hydrophobic interaction. ANS did not alter TLN activity. The dissociation constants (K_d) of the complex between ANS and TLN was $33 \pm 2 \mu\text{M}$ at 0 M NaCl at pH 7.5, decreased with increasing NaCl concentrations, and reached $9 \pm 3 \mu\text{M}$ at 4 M NaCl. The K_d values were not varied (31–34 μM) in a pH range of 5.5–8.5. This suggests that at high NaCl concentrations, Na^+ and/or Cl^- ions bind with TLN and affect the binding of ANS with TLN. Our results also suggest that the activation and stabilization of TLN by NaCl are partially brought about by the binding of Na^+ and/or Cl^- ions with TLN.

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Vimbai Netsai Charity Samukange

List of Publications

Original papers

1. Samukange, V., Yasukawa, K., and Inouye, K. (2012) Interaction of 8-anilinonaphthalene 1-sulphonate (ANS) and human matrix metalloproteinase 7 (MMP-7) as examined by MMP-7 activity and ANS fluorescence. *J. Biochem.* **151**, 533–540
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3. Samukange, V., Kamo, M., Yasukawa, K., and Inouye, K. (2014) Effects of salts on the interaction of 8-anilinonaphthalene 1-sulphonate (ANS) and thermolysin. *Biosci. Biotechnol. Biochem.* **78**, 1522–1528