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Effects of Dimethyl Sulfoxide, Temperature, and Sodium Chloride on the Activity of Human Matrix Metalloproteinase 7 (Matrilysin)[1]

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Effects of dimethyl sulfoxide (DMSO), temperature, and sodium chloride on the matrilysin-catalyzed hydrolysis of (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR] were examined. DMSO inhibited the matrilysin activity competitively with the inhibitor constant (K_i) of 0.59±0.04 M, and the binding between them was endothermic and entropy-driven. The binding of matrilysin with MOCAc-PLGL(Dpa)AR was also found to be entropy-driven. The matrilysin activity was increased in a biphasic exponential fashion with increasing concentration of NaCl, and was 5.3 times higher in the presence of 4 M NaCl than that in its absence. The first and second phases were separated at 0.5 M NaCl, and the activation at x M NaCl compared with the activity in the absence of NaCl was expressed as 2.1^x at [NaCl] < 0.5 M and 1.4^x at [NaCl] > 0.5 M. The activation was brought about solely through a decrease in the Michaelis constant (K_m), and the catalytic constant (k_{cat}) was not much altered. This suggests that the decrease in the electrostatic interaction and the increase in the hydrophobic interaction between matrilysin and the substrate might enhance the enzyme activity by reducing the K_m value.

Key words: dimethyl sulfoxide, enzyme activity, matrilysin, matrix metalloproteinase, salt activation, temperature.

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that are believed to degrade extracellular matrix (ECM) and take part in both normal and pathological processes, including development, reproduction, maintenance, tissue destruction, and fibrotic diseases (*1*, *2*).

Matrilysin (MMP-7)[EC 3.4.24.23] is the smallest MMP consisting of a signal peptide, a propeptide, and a catalytic domain, and the molecular mass of the latent pro-form is 28 kDa and that of its mature form is 19 kDa (*3*, *4*). Matrilysin has been detected in lesions of prostate (*5*), colon (*6*), brain (*7*), stomach (*8*), lung (*9*), and breast (*10*), and degrades the ECM components such as gelatins of type I, III, IV, and V, type IV basement membrane collagen, fibronectin, vitronectin, proteoglycan, laminin, and elastin (*4*, *11–13*), suggesting that matrilysin may play a role in tumor invasion and metastasis.

The X-ray crystallographic analysis of matrilysin in complex with several different inhibitors demonstrated that matrilysin is composed of a five-stranded β-sheet, three α-helices, a catalytic zinc, a structural zinc, and two structural calcium ions (*14*). The three His residues are bound coordinately to the catalytic zinc, whereas the third zinc ligand of a well-studied metalloproteinase, thermolysin, is a Glu residue (*15*, *16*). We reported remarkable activation of thermolysin by high concentration (1–4 M) of neutral salts in the hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, a precursor of a synthetic sweetener (*17*), and in the hydrolysis of *N*-[3-(2-furyl)acryloyl]-dipeptide amides (*18*). The peptidase activity of thermolysin increases in an exponential fashion with increasing concentration of salt, and the degree of activation at x M NaCl was expressed by 1.9^x (*18*). It is noted that the activation is brought about solely through an increase in the catalytic constant (k_{cat}), and the Michaelis constant (K_m) is not affected at all. Recently, we have proposed an effective procedure to prepare active matrilysin from inclusion bodies expressed by *Escherichia coli* in good yield and at reasonable cost (*19*), and examined the effect of salt on its azocoll-hydrolyzing activity (*20*). The activity decreased to 40–50% with addition of 0.2–0.5 M NaCl, increased to 200% with increasing [NaCl] from 0.5 to 3 M, and decreased again with increasing [NaCl] from 3 to 4 M. This complex behavior seems to reflect the complex interaction between the collagen substrate and matrilysin. The salt effect on matrilysin should be re-examined with a simple synthetic substrate.

In this paper, effects of temperature and salt on the peptidase activity of matrilysin are studied using a fluorescent artificial substrate, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR]. Although this substrate has been used commonly for studies on MMPs (*21*), a small amount of an organic solvent such

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Abbreviations: DMSO, dimethyl sulfoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MMP, matrix metalloproteinase; MOCAc-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; L(Dpa)AR, L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2.

as dimethyl sulfoxide (DMSO) must be added to the reaction solution to dissolve it. Here, the inhibitory effect of DMSO on the matrilysin activity, the thermodynamic properties of the interactions of matrilysin with the substrate and DMSO, and the characteristic activation of matrilysin by a high concentration of NaCl are described.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human pro-matrilysin was prepared according to the methods previously reported (*19*). Pro-matrilysin was incubated with 1 mM *p*-aminophenylmercuric acetate at pH 7.5, 37°C, for 30 min and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.05% Brij-35 (buffer A), 4°C. The preparation was applied to metal chelation HPLC on a TSKgel Chelate-5PW column [7.5 mm (inner diameter) × 75 mm] (Tosoh, Tokyo) equilibrated with buffer A (pH 7.5) containing 0.5 M NaCl, 25°C. After charging the column with 1 mM $ZnCl_2$, a linear gradient was generated from 0 M to 0.5 M glycine over 30 min at a flow-rate of 1 ml/min at 25°C. Matrilysin was eluted at 0.24 M glycine, and the fractions were collected for further analysis. The concentration of matrilysin was determined with a Shimadzu UV-2200 spectrophotometer (Kyoto) using the molar absorption coefficient at 280 nm of 31.8 $mM^{-1}\cdot cm^{-1}$ calculated from amino acid composition (*3*). A substrate of matrilysin, MOCAc-PLGL(Dpa)AR, is known to be cleaved at the peptide bond between glycine and leucine residues (*21*). MOCAc-PLGL(Dpa)AR (Lot 480429) and MOCAc-L-Pro-L-Leu-Gly (MOCAc-PLG, Lot 471218) were purchased from the Peptide Institute (Osaka), and their concentrations were determined using the molar absorption coefficients ε_{410}=7.5 $mM^{-1}\cdot cm^{-1}$ and ε_{324}=12.9 $mM^{-1}\cdot cm^{-1}$, respectively (*21*). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto). The HPLC apparatus consisted of a solvent-delivery system CCPM-II, a UV monitoring system UV-8020, a computer-control system PX-8020, a degasser SD-8020, a column oven CO-8020 and an integrator Chromatocorder 21, purchased from Tosoh (Tokyo).

HPLC of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The hydrolysis of MOCAc-PLGL(Dpa)AR by matrilysin was initiated by adding 30 μl of the substrate solution (0.11–3.0 mM) in DMSO to 970 μl of the matrilysin solution (8.1 nM) in 50 mM HEPES buffer containing 10 mM $CaCl_2$ at pH 7.5. The initial concentration of matrilysin in the reaction solution was 7.88 nM. The reaction was stopped by mixing 100 μl of the reaction solution with 400 μl of 1% trifluoroacetic acid (TFA). The mixture (100 μl) was applied to HPLC, which was performed on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) × 150 mm] (Tosoh, Tokyo) equilibrated with 0.1% TFA. A linear gradient was generated from 20 to 70% acetonitrile at time 5 min over 15 min at a flow-rate of 1 ml/min, and absorption of the eluate was measured at 335 nm. The amount of the product, MOCAc-PLG, was determined from the peak area. The initial reaction rate, v, at a substrate concentration was determined from the time course of the amount of MOCAc-PLG formed, and the kinetic parameters, the catalytic (k_{cat}) and Michaelis (K_m) constants, were determined according to the Michaelis-Menten equation by using the nonlinear least-squares method (*22*).

Fluorometric Analysis of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was performed by mixing 1,242 μl of the matrilysin solution (3.8 nM) in 50 mM HEPES buffer containing 10 mM $CaCl_2$ and 8 μl of MOCAc-PLGL(Dpa)AR (62.5 μM) dissolved in DMSO at pH 7.5, 25°C. The hydrolysis of MOCAc-PLGL(Dpa)AR was measured by following the increase in the fluorescence intensity at 393 nm by excitation at 328 nm with a JASCO FP-777 fluorescence spectrophotometer (Tokyo). The reaction was carried out under pseudo-first order conditions, where the substrate concentration is much lower than the K_m value, in order to avoid its absorptive quenching effects.

Thermodynamic Analysis of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The activation energy (E_a) of the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was determined from the slope ($-E_a/R$) of the Arrhenius plot of k_{cat} against $1/T$, and the activation parameters of the hydrolysis were determined according the following equations (*23*):

$$\Delta G^{\ddagger} = -RT\{\ln k_{cat} - \ln(kT/h)\} \quad (1)$$

$$\Delta H^{\ddagger} = E_a - RT \quad (2)$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T \quad (3)$$

where k, h, and R are the Boltzmann, Planck, and gas constants, respectively, and T is the temperature in degrees Kelvin. The standard enthalpy change ($\Delta H^{\circ\prime}$) for the binding of matrilysin with the substrate was determined from the slope ($\Delta H^{\circ\prime}/R$) of the plot of the K_m value at 0% DMSO, which is designated as K_{mo}, against $1/T$ (Van't Hoff plot). The other thermodynamic parameters for the binding of matrilysin with the substrate were determined according to the following equations (*23*):

$$\Delta G^{\circ\prime} = -RT \ln (1/K_{mo}) \quad (4)$$

$$\Delta S^{\circ\prime} = (\Delta H^{\circ\prime} - \Delta G^{\circ\prime})/T \quad (5)$$

The thermodynamic parameters for the binding of matrilysin with DMSO were determined by replacing K_{mo} in Eq. 4 with the inhibitor constant, K_i, of DMSO against matrilysin.

RESULTS

HPLC of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The hydrolysis was monitored by HPLC rather than fluorometrically because of the absorptive quenching effect at high concentration of MOCAc-PLGL (Dpa)AR. Although the substrate was sparsely soluble in aqueous solution, its solubility increased to 90 μM by adding 3% DMSO. The substrate and its two products, MOCAc-PLG and L(Dpa)AR, were separated by HPLC with TSKgel ODS-80Ts, and MOCAc-PLGL(Dpa)AR, MOCAc-PLG, and L(Dpa)AR were eluted at 16.6, 13.4, and 6.17 min, respectively (Fig. 1A). The reaction rate, v, was determined from the time course of the production of MOCAc-PLG, and the k_{cat} and K_m values for the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR in the presence of 3% DMSO at pH 7.5, 25°C were determined to be 5.0 ± 0.2 s^{-1} and 45 ± 4 μM, respectively (Fig. 1B).

Effect of DMSO on the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was examined at DMSO

concentrations ranging from 3 to 10% at pH 7.5, 25°C, and the k_{cat} and K_m values were determined in the same way as in Fig. 1. When the DMSO concentration increased from 3 to 10%, the matrilysin activity decreased, and K_m increased significantly from 45 ± 4 to 87 ± 8 μM, while k_{cat} increased only slightly from 5.0 ± 0.2 to 5.8 ± 0.2 s^{-1} (Fig. 2). It is noted that the catalytic activity is enhanced by DMSO, although the activation was small. It is obvious that the inhibition of matrilysin by DMSO is derived from the increase in the K_m value. Accordingly, the inhibition is considered to be substantially competitive. The inhibitor constant (K_i) of DMSO against matrilysin was calculated to be 4.64 ± 0.28%, namely, 0.59 ± 0.04 M, using the rate equation (Eq. 6) for the competitive inhibition.

$$v = \{k_{cat}[S][E]_o\}/\{K_m(1 + [I]/K_i) + [S]\} \quad (6)$$

The K_m value at 0% DMSO (which is designated as K_{mo}) was estimated to be 28 ± 3 μM by extrapolating the K_m values at various DMSO concentrations (Fig. 2B).

Temperature Dependence of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—Temperature dependence of the kinetic parameters, k_{cat} and K_m, of the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was examined at temperatures ranging from 20 to 37°C (Fig. 3). The rapid equilibrium between the (E+S) mixture and the ES complex is generally maintained in the Michaelis-Menten type enzyme kinetics, and even in the steady-state conditions, the rate constant (k_2) for the reaction of the ES complex to the (E+P) mixture is much smaller than that (k_{-1}) for the reaction of the ES complex to the (E+S) mixture. Therefore, it is reasonable to set K_m [= $(k_{-1} + k_2)/k_1$] equal to K_s (= k_{-1}/k_1), where k_1 is the rate constant for the formation of the ES complex from the E and S mixture. It should be mentioned that K_m was used to analyze formation of the ES complex, assuming that K_m represents the dissociation constant (K_s) of the ES complex. The thermodynamic parameters for the formation of matrilysin-DMSO and matrilysin-[MOCAc-PLGL(Dpa)AR] complexes are summarized in Table I. The large positive $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values for the binding of matrilysin with DMSO indicate that the binding is endothermic and entropy-driven. Consequently, it is considered that the K_m value for the hydrolysis of MOCAc-PLGL(Dpa)AR by matrilysin in the presence of DMSO may increase with increasing temperature. The binding of matrilysin with MOCAc-PLGL(Dpa)AR was also analyzed thermodynamically using K_{mo}. A small change was observed in K_{mo} between 20 and 37°C, leading to a small negative $\Delta H^{\circ\prime}$ value. The $\Delta S^{\circ\prime}$ value is positive, indicating that the binding is entropy-driven.

The activation energy (E_a) of the ES complex in the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was determined to be 60.6 ± 3.3 kJ·mol^{-1}. The Gibbs free energy of activation ($\Delta G^{\ddagger}$), enthalpy of activation ($\Delta H^{\ddagger}$), and entropy of activation ($\Delta S^{\ddagger}$) were calculated to be 68.9 ± 0.1

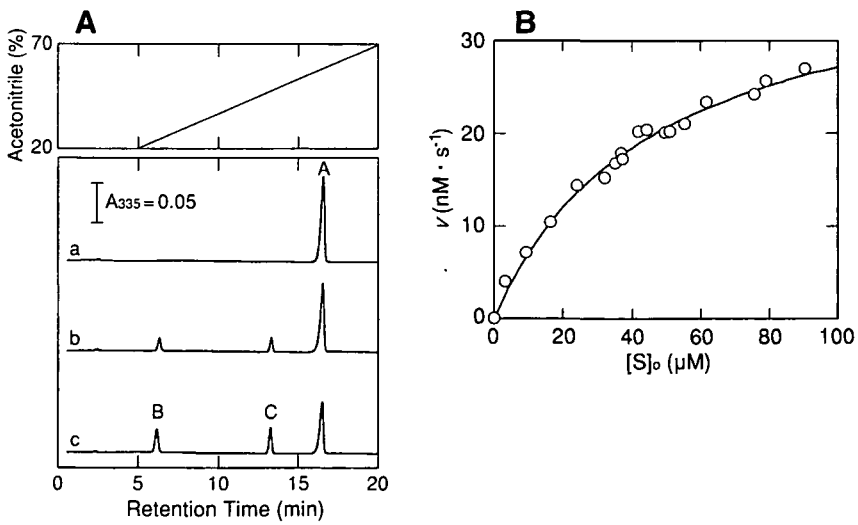

Fig. 1. **HPLC analysis of the matrilysin-catalyzed hydrolysis of MOCAc-PLGL-(Dpa)AR.** The reaction was performed in 50 mM HEPES (pH 7.5) plus 10 mM $CaCl_2$ and 3% DMSO at 25°C. The initial concentration of matrilysin was 7.88 nM. Panel A: Separation and determination of the products of the hydrolysis of MOCAc-PLGL(Dpa)AR by HPLC with TSKgel ODS-80Ts. The initial concentration of MOCAc-PLGL(Dpa)AR was 85.9 μM. The reaction times were: a, 0 min; b, 10 min; and c, 20 min. MOCAc-PLGL(Dpa)-AR, L(Dpa)AR, and MOCAc-PLG were eluted at peaks A (16.6 min), B (6.17 min), and C (13.4 min), respectively. Panel B: Dependence on the substrate concentration ([S]) of the reaction rate (v) of the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. The catalytic constant (k_{cat}) and the Michaelis constant (K_m) were determined to be 5.0 ± 0.2 s^{-1} and 45 ± 4 μM, respectively.

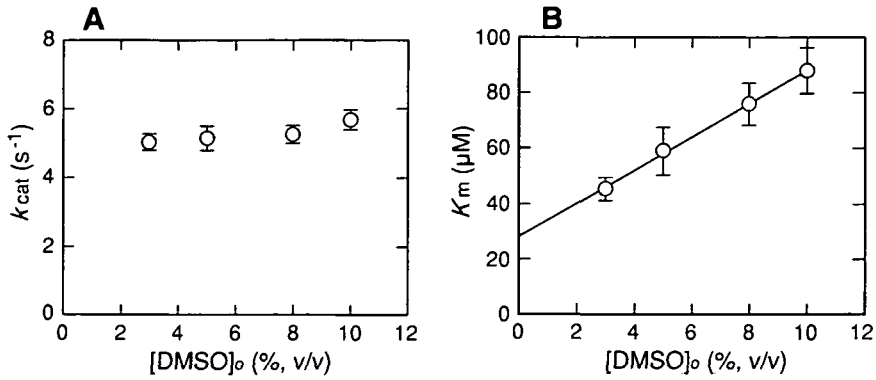

Fig. 2. **Effect of DMSO on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL-(Dpa)AR.** The reaction was performed in 50 mM HEPES (pH 7.5) plus 10 mM $CaCl_2$ and various concentrations of DMSO at 25°C. The initial concentration of matrilysin was 7.88 nM. Panel A: Effect of DMSO on the catalytic constant (k_{cat}). Panel B: Effect of DMSO on the Michaelis constant (K_m). The competitive inhibitor constant (K_i) of DMSO against matrilysin, and the K_{mo} value (K_m at 0% DMSO) were determined to be 4.64 ± 0.28% or 0.59 ± 0.04 M, and 27.7 μM, respectively.

kJ·mol^{-1}, 58.1 ± 3.3 kJ·mol^{-1}, and −36.2 ± 11.5 J·mol^{-1}·K^{-1}, respectively. The positive $\Delta H^{\ddagger}$ value suggests that the activation process of the ES complex is endothermic, and the positive $\Delta G^{\ddagger}$ value and the negative $\Delta S^{\ddagger}$ value suggest that the process shifts the complex to the more energy-rich and highly-ordered state.

Activation of the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR by NaCl—The matrilysin activity at high concentrations (1–4 M) of NaCl was measured under the pseudo-first order conditions ([S] ≪ K_m), because the solubility of MOCAc-PLGL(Dpa)AR decreases in the presence of NaCl. The substrate concentration used (400 nM) was low enough to eliminate the self-absorptive quenching effect, and the matrilysin activity was determined by measuring the fluorescence change continuously. Under the conditions used, the reaction rate, v, is expressed as k_{cat} [E]$_o$[S]/K_m (Michaelis-Menten equation). The matrilysin activity increased in a biphasic exponential fashion with an increase in the NaCl concentration, as is clearly shown by plotting the logarithmic value of v (ln v) against the initial NaCl concentration ([NaCl]$_o$) in the reaction solution (Fig. 4B). The plot is composed of two straight lines with slopes (ln v /[NaCl]$_o$) of 0.742 and 0.336 M^{-1}. The v values at 0 and 4 M NaCl were 0.24 and 1.26 nM·s^{-1}, respectively and thus the degree of activation at 4 M NaCl was calculated to be 5.3. The activation behavior was analyzed by the method previously applied (*18*). The activity (v) in the presence of x M NaCl is expressed as:

$$\ln v = \ln v_o + a \cdot x \quad (7)$$

where v_o is the activity in the absence of NaCl, and a is the slope of the straight line in Fig. 4B. Equation 7 can be rewritten as Eq. 8.

$$\ln (v/v_o) = a \cdot x \quad (8)$$

Thus, the degree of activation (v/v_o) values were determined to be 2.1^x when [NaCl]$_o$ < 0.5 M (phase 1), and 1.4^x when [NaCl]$_o$ > 0.5 M (phase 2).

The k_{cat} and K_m values in the presence of NaCl were determined independently by HPLC analysis (Fig. 5). A concentration of NaCl higher than 2 M could not be employed

TABLE I. **Thermodynamic parameters for the binding of matrilysin and DMSO or MOCAc-PLGL(Dpa)AR at pH 7.5, 25°C.** $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, and $\Delta S^{\circ\prime}$ are standard free energy change, standard enthalpy change, and standard entropy change, respectively.

	$\Delta G^{\circ\prime}$ (kJ·mol^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})	$\Delta S^{\circ\prime}$ (J·mol^{-1}·K^{-1})
DMSO	−1.31 ± 0.17	71.5 ± 14.0	244 ± 48
MOCAc-PLGL(Dpa)AR	−26.1 ± 0.1	−1.90 ± 2.43	80.9 ± 8.6

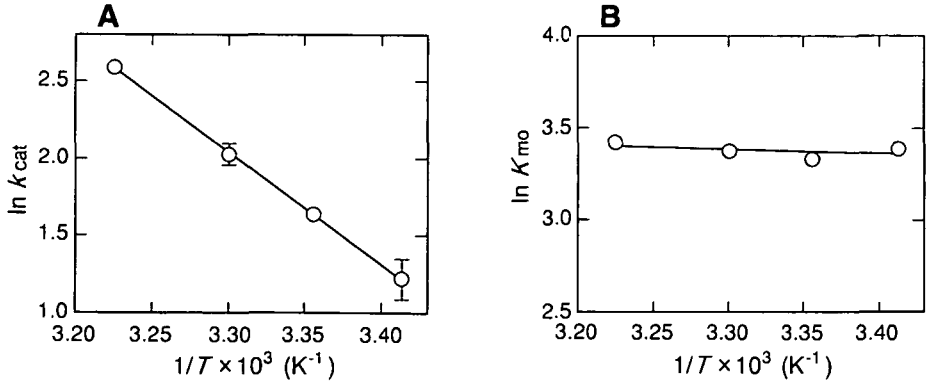

Fig. 3. **Effect of temperature on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR.** The reaction was performed in 50 mM HEPES (pH 7.5) plus 10 mM $CaCl_2$ and DMSO (3–10%) with the initial concentration of matrilysin of 7.88 nM. Panel A: Arrhenius plot of the catalytic constant [k_{cat} (s^{-1})]. The activation energy (E_a) was determined to be 60.6 ± 3.3 kJ·mol^{-1}. Panel B: Van't Hoff plot of the K_{mo} value [K_m (μM) at 0% DMSO]. By assuming that K_{mo} represents the dissociation constant of the ES complex, the standard enthalpy change ($\Delta H^{\circ\prime}$) for the binding between matrilysin and the substrate was determined to be −1.90 ± 2.43 kJ·mol^{-1}.

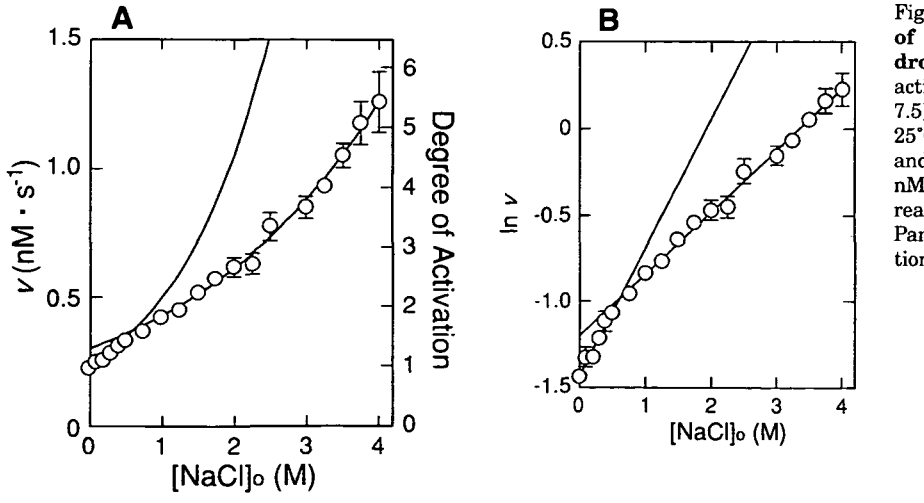

Fig. 4. **Fluorometric analysis of the effect of NaCl on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR.** The reaction was performed in 50 mM HEPES (pH 7.5) plus 10 mM $CaCl_2$ and 0.6% DMSO at 25°C. The initial concentrations of matrilysin and MOCAc-PLGL(Dpa)AR were 3.78 and 400 nM, respectively. Panel A. Effect of NaCl on the reaction rate (v) and the degree of activation. Panel B. Logarithmic relationship of the reaction rate with the NaCl concentration.

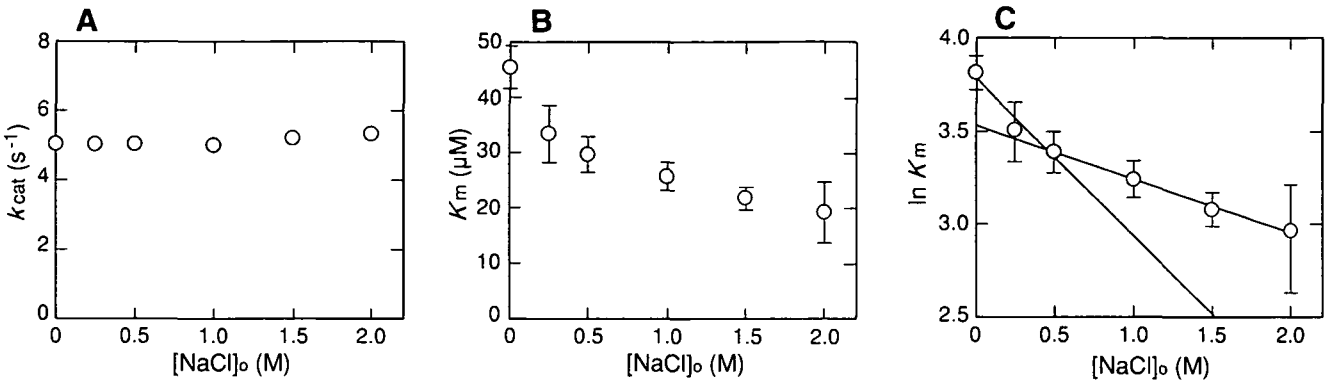

Fig. 5. **HPLC analysis of effect of NaCl on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR.** The reaction was performed in 50 mM HEPES (pH 7.5) plus 10 mM $CaCl_2$ and 3% DMSO at 25°C, and the substrate and the products were determined by HPLC as described in Fig. 1. The initial concentration of matrilysin was 7.88 nM. Panel A: Effect of NaCl on the catalytic constant (k_{cat}). Panel B: Effect of NaCl on the Michaelis constant (K_m). Panel C: Logarithmic relationship of the K_m value with the NaCl concentration.

because of the limited solubility of MOCAc-PLGL(Dpa)AR. The K_m value decreased with increasing [NaCl], and the k_{cat} value was not significantly affected (Fig. 5), suggesting that activation of the matrilysin by NaCl is brought about solely through a decrease in the K_m value. Furthermore, the K_m value decreased more sharply at $[NaCl]_0 < 0.5$ M than at $[NaCl]_0 > 0.5$ M. It is indicated that the different response of the K_m value depending on [NaCl] could be reflected in the biphasic activation of matrilysin by NaCl. The plot of (ln K_m) against $[NaCl]_0$ is also composed of two lines with slopes of –0.844 M^{-1} ($[NaCl]_0 < 0.5$ M) and –0.288 M^{-1} ($[NaCl]_0 > 0.5$ M) (Fig. 5C). The K_m values were analyzed by the same procedure as was used for the reaction rate using Eqs. 7 and 8. The ratios of the K_m value at x M NaCl to that at 0 M NaCl were determined to be 0.43^x when $[NaCl]_0 < 0.5$ M, and 0.75^x when $[NaCl]_0 > 0.5$ M. Because the reaction rate, v, is expressed as k_{cat} $[E]_0$ $[S]/K_m$, the degree of activation (v/v_0) was calculated to be 2.3^x when $[NaCl]_0 < 0.5$ M, and 1.3^x when $[NaCl]_0 > 0.5$ M. These values obtained by kinetics followed by HPLC in the presence of 3% DMSO were in good agreement with those obtained in the presence of 0.6% DMSO followed by fluorescence measurements. It also suggests that the degree of activation of matrilysin by NaCl is not influenced by the presence of DMSO, and that the activation by NaCl and inhibition by DMSO might be independent.

DISCUSSION

We have reported that a bacterial thermophilic metalloproteinase, thermolysin, is markedly activated (*17*, *18*) and stabilized (*24*) by high concentrations (1–4 M) of neutral salts, and is strongly inhibited by various organic solvents (*25*). Evaluation of the activity and thermal stability of matrilysin in comparison with thermolysin is expacted to further our understanding of the structure–activity relationship of metalloproteinases. In this study, 3% DMSO was added in the reaction solution in order to dissolve enough of the substrate MOCAc-PLGL(Dpa)AR to determine separately k_{cat} and K_m. The matrilysin activity is inhibited competitively by DMSO with K_i of 0.59 ± 0.04 M. Thermodynamic parameters for the formation of the matrilysin-DMSO complex (Table I) suggest that the binding of matrilysin with DMSO is endothermic and entropy-driven. The large positive entropy change ($\Delta S^{\circ\prime}$) suggests that a number of water molecules which are in ordered arrays around the hydrophobic surface of matrilysin must be released when DMSO binds to matrilysin (*26*). Accordingly, binding of DMSO to the active site of matrilysin might be mainly through hydrophobic interactions. On the other hand, the thermodynamic parameters for the binding of matrilysin with MOCAc-PLGL(Dpa)AR also indicate that the binding is entropy-driven.

We have reported that the activation of the thermolysin-catalyzed hydrolysis of *N*-[3-(2-furyl)acryloyl](FA)-dipeptide amide and *N*-carbobenzoxyl-L-phenylalanine methyl ester by high concentration of neutral salt is due to solely an increase in the catalytic constant (k_{cat}). The activity increases apparently in an exponential fashion with increasing concentration of salt, and the degree of activation at 4 M NaCl is in the range of 11–17-fold, depending on the substrate (*18*). For example, the degree of activation at x M NaCl for FA-dipeptide amides is equal to 1.9^x, where x is in the range of 0 to 5. The effect of NaCl on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR has been examined in comparison with the halophilicity of thermolysin. The matrilysin activity observed in the fluorometric analysis at 0.6% DMSO also seems to increase apparently in an exponential fashion with increasing NaCl concentration (Fig. 4). The degree of activation at 4 M NaCl is 5.3, considerably smaller than that of thermolysin with FA-dipeptides. It should be noted that the activation of matrilysin is biphasic depending on [NaCl], and the activation curve is composed of two exponential curves, while that of thermolysin is expressed by a single exponential curve. The degree of activation of matrilysin is expressed as 2.1^x at [NaCl] < 0.5 M and 1.4^x at [NaCl] > 0.5 M. This activation was derived from a biphasic decrease in K_m (Fig. 5). The dependence of K_m on [NaCl] is changed around 0.5 M NaCl. A similar type of salt-dependence was previously reported in the affinity of hydrophobic amino acids to the gel-filtration matrix Toyopearl, and the effect of NaCl on the electrostatic and hydrophobic interactions between the amino acids and the matrix was precisely analyzed (*27*). The isoelectric con-

stant (p*I*) of matrilysin is considered to be 5.9 (*28*). Accordingly, the decrease in K_m observed with increasing [NaCl] up to 0.5 M is supposed to be due to weakened repulsive electrostatic interactions between MOCAc-PLGL(Dpa)AR and matrilysin as a result of shielding of a positive charge on the substrate and negative charges on the enzyme. On the other hand, the decrease in the K_m value observed at [NaCl] > 0.5 M is considered to be derived from the strengthening of hydrophobic interactions with increase in [NaCl]. The increased hydrophobic interaction would favor the ES complex formation. It has been reported that matrilysin shows preference for a hydrophobic residue, especially a leucyl residue, in the P1′ position (*4*). A large positive value of the entropy change in the interaction between matrilysin and MOCAc-PLGL(Dpa)AR may be consistent with the involvement of hydrophobic interactions in the ES complex formation.

The activation of matrilysin by NaCl observed with the substrate MOCAc-PLGL(Dpa)AR (Fig. 4) is different from that observed with the azo-dye-coupled collagen substrate, azocoll (*20*). With azocoll, the activity decreases with increasing [NaCl] up to 0.5 M to 40–50% of that in the absence of NaCl, and increases to 200% with increasing [NaCl] from 0.5 to 3 M, but decreases again to the level observed at 0 M NaCl with increasing [NaCl] from 3 to 4 M. When [NaCl] increases from 0 to 3 M, the k_{cat} value increases progressively to reach 3 times its original level, while the K_m value increases only 1.6 times. This behavior is different from that observed with MOCAc-PLGL-(Dpa)AR: in particular, the decrease in K_m observed with MOCAc-PLGL(Dpa)AR is not observed with the azocoll substrate. The increase in K_m observed with azocoll with increasing [NaCl] is almost saturated at 1 M NaCl, suggesting that the increase is due to neutralization of electrostatic interactions between azocoll and matrilysin. The molecular activity increases with the increase in [NaCl] from 0 to 3 M, but it decreases at 4 M, probably due to denaturation or aggregation of the azocoll substrate as well as the conformational change of the enzyme. Azocoll is one of the most widely used substrates and is considered to be biologically more relevant than synthetic peptide substrates (*29*, *30*). Hydrophobic and bulky groups, sulfanilic acid and sulfanilamide, are thought to be coupled with lysine, histidine, and tyrosine residues through azo-bonds in azocoll. Accordingly, the electrostatic and hydrophobic characters of the azocoll substrate might be changed considerably from those of the parental collagen. Mechanistic studies of the salt effects on the matrilysin-catalyzed hydrolysis of the azocoll substrate as well as synthetic peptide substrates must be performed more precisely.

The degree of activation of matrilysin by NaCl is independent of the presence of 0.6 and 3% DMSO (Figs. 4 and 5). On the other hand, the degree of activation of thermolysin with FA-dipeptide amides decreases drastically in the presence of various organic solvents (*31*). This difference between matrilysin and thermolysin might be due to the difference in the salt-activation mechanism: thermolysin is activated by salt through an increase in k_{cat}, while matrilysin is activated through a decrease in K_m. Interestingly, it has been reported that K_m for the hydrolysis of dansyl (Dns)-Gly-Phe-Ala and Dns-Ala-Phe-Ala by thermolysin decreases with increasing [NaCl], but k_{cat} is not affected. However, both k_{cat} and K_m for the hydrolysis of Dns-Ala-Ala-Phe-Ala decrease equally with increasing [NaCl], leaving the k_{cat}/K_m value unaltered (*31*). It has been also reported that aspartic acid proteases of avian myeloblastosis virus and human immunodeficiency virus type I and porcine pepsin are activated remarkably in the presence of NaCl up to 5 M (*32*). For these enzymes, the k_{cat}/K_m value increases exponentially with the increase of [NaCl], where K_m is reduced progressively but k_{cat} is not changed. These observations suggest that halophilic properties are quite different and complicated depending on the species of enzyme, substrate and salts. It should be noted that activation of enzymes by the increase in k_{cat} and the decrease in K_m is achieved through stabilization of the transition state and the ES complex, respectively, and the difference between them seems to be delicately controlled by the addition of salts. This paper demonstrates the significance of hydrophobic interaction at the active site of matrilysin with substrates and inhibitors, and this could be suggestive for developing and searching for effective inhibitors of matrilysin for cancer diagnosis and therapy.

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