Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts

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The activity of thermolysin in the hydrolysis of *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide and *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester is remarkably enhanced in the presence of high concentrations (1–5 M) of neutral salts [Inouye (1992) *J. Biochem. (Tokyo)* **112**, 335–340]. In this study, the effect of salts on such activity has been examined using a series of substrates, furylacryloyl dipeptide amides, which have various hydrophobic amino acids at the cleavable bond. Although the enzyme activity varies widely depending on the substrate employed, the degree of activation at a given con-

centration of NaCl is considerably similar. This indicates that the degree of activation is not dependent on the hydrophobicity of the amino acid side chains at the scissile bond of the substrates. The molecular activity, k_{cat} , and Michaelis constant, K_m , were evaluated separately for substrates *N*-[3-(2-furyl)acryloyl]-L-leucyl-L-alanine amide and *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-alanine amide, and the activation was found to be brought about only by an increase in k_{cat} . The effectiveness of monovalent cations on the increase of k_{cat} was determined to follow the order of $Na^+ > K^+ > Li^+$.

INTRODUCTION

Thermolysin (EC 3.4.24.4) is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* [1,2]. It requires essentially one zinc ion for enzyme activity and four calcium ions for structural stability [3–5], and catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues, especially at the P1' site [6] (the nomenclature used for the amino acid residues (P) of the substrate and for naming the subsites (S) of the active site being that of Schechter and Berger [7]). The amino acid sequence [8] and three-dimensional structure [9] are known, and the reaction mechanism has been proposed [10,11]. Holmquist and Vallee [12] first reported that some neutral salts, such as NaBr and NaCl, significantly activated the thermolysin-catalysed hydrolysis of *N*-[3-(2-furyl)acryloyl] (FA)-glycyl-L-leucine amide (FAGLA) and its ester analogue FA-Gly-OLeu-NH₂. We have reported the activation of thermolysin by high concentrations of neutral salts in the hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZAPM), a precursor of a synthetic sweetener, as well as in the hydrolysis of FAGLA [13]. NaCl and NaBr are the most effective in this activation, and the activity increases with an increase of salt concentration in a pseudo-exponential fashion. The degree of activation in the hydrolysis of ZAPM is 6–7-fold at 3.8 M NaBr and NaCl. The molecular activity, k_{cat} , and Michaelis constant, K_m , can be evaluated separately in the case of ZAPM, and the activation has been demonstrated to be induced solely by increasing k_{cat} . Recently, we have described a characteristic absorption difference spectrum that is observed on mixing thermolysin with NaCl and NaBr, suggesting a conformational change of the enzyme in the interaction with the salts [13,14]. The cause of the salt activation is currently not known, and there remains a possibility that the activation correlates with the difference spectrum.

For FAGLA and ZAPM, the activity of thermolysin increases progressively with increasing salt concentration, and does not show any saturating behaviour even at the saturated concentra-

tion of salts. Questions are thus raised: can the activation be observed with other substrates? If the activity is enhanced even with favourable substrates, is there an upper limit to the activity? In the present paper, we aim to give answers to the questions by examining the activation, using a series of FA-dipeptide amide substrates bearing various hydrophobic amino acids at the scissile bond. We report that the degree of activation is not dependent on the hydrophobicity of the substrates, although the activity of the enzyme depends strongly on it.

EXPERIMENTAL

Materials

A three-times-crystallized-and-lyophilized preparation of thermolysin (Lot T8BA51; 8360 proteinase units/mg according to the supplier) was purchased from Daiwa Kasei, Osaka, Japan. This preparation was used without further purification. The thermolysin solution was prepared in 40 mM Tris/HCl buffer containing 10 mM CaCl₂, pH 7.5 (standard buffer), and filtered with a Millipore membrane filter, Type HA (pore size 0.45 µm), before use. The concentration was determined spectrophotometrically using the calculation that 1 mg/ml gave an absorbance value at 277 nm of 1.83 [13] and given a molecular mass of 34.6 kDa [8]. FA-dipeptide amides [FA-Gly-Y-NH₂; Y = Ala, (S)-2-amino-*n*-butyric acid (Abu), Val, (S)-2-amino-*n*-pentanoic acid (Nva) or Phe], dipeptide amides (Leu-Ala-NH₂ and Phe-Ala-NH₂) and FA-succinimide ester were purchased from Bachem (Bubendorf, Switzerland). FAGLA (Lot 370513) was obtained from the Peptide Institute (Osaka, Japan). All other reagents were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). All amino acids were the L-forms.

Synthesis of FA-Leu-Ala-NH₂ and FA-Phe-Ala-NH₂

Furylacryloyl substrates (FA-X-Ala-NH₂; X = Leu or Phe) were synthesized by reacting FA-succinimide ester with appropriate

Abbreviations used: Dns, 5-(dimethylamino)-naphthalenyl-1-sulphonyl; FA, 3-(2-furyl)acryloyl; FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; ZAPM, *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester; Abu, (S)-2-amino-*n*-butyric acid; Nva, (S)-2-amino-*n*-pentanoic acid (L-norvaline).

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dipeptide amides according to the procedures described previously [15]. Examination of the homogeneity of the synthesized peptides was performed by thin-layer chromatography on fluorescent silica gel 60 F254 (Merck, Darmstadt, Germany) in chloroform/methanol/acetic acid (85:10:5 and 90:10:0, by vol.) to reveal a single fluorescent quenching spot. The concentrations of all FA-dipeptide amides were determined spectrophotometrically using the molar absorption coefficient ϵ_{345} $766 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13,16].

Hydrolysis of FA-dipeptide amide substrates

Hydrolysis of FA-dipeptide amides by thermolysin was measured by following the decrease in absorbance at 345 nm with the Shimadzu spectrophotometer UV-2200. The amount of FA-dipeptide amides hydrolysed was estimated by using the molar absorption difference on the hydrolysis $\Delta\epsilon_{345} - 310 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13] in the standard buffer at 25 °C. The $\Delta\epsilon_{345}$ value was confirmed not to be changed by the addition of NaCl, KCl and LiCl up to 5 M. Dependence of the specificity constant k_{cat}/K_m for the hydrolysis of FA-X-Ala-NH₂ and FA-Gly-Y-NH₂ on the hydrophobicity parameter π of the side chains of the amino acids X and Y was examined according to the treatment of Fujita et al. [17]. The π values for the side chains of Gly, Ala, Val, Leu and Phe of 0.00, 0.32, 1.27, 1.81 and 1.95 respectively, evaluated by Akamatsu et al. [18], were used. The π values for the side chains of Abu and Nva were estimated to be 0.86 and 1.40 respectively, by subtracting the group branching factor (0.22) from the respective hydrophobic parameters, 1.08 and 1.62, estimated for ethyl and *n*-propyl substituents in aliphatic amines respectively [19]. Kinetic parameters (k_{cat} and K_m) were determined by using the non-linear least-squares method with Taylor expansion [20].

RESULTS AND DISCUSSION

Effect of NaCl on the hydrolysis of FA-dipeptide amides

The effect of NaCl on the reaction rate of thermolysin-catalysed hydrolysis of eight kinds of FA-dipeptide amides with different amino acids at the P1 and P1' sites of the scissile bond was examined. The reaction was carried out at substrate concentrations lower than the respective K_m values (pseudo-first-order conditions). The first-order rate constant for substrate hydrolysis equals $(k_{\text{cat}}/K_m) \cdot [E]_0$, where $[E]_0$ is the total enzyme concentration, allowing us to estimate the specificity constant k_{cat}/K_m . Figure 1 shows dependence on the NaCl concentration of the relative activity for the hydrolysis of three representative substrates (FA-Gly-Ala-NH₂, FA-Gly-Leu-NH₂ and FA-Leu-Ala-NH₂). The activity increased apparently in an exponential fashion with increasing NaCl concentration, and the degree of the activation at 4 M NaCl was in the range of 11–17-fold, depending on the substrates. The activity seemed to increase further with [NaCl] higher than 4 M as long as its solubility was permitted. The $\log(k_{\text{cat}}/K_m)$ values for eight substrates increased linearly with an increase in NaCl concentration, and the slopes of the lines were similar, whereas the $\log(k_{\text{cat}}/K_m)$ value at 0 M NaCl varied considerably depending on the hydrophobicity of the amino acid side chains at the scissile bond of the substrates (Figure 2). However, it should be pointed out that there was a slight but distinct tendency for the slopes to increase in accordance with an increase in hydrophobicity of aliphatic side chains at the P1 and P1' amino acids. As for the P1' amino acid Y in FA-Gly-Y-NH₂, its hydrophobicity parameter (π) and the slope (a) of the straight line obtained from Figure 2(A) are as follows: Ala (π 0.32, a 0.26 M⁻¹); Abu (π 0.86, a 0.27); Val (π 1.27, a 0.27); Nva (π 1.40, a 0.28); Leu (π 1.81, a 0.28); and Phe

Figure 1 Activation of the peptidase activity of thermolysin by NaCl

The reaction was performed with thermolysin at 0.20 μM in the presence of NaCl at the indicated concentrations in the standard buffer (pH 7.5) at 25 °C. Substrates were 0.4 mM FA-Gly-Ala-NH₂ (○), 0.4 mM FA-Gly-Leu-NH₂ (□) and 0.1 mM FA-Leu-Ala-NH₂ (△). The relative activity shown on the ordinate axis is defined as the ratio of the (k_{cat}/K_m) value at a given NaCl concentration to the (k_{cat}/K_m) value obtained in the absence of NaCl. The (k_{cat}/K_m) values at 0 M NaCl are 43 , 2.2×10^4 and $6.9 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ for FA-Gly-Ala-NH₂, FA-Gly-Leu-NH₂ and FA-Leu-Ala-NH₂ respectively. $[\text{NaCl}]_0$ is the initial substrate concentration.

(π 1.95, a 0.26). Similarly, for the P1 amino acid X in FA-X-Ala-NH₂ the values are as follows: Gly (π 0.00, a 0.26); Leu (π 1.81, a 0.30); and Phe (π 1.95, a 0.24). The degree of activation for substrates containing Phe, such as FA-Gly-Phe-NH₂ and FA-Phe-Ala-NH₂, is the lowest among eight substrates, although Phe is the most hydrophobic. Aromatic amino acids, including Phe, often behave differently from aliphatic amino acids in events as far as hydrophobicity is concerned [21], and such a phenomenon is also observed in Figure 3.

The a values for eight different substrates are considerably similar and average $0.269 \pm 0.016 \text{ M}^{-1}$, even though there is a large difference ranging 500-fold in the activity (k_{cat}/K_m) among them (Figure 2). In fact, the activity (k_{cat}/K_m) of thermolysin towards favourable substrates, FA-Gly-Leu-NH₂ and FA-Gly-Phe-NH₂, is $2.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, and that towards a poor substrate, FA-Gly-Ala-NH₂, is $40 \text{ M}^{-1} \cdot \text{s}^{-1}$. Instead of this large difference in the activity, the degree of activation for these substrates at 4 M NaCl is in a considerably narrower range of 11–17-fold: 11-fold with FA-Gly-Leu-NH₂ and 17-fold with FA-Leu-Ala-NH₂ (Figure 1). Figure 2 shows that the activity (k_{cat}/K_m at $x \text{ M NaCl}$) is expressed as

$$\log(k_{\text{cat}}/K_m)_x = \log(k_{\text{cat}}/K_m)_0 + a \cdot x \quad (1)$$

where x and 0 refer to NaCl concentrations of x and 0 M, respectively, and a is the slope of the straight line in Figure 2, being equal to $0.269 \pm 0.016 \text{ M}^{-1}$. Eqn. (1) is converted into

$$\log[(k_{\text{cat}}/K_m)_x / (k_{\text{cat}}/K_m)_0] = a \cdot x \quad (2)$$

Thus,

$$(k_{\text{cat}}/K_m)_x / (k_{\text{cat}}/K_m)_0 = (10^a)^x = 1.9^x \quad (3)$$

This equation indicates that the degree of activation at $x \text{ M NaCl}$, $(k_{\text{cat}}/K_m)_x / (k_{\text{cat}}/K_m)_0$, is equal to 1.9^x .

Figure 2 Logarithmic relationship of the peptidase activity of thermolysin with NaCl concentration

The reaction was carried out in the standard buffer (pH 7.5) at 25 °C containing NaCl at the indicated concentrations with thermolysin of 0.1–0.85 μ M and substrates of 14 μ M–0.4 mM. The peptidase activity was expressed as k_{cat}/K_m on the ordinate axis. (A) Substrates are in the form of FA-Gly-Y-NH₂, where Y is Ala (○), Abu (□), Val (△), Nva (●), Leu (▼) or Phe (▽). (B) Substrates are in the form of FA-X-Ala-NH₂, where X is Gly (○), Leu (□) or Phe (△). [NaCl]₀ is the initial substrate concentration.

Hydrophobic effect on the NaCl-dependent activation of thermolysin

The $\log(k_{\text{cat}}/K_m)$ values obtained at a constant NaCl concentration were plotted against the hydrophobicity parameter π of the side chain of the P1' amino acid (Figure 3A). For linear aliphatic amino acids (Ala, Abu and Nva), a linear relationship was obtained at the respective NaCl concentrations, and the slopes are substantially the same (1.96 ± 0.01). The data points for branched aliphatic amino acids (Val and Leu) lie under the lines drawn for the linear aliphatic amino acids, and the lines connecting the points for Val and Leu are parallel to the lines for

Figure 3 Relationship between $\log(k_{\text{cat}}/K_m)$ of thermolysin-catalysed hydrolysis of FA-dipeptide amides and hydrophobicity of the amino acid side chains at the scissile bond

The reaction was carried out in the presence of NaCl at 0 M (○), 1 M (△), 2 M (□), 3 M (▽) and 4 M (◇) in the standard buffer (pH 7.5) at 25 °C with thermolysin at 0.10–0.85 μ M and substrates of 14 μ M–0.4 mM. (A) Substrates were FA-Gly-Y-NH₂, where Y is Ala, Abu, Val, Nva, Leu or Phe. (B) Substrates were FA-X-Ala-NH₂, where X is Gly, Leu or Phe. Hydrophobicity parameters (π): Gly, 0.00; Ala, 0.32; Abu, 0.86; Val, 1.27; Nva, 1.40; Leu, 1.81; and Phe, 1.95.

the linear aliphatic amino acids. The points for Phe are all under the lines for the branched aliphatic amino acids. An interesting feature is pointed out: all data points examined lie on six representative linear lines. Intervals between lines are all equal, as 0.25 unit in $\log(k_{\text{cat}}/K_m)$. Points for Val and Leu at 0 M NaCl and Phe at 1 M NaCl lie on the first line (ordinate-axis intercept 0.75). On the second line (1.00), points for Ala, Abu and Nva lie at 0 M, Val and Leu lie at 1 M and Phe lies at 2 M NaCl. On the third line (1.25), points for Ala, Abu and Nva lie at 1 M, Val and Leu lie at 2 M, and Phe lies at 3 M NaCl. On the fourth line (1.50), points for Ala, Abu and Nva lie at 2 M, Val and Leu lie at 3 M, and Phe lies at 4 M NaCl. On the fifth line (1.75), points for Ala, Abu and Nva lie at 3 M, and Val and Leu lie at 4 M NaCl. And on the sixth line (2.00), points for Ala, Abu and Nva lie at 4 M NaCl. The point for Phe at 0 M NaCl is expected on a line with a slope of 1.96 and an ordinate-axis intercept of 0.50. This result indicates that the enzyme activity k_{cat}/K_m increases with an increase in the hydrophobicity at the P1' amino acid side chain in the presence of 0–4 M NaCl. If a linear aliphatic side

chain of the P1' amino acid is replaced with a branched one with the corresponding hydrophobicity, $\log(k_{\text{cat}}/K_m)$ may decrease by 0.25, and the same amount of decrease in $\log(k_{\text{cat}}/K_m)$ may be given by a 1 M decrease in [NaCl]. When a benzyl group is introduced in place of a linear aliphatic side chain of the corresponding hydrophobicity, $\log(k_{\text{cat}}/K_m)$ decreases by 0.50, and the effect is the same as that induced by decreasing [NaCl] by 2 M. Thermolysin favours hydrophobic amino acids at the P1' residue, as has been previously reported [6], and it favours linear aliphatic side chains rather than branched aliphatic or aromatic ones. In the same way, $\log(k_{\text{cat}}/K_m)$ was plotted against P of the P1 amino acid side-chain in FA-X-Ala-NH₂ (Figure 3B). Straight lines were drawn by assuming linearity between data points for X = Gly or Leu at the respective NaCl concentrations. The lines are almost parallel, and the slope at 0 M NaCl is 0.650, whereas those at 1, 2, 3 and 4 M NaCl are 0.750. Thermolysin favours substrates having hydrophobic amino acids at the P1 residue, too. Points for Phe exist over the respective lines, and the point at 0 M NaCl is on the line connecting the points of Gly and Leu at 3 M NaCl, indicating that thermolysin favours an aromatic amino acid, Phe, rather than a linear aliphatic amino acid, Leu, at the P1 residue, and that the effect of replacing a linear aliphatic amino acid at the P1 residue with Phe is almost equivalent to adding 3 M NaCl. By replacing Gly at the P1 residue with Leu, $\log(k_{\text{cat}}/K_m)$ increases by 1.20–1.35. With every increase of 1 M NaCl, the increment of $\log(k_{\text{cat}}/K_m)$ is 0.29 ± 0.01 , which is slightly larger than the value (0.25) obtained with FA-Gly-Y-NH₂.

Pank et al. [22] reported a plot similar to that of Figure 3(A) for the thermolysin-catalysed hydrolysis of FA-Gly-Y-NH₂ with a slope of 1.9 ± 0.3 in 0.17 M Tris/maleate/NaOH buffer, pH 7.2, at 25 °C without any other additional salts. The hydrophobicity scale used by Pank et al. was originally reported by Fujita et al. [17]. The scale used in this study is a corrected one [18,19], and with this scale, the data of Pank et al. [22] are in reasonable agreement with our data obtained at 0 M NaCl. The result shown in Figure 3 demonstrates that the linear relationship is maintained in the presence of NaCl up to 4 M. If a chemical reaction is controlled only by hydrophobic effect, the slope is theoretically expected to be unity and is known empirically to fall in a range between 0.5 and 1 [23–25]. The value of about 2 (Figure 3) suggests that some other effect (such as a steric one) in addition to the hydrophobic one might be involved in participation of the P1' residue in the activity k_{cat}/K_m . On the other hand, the plot of $\log(k_{\text{cat}}/K_m)$ against π for FA-X-Ala-NH₂ gives a straight line with a slope of 0.65–0.75 by connecting the data for Gly and Leu (Figure 3B), suggesting that hydrophobic effect contributes largely to participation of the P1 residue in the activity.

k_{cat}/K_m corresponds to the apparent second-order rate constant when free enzyme (E) and free substrate (S) form products [26]. Therefore, it is considered to be the equilibrium constant between the ground state (E+S) and the transition state ES[‡]. When the difference in the Gibbs free energy between the ground state and the transition state is given by $\Delta G_{\text{‡}}^\ddagger$, k_{cat}/K_m and $\Delta G_{\text{‡}}^\ddagger$ are related in eqn. (4):

$$k_{\text{cat}}/K_m = (k \cdot T/h) \exp(-\Delta G_{\text{‡}}^\ddagger / R \cdot T) \quad (4)$$

Eqn. (4) is converted to eqn. (5):

$$\log(k_{\text{cat}}/K_m) = \log(k \cdot T/h) - (\Delta G_{\text{‡}}^\ddagger / 2.303 R \cdot T) \quad (5)$$

where k and h are the Boltzmann and Planck constants, respectively, R is the gas constant and T is the temperature in Kelvin.

From Figure 2, an increment of $\log(k_{\text{cat}}/K_m)$ given by an increase of 1 M NaCl is $0.269 \pm 0.016 \text{ M}^{-1}$, indicating that $\Delta G_{\text{‡}}^\ddagger$

decreases by $0.37 \pm 0.02 \text{ kcal/mol}$ with an increase of 1 M NaCl, namely a decrease in $\Delta G_{\text{‡}}^\ddagger$ of 1.5 kcal/mol by addition of 4 M NaCl. Similarly, increments of $\log(k_{\text{cat}}/K_m)$ with an increase by 1 unit in hydrophobicity of the P1' and P1 residues are 1.96 and 0.65–0.75 respectively (Figure 3), which correspond to a decrease in $\Delta G_{\text{‡}}^\ddagger$ to the respective amounts of 2.6 and 1.0 kcal/mol.

Effect of NaCl on the kinetic parameters

The kinetic parameters, k_{cat} and K_m , for the thermolysin-catalysed hydrolysis of FA-dipeptide amides employed in this study, except for FA-Leu-Ala-NH₂ and FA-Phe-Ala-NH₂, could not be determined separately because of the sparing solubility in comparison with the K_m values, especially in the presence of NaCl. Therefore the enzyme reaction was inevitably conducted under the pseudo-first-order conditions ($[S] \ll K_m$), so that the initial rates are proportional to k_{cat}/K_m . The parameters for FA-Leu-Ala-NH₂ were barely evaluated in NaCl concentrations of only up to 2 M, $[S]_0$ being set to $0.1\text{--}1.5 \times K_m$. By non-linear least-squares regression for the Michaelis–Menten (v versus $[S]$) plots, k_{cat} and K_m were determined to be $0.56 \pm 0.08 \text{ s}^{-1}$ and $0.80 \pm 0.04 \text{ mM}$, respectively, at 0 M NaCl; $1.20 \pm 0.08 \text{ s}^{-1}$ and $0.80 \pm 0.06 \text{ mM}$ at 1 M NaCl; and $2.64 \pm 10.15 \text{ s}^{-1}$ and $0.82 \pm 0.10 \text{ mM}$ at 2 M NaCl. With FA-Phe-Ala-NH₂, the parameters were evaluated safely in [NaCl] up to 1 M, and k_{cat} and K_m were $5.7 \pm 0.3 \text{ s}^{-1}$ and $83 \pm 5 \mu\text{M}$, respectively, at 0 M NaCl, and $10.1 \pm 0.4 \text{ s}^{-1}$ and $84 \pm 8 \mu\text{M}$ at 1 M NaCl. Only k_{cat} increased, whereas K_m remained unaltered, with increasing NaCl concentration, which resulted in the increase in k_{cat}/K_m shown in Figures 1 and 2. Being consistent with the results obtained with ZAPM [13], the activation of thermolysin in the hydrolysis of FA-Leu-Ala-NH₂ and FA-Phe-Ala-NH₂ has been demonstrated to be brought about only by the enhancement of k_{cat} , and K_m is not altered in the presence of salts. Let us suppose that this could be adopted reasonably to FA-dipeptide amide substrates. Eqn. (3) is turned into eqn. (6), indicating the degree of increase in k_{cat} in the presence of x M NaCl:

$$(k_{\text{cat}})_x / (k_{\text{cat}})_0 = 1.9^x \quad (6)$$

The parameters for six other substrates could not be determined definitely even in the absence of NaCl. In the case of FAGLA, k_{cat} and K_m were narrowly estimated to be $> 60 \text{ s}^{-1}$ and $> 3 \text{ mM}$, respectively, at 0 M NaCl, and $> 120 \text{ s}^{-1}$ and $> 3 \text{ mM}$ at 1 M NaCl. In the presence of 5–20% organic solvents such as ethanol, dimethyl sulphoxide and ethylene glycol, the substrate solubility increased and the parameters could be evaluated. The organic solvents, however, inhibit the hydrolysis in a complicated manner (K. Inouye, unpublished work), suggesting that it is not easy to estimate the effect of NaCl on the kinetic parameters in the presence of the organic solvents. Substrates containing oligopeptides larger than FA-dipeptide amides may give smaller K_m values, allowing us to determine k_{cat} and K_m values separately. With 5-(dimethylamino)-naphthalenyl-1-sulphonyl (Dns)-Ala-Phe-Ala and Dns-Gly-Phe-Ala, however, dependence of the thermolysin activity on NaCl concentration is different from that observed with FA-dipeptide amides [27] (see below). This suggests that the effect of NaCl on the activity of thermolysin could be changed depending on substrate structure and interaction between subsites of thermolysin and substrates.

Effectiveness of monovalent cations on the salt activation of thermolysin

Thermolysin-catalysed hydrolysis of FA-Leu-Ala-NH₂ was examined at 1 M NaCl, KCl and LiCl. The K_m and k_{cat} values in

the absence of salt were 0.80 ± 0.04 mM and 0.56 ± 0.08 s⁻¹ respectively. The K_m values were 0.80 ± 0.06 , 0.81 ± 0.06 and 0.78 ± 0.08 mM with 1 M NaCl, KCl and LiCl respectively, showing that the K_m value is not changed on the addition of salts in the reaction medium. The k_{cat} values were changed depending on the salts, and were 1.20 ± 0.08 , 1.12 ± 0.07 and 0.91 ± 0.08 s⁻¹ with 1 M NaCl, KCl and LiCl respectively.

It has been shown that the activation of thermolysin through increasing k_{cat} in the hydrolysis of FA-Leu-Ala-NH₂ is dependent on the species of monovalent cations employed, and that the effectiveness follows the order of Na⁺ > K⁺ > Li⁺, which is the same as that observed in the hydrolysis of FAGLA and ZAPM [13]. This order is different from that of the ionic radius of the monovalent cations, K⁺ > Na⁺ > Li⁺, and that of the Hofmeister's series, K⁺ < Na⁺ < Li⁺ [28], suggesting a possibility that the activation of thermolysin is induced by specific interaction between the cations and the enzyme. As for anions, we have demonstrated that the difference between NaCl and NaBr or between KCl and KBr is not significant in the effects of activation of thermolysin [13], and that tryptophyl residue 115 interacts favourably with Cl⁻ but not with Br⁻ [14]. Further studies are needed to elucidate the relationship between binding of ions, especially cations, and the activation of the enzyme.

Dependence of the salt activation of thermolysin on the substrate structure

The variations in the thermolysin activity as a function of increasing NaCl concentration exhibit exponential behaviour for all FA-dipeptide amide substrates examined. The degree of activation is not much dependent on the structure of the substrates ($a = 0.269 \pm 0.016$ M⁻¹), and ranges from 11- to 17-fold at 4 M NaCl. Rather different values have been reported, however. The value is 6–7 times for ZAPM at 3.8 M NaCl ($a = 0.22$ M⁻¹) [13]. The thermolysin activity toward FAGLA and its ester analogue FA-Gly-OLeu-NH₂ increases progressively with increasing NaBr concentration until 5 M, and it becomes 25-fold that observed in the absence of salt; in contrast, the activity towards benzoyl-Gly-Phe-Ala and benzoyl-Gly-OPhe-Ala increases only 5-fold at 5 M NaBr [12]. It is evident that the degree of activation depends on the substrates, and the discrepancy in the degree of activation could be derived from the difference in the substrate structure at either P2 or P2' residues, or both. Yang et al. [27] observed activation by 8- and 18-fold at 4 M NaCl with Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala respectively. Interestingly, they have reported that increasing [NaCl] increases k_{cat}/K_m in a linear fashion but not progressively, and that it decreases K_m but leaves k_{cat} unaltered for both substrates. With ZAPM [13] and the FA-dipeptide amides used in this study, the activation of thermolysin by NaCl is brought about solely through an increase in k_{cat} , and K_m is not affected at all by the addition of NaCl. It has been discussed that the different results between ZAPM and Dns-tripeptides were related to the fact that the Asp residue of ZAPM has a negatively charged side chain, and that its interactions with the enzyme are modulated by the cation [27]. This, however, cannot be the case, because the FA-dipeptide amides show an exponential behaviour in the salt activation brought about solely from an increase of k_{cat} , although they do not carry negatively charged amino acids. The discrepancy must be derived from some other difference in the structure of substrates. The P2' residue (Ala) of the Dns-tripeptide substrates can contact directly the subsite S2', while this contact might be less sufficient in the case of FA-dipeptide amides and ZAPM because of the smaller size of the amide and methyl moieties. The Dns group, being bulkier than the corresponding FA and Z groups, may interact

with not only the S2 but also the S3 subsites. Because of the bulkiness, the salt effect on the thermolysin-catalysed hydrolysis of Dns-tripeptides might be different from that of FA-dipeptide amides and ZAPM.

Some reports on salt activation of enzymes other than thermolysin are found in the literature [29]. The activity of nuclease H of *Micrococcus varians* in the hydrolysis of thymidine-5'-monophospho-*p*-nitrophenyl ester increases with increasing [NaCl] in an exponential fashion, and at 5 M NaCl it is 50-fold higher than that in the absence of NaCl, whereas the activation profile toward DNA is bell-shaped with an optimal [NaCl] at 2.5 M [30]. The activity of a serine proteinase, halolysin, of an archaebacterium in the hydrolysis of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide is enhanced exponentially with an increase in [NaCl], while the activation of *N*-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methyl-coumarin shows a bell-shaped effect [31]. The activation behaviour of these enzymes is dependent on substrates, and the effect of cations is in the order of K⁺ > Na⁺ > Li⁺, which is different from that for thermolysin. Recently, it has been reported that aspartic acid proteases of avian myeloblastosis virus and human immunodeficiency virus type I and porcine pepsin are activated markedly in the presence of NaCl up to 5 M [32]. For these enzymes, k_{cat}/K_m increases exponentially with an increase of NaCl, where K_m is reduced progressively but k_{cat} is not altered. Although they show an exponential activation behaviour like thermolysin, the cause of the activation is different. Varieties in the activation behaviour suggest that the activation may be attributable not only to a change in the characteristics of the medium by adding salts but also to specific interaction of salts with enzymes and substrates. Actually, the intensity of the absorption difference spectrum induced by adding NaCl or NaBr to thermolysin is 10-fold or greater than that expected from the solvent perturbation difference spectrum using model compounds [14]. This suggests that ions of salts interact preferably with thermolysin. The effects of the substrate properties on the activation of and interaction between salts and thermolysin are currently under examination.

Influence of ionic strength

Theoretical treatments of the influence of ionic strength (μ) on the rates of reaction between a charged molecule and a neutral one gave the relationship:

$$\log(k/k_0) = b \cdot \mu \quad (7)$$

where b is a constant, so that the logarithm of the rate constant becomes a linear function of the first power of the ionic strength [33]. It is noteworthy that the relationship shown in Figure 2 is similar to that expressed by eqn. (7). As has been discussed above, the relationship in Figure 2 is reasonably expressed by eqn. (6), so $1.9^x = 10^{b \cdot \mu}$. If the ionic strength μ of the NaCl solution is expressed anyway with the NaCl molarity x , the b value is calculated to be 0.279, although the meaning of this value is unclear.

Generally, physicochemical treatments have been carried out so far in considerably dilute solutions, and avoided events in the concentrated salt solutions such as those studied in the present study. Concepts of ionic strength, activity coefficient, dielectric constant, hydration of ions or aqueous solution are all vague in such concentrated solutions. In order to get away from confusion brought about by the use of these physicochemical quantities, we use molarity in order to describe amounts of salts in the solution in the present paper. Studies on halophilic enzymes may be expected to expand the physicochemical limitations, so that

physical chemistry can interpret and predict the events in such concentrated salt solutions.

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REFERENCES

- 1 Endo, S. (1962) *J. Ferment. Technol.* **40**, 346–353
- 2 Matsubara, H. and Feder, J. (1971) in *The Enzymes*, 3rd edn. (Boyer, P. D., ed.), vol. 3, pp. 721–795, Academic Press, New York
- 3 Latt, S. A., Holmquist, B. and Vallee, B. L. (1969) *Biochem. Biophys. Res. Commun.* **37**, 333–339
- 4 Feder, J., Garrett, L. R. and Wildi, B. S. (1971) *Biochemistry* **10**, 4552–4555
- 5 Tajima, M., Urabe, I., Yutani, K. and Okada, H. (1976) *Eur. J. Biochem.* **64**, 243–247
- 6 Morihara, K. and Tsuzuki, H. (1970) *Eur. J. Biochem.* **15**, 374–380
- 7 Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- 8 Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A. and Neurath, H. (1972) *Nature (London)* **238**, 35–37
- 9 Holmes, M. A. and Matthews, B. W. (1982) *J. Mol. Biol.* **160**, 623–639
- 10 Hangauer, D. G., Monzingo, A. F. and Matthews, B. W. (1984) *Biochemistry* **23**, 5730–5741
- 11 Izquierdo, M. C. and Stein, R. L. (1990) *J. Am. Chem. Soc.* **112**, 6054–6062
- 12 Holmquist, B. and Vallee, B. L. (1976) *Biochemistry* **15**, 101–107
- 13 Inouye, K. (1992) *J. Biochem. (Tokyo)* **112**, 335–340
- 14 Inouye, K., Kuzuya, K. and Tonomura, B. (1994) *J. Biochem. (Tokyo)* **116**, 530–535
- 15 Blumberg, S. and Vallee, B. L. (1975) *Biochemistry* **14**, 2410–2419
- 16 Feder, J. (1968) *Biochem. Biophys. Res. Commun.* **32**, 326–332
- 17 Fujita, T., Iwasa, J. and Hansch, C. (1964) *J. Am. Chem. Soc.* **86**, 5175–5180
- 18 Akamatsu, M., Okutani, S., Nakao, K., Hong, N. J. and Fujita, T. (1990) *Quant. Struct.-Act. Relat.* **9**, 189–194
- 19 Takayama, C., Akamatsu, M. and Fujita, T. (1985) *Quant. Struct.-Act. Relat.* **4**, 149–160
- 20 Sakoda, M. and Hiromi, K. (1976) *J. Biochem. (Tokyo)* **80**, 547–555
- 21 Inouye, K. (1991) *Agric. Biol. Chem.* **55**, 2129–2139
- 22 Pank, M., Kirret, O., Paberit, N. and Aaviksaar, A. (1982) *FEBS Lett.* **142**, 297–300
- 23 Järv, J., Kesvatera, T. and Aaviksaar, A. (1976) *Eur. J. Biochem.* **67**, 315–322
- 24 Dorovska, V. N., Varfolomeyev, S. D., Kazanskaya, N. F., Klyosov, A. A. and Martinek, K. (1972) *FEBS Lett.* **23**, 122–124
- 25 Hansch, C. and Leo, A. (1995) in *Exploring QSAR: Fundamentals and Applications in Chemistry and Biology*, pp.170–183, American Chemical Society, Washington, DC
- 26 Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd edn., pp. 98–120, Freeman, New York
- 27 Yang, J. J., Artis, D. R. and Van Wart, H. E. (1994) *Biochemistry* **33**, 6516–6523
- 28 Shannon, R. D. (1976) *Acta Crystallogr.* **A32**, 751–767
- 29 Inouye, K. (1994) *Seikagaku* **66**, 446–450
- 30 Kamekura, M. and Onishi, H. (1978) *J. Bacteriol.* **133**, 59–65
- 31 Kamekura, M. and Seno, Y. (1990) *Biochem. Cell Biol.* **68**, 352–359
- 32 Tropea, J. E., Nashed, N. T., Louis, J. M., Sayer, J. M. and Jerina, D. M. (1992) *Bioorg. Chem.* **20**, 67–76
- 33 Laidler, K. J. (1965) in *Chemical Kinetics*, 2nd edn., pp. 198–255, McGraw-Hill, New York