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Effects of Nitration and Amination of Tyrosyl Residues in Thermolysin on Its Hydrolytic Activity and Its Remarkable Activation by Salts[1]

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Thermolysin is remarkably activated in the presence of high concentrations (1-5 M) of neutral salts and its activity is enhanced 15 times by 4 M NaCl at pH 7.0 and 25°C [Inouye, K. (1992) *J. Biochem.* 112, 335-340]. In this study, the effects of nitration and amination of tyrosyl residues in thermolysin on its halophilic properties were examined. Nitration and successive amination inactivate thermolysin progressively as the degree of modification increases. When 16 tyrosyl residues were nitrated, the activity decreased to 10% of that of the native enzyme, whereas it recovered to 30% when they were aminated. The decrease in the activity by the nitration and amination was shown to be brought about only by a decrease in the molecular activity, k_{cat}; the Michaelis constant, K_m, was unaltered. When 14 tyrosyl residues of thermolysin were nitrated, the degree of activation by 4 M NaCl at pH 7.0 decreased from 15 to 10, and this decreased further to 5 when the pH of the reaction medium was raised to 8.5. However, when the nitrated tyrosyl residues were reduced to aminotyrosyl residues, the degree of activation was restored to that of the native enzyme. The change in the degree of activation by nitration and amination of thermolysin could be due to the change in the ionization of tyrosyl residues, and it was suggested that removing negative charges from tyrosyl residues of thermolysin enhances its halophilicity.

Key words: chemical modification, halophilicity, metalloproteinase, salt-activation, thermolysin, tyrosine.

Thermolysin [EC 3.4.24.27] 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 catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues. The amino acid sequence (*6, 7*) and three-dimensional structure (*8*) are known, and the kinetic mechanism of the reaction has been proposed (*9, 10*).

We have previously reported remarkable activation of thermolysin of up to 10-15 times by high concentrations (1-5 M) of neutral salts in the hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZAPM), a precursor of a synthetic sweetener (*11, 12*), and in the hydrolysis of *N*-[3-(2-furyl)acryloyl](FA)-dipeptide amides with different amino acids at the scissile bond (*13*). The activation is brought about most effectively by NaCl and NaBr, and the activity increases exponentially with increasing NaCl concentration. The molecular activity, k_{cat}, and Michaelis constant, K_m, can be evaluated separately in the cases of ZAPM, FA-L-leucyl-L-alanine amide and FA-L-phenylalanyl-L-alanine amide, and the activation has been demonstrated to be induced solely by an increase in k_{cat} (*11, 13*). We have previously observed a characteristic absorption difference spectrum on mixing thermolysin with NaCl and NaBr, suggesting changes in the states of tyrosyl and tryptophyl residues (*11, 14*). Specific interaction between cations and enzyme might be involved in the activation of thermolysin, and the effectiveness of cations is in the order of $Na^+ > K^+ > Li^+$ (*11-13*). The degree of activation shows bell-shaped pH dependence with the optimum pH around 7, and it decreases significantly with increasing temperature and increasing alcohol concentration added to the reaction medium (*15*). Accordingly, the salt-dependent activation might be related to the electrostatic interaction of thermolysin with ions in the medium, although the exact mechanism is not yet clear. Addition of NaCl might modify the structure of the enzyme, *e.g.*, by shielding surface charges, promoting the binding of hydrated ions to the enzyme surface, and causing disintegration of the water structure. Because the shielding effect is generally saturated at 0.5-1.0 M NaCl, it is not a major factor in the activation, which might rather be caused by a conformational factor of the enzyme and/or an envi-

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Abbreviations: FA, 3-(2-furyl)acryloyl; FAGLA, *N*-[3-(2-furyl)-acryloyl]-glycyl-L-leucine amide; Mes, 4-morpholinoethanesulfonic acid; TNM, tetranitromethane; ZAPM, *N*-carbobenzoxy-L-phenylalanine methyl ester.

ronmental factor of the medium. The states of tyrosyl residues of thermolysin have been estimated by means of nitration and pH-dependent ionization (*16*). In the present paper, we report that the degree of activation of thermolysin by NaCl is dependent on the ionization states of tyrosyl residues, which were modified by means of nitration and amination.

EXPERIMENTAL PROCEDURES

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. This preparation was used without further purification. The thermolysin solution was filtered with a Millipore membrane filter, Type HA (pore size 0.45 μm), before use. The concentration of thermolysin was determined spectrophotometrically using an absorbance value, A (1 mg/ml), at 277 nm of 1.83 (*11*) and a molecular mass of 34.6 kDa (*6*). FA-succinimide ester and Leu-Ala-NH_2 were purchased from Bachem (Bubendorf, Switzerland). FA-Leu-Ala-NH_2 was synthesized by treating FA-succinimide ester with Leu-Ala-NH_2 according to the procedures described previously (*17*). FAGLA (Lot 370513) was obtained from the Peptide Institute (Osaka). The concentrations of FA-dipeptide amides were determined spectrophotometrically using the molar absorption coefficient of $\varepsilon_{345} = 766\ M^{-1} \cdot cm^{-1}$ (*11*). All other reagents were of reagent grade and purchased from Nacalai Tesque (Kyoto). All spectrophotometric measurements were carried out with a Shimadzu UV-2200 spectrophotometer.

Nitration and Amination of Thermolysin—Nitration of thermolysin was performed by adding an appropriate portion of 1 or 10% solution of tetranitromethane (TNM) in methanol to the thermolysin solution (7-10 μM) in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.0. The reaction mixture was maintained at 25°C for a suitable period, then the reaction was terminated by gel-filtration on a Bio-Gel P-4 column [1.5 cm (diameter) × 25 cm] equilibrated with the same buffer. The degree of nitration of tyrosyl residues in thermolysin was determined spectrophotometrically from the absorbance at 381 nm, the nitrophenol-nitrophenolate isosbestic point of 3-nitro-L-tyrosine, using a molar absorption coefficient of $2.2 \times 10^3\ M^{-1} \cdot cm^{-1}$ (*18, 19*). The concentration of nitrated thermolysin was determined by measuring the absorbance at 275 nm, pH 8.0, using a molar absorption coefficient for native thermolysin of $\varepsilon_{275} = 62.25 \times 10^3\ M^{-1} \cdot cm^{-1}$ according to the procedures described previously (*20*). The nitrotyrosine in thermolysin was reduced to aminotyrosine with a 60-fold molar excess of sodium hydrosulfite ($Na_2S_2O_4$) in the same buffer at pH 8.0, 25°C (*21*). The amination was monitored by the decrease in the absorbance of nitrotyrosine at 381 nm, which was complete within 10 min. The nitrotyrosyl residues were entirely reduced to aminotyrosyl residues at the end of the amination, which was confirmed spectrophotometrically by the disappearance of the absorbance at 381 nm.

Hydrolysis of FA-Dipeptide Amide Substrates—Hydrolysis of FAGLA and FA-Leu-Ala-NH_2 by thermolysin was measured by following the decrease in absorbance at 345 nm (*11, 13*). The amount of FA-dipeptide amides hydrolyzed was evaluated by using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1} \cdot cm^{-1}$, at 25°C (*11*). The reaction was carried out in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.0-8.5, and in 40 mM Mes-NaOH buffer containing 10 mM $CaCl_2$ at pH 5.5-7.0. The reaction with FAGLA was carried out under pseudo-first order conditions, where the substrate concentration is lower than K_m, because of the sparing solubility of FAGLA, and the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m. Kinetic parameters (k_{cat} and K_m) for the hydrolysis of FA-Leu-Ala-NH_2 were determined by using the linear least-squares method (*22*).

Thermal Inactivation—Seven micromolar thermolysin in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 8.0) was incubated at 60, 70, and 80°C, for various periods, followed by incubation at 25°C for 3 min, then the hydrolysis of FAGLA was measured at pH 7.5, 25°C as described above.

RESULTS

Effect of Nitration and Amination of Tyrosyl Residues on the Hydrolysis of FA-Dipeptide Amides—The activity of thermolysin in the hydrolysis of FAGLA decreased with the increase in the extent of treatment with TNM, namely, the molar ratio of TNM to thermolysin, [TNM]/[TLN] (M/M), and the reaction time of nitration. The enzyme activity observed after nitration for 1 h decreased gradually as the molar ratio [TNM]/[TLN] was increased, falling to 50% of that of the native enzyme at 2,000-fold molar excess of TNM (Fig. 1A). The activity also decreased with increasing reaction time. At [TNM]/[TLN] = 2,000 (M/M), it progressively decreased to 25% of that of the native enzyme within 3-4 h and then gradually to 10% in 12 h, although complete inactivation was not observed when the reaction time was further prolonged (Fig. 1B). The number of nitrotyrosyl residues per mole of the nitrated thermolysin with the residual activity of 10% was 16, and the k_{cat}/K_m value was determined to be $0.22 \times 10^4\ M^{-1} \cdot s^{-1}$. The activity of thermolysin also decreased as the number of nitrotyrosyl and aminotyrosyl residues was increased (Fig. 2). Nitration of 5, 10, and 16 tyrosyl residues inactivated thermolysin to 70, 30, and 10% of the activity of the native enzyme, respectively. Conversion of nitrotyrosyl residues to aminotyrosyl residues did not significantly affect the progressive decrease in the activity of thermolysin. However, the aminated thermolysin showed a slightly higher activity than the nitrated enzyme at pH 7.5. The difference in activity between the aminated and nitrated enzymes increased with the increase in the degree of modification. When 16 tyrosyl residues were modified, the activity of the aminated enzyme was 30% of that of the native one, being three times higher than that of the nitrated one.

The effect of the nitration and amination of tyrosyl residues on the kinetic parameters, k_{cat} and K_m, for the thermolysin-catalyzed hydrolysis of FA-Leu-Ala-NH_2 is shown in Fig. 3. The k_{cat} and K_m values were determined to be $0.54\ s^{-1}$ and 0.80 mM, respectively, for the native thermolysin; $0.34\ s^{-1}$ and 0.79 mM for the nitrated thermolysin containing 5 mol of nitrotyrosyl residues per mole of the enzyme, 5-NO_2-thermolysin; $0.25\ s^{-1}$ and 0.80 mM for 7-NO_2-thermolysin; $0.14\ s^{-1}$ and 0.85 mM for 10-NO_2-thermolysin; and $0.074\ s^{-1}$ and 0.78 mM for 15-NO_2-ther-

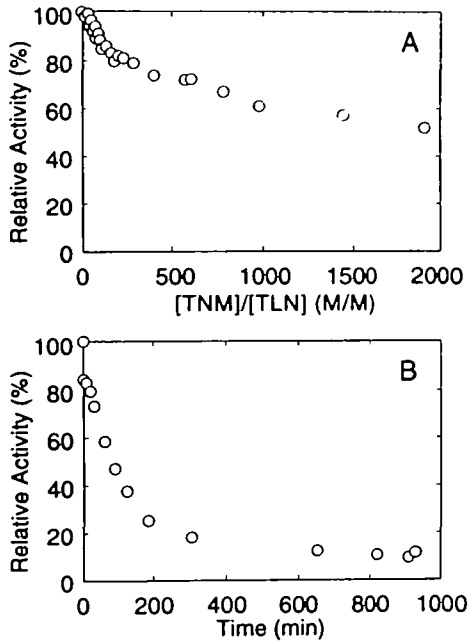

Fig. 1. **Effect of nitration of thermolysin (TLN) with tetranitromethane (TNM) on the peptidase activity of thermolysin.** (A) Effect of variation of the molar excess of TNM on the peptidase activity. The nitration was carried out by adding an appropriate volume of 1% TNM solution in methanol to 7-10 μM thermolysin solution in 40 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ for 1 h at 25°C. The hydrolytic reaction was carried out with 0.06-0.1 μM thermolysin and 0.2 mM FAGLA in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 25°C. The relative activity shown on the ordinate is defined as the percent ratio of the activity (k_{cat}/K_m) of the modified thermolysin to that (which is 2.2×10^4 $M^{-1}\cdot s^{-1}$) of the native enzyme. The molar ratio of the TNM to thermolysin concentrations under the modification conditions is shown on the abscissa. (B) Time-course of the inactivation of thermolysin by TNM. The nitration and the hydrolytic reaction were carried out under the above conditions. The initial concentrations of the native thermolysin and TNM in the nitration reaction were 7 μM and 14 mM, respectively, namely, [TNM]/[TLN] = 2,000 (M/M).

molysin (Fig. 3A). The corresponding values were 0.32 s^{-1} and 0.78 mM, respectively, for the aminated thermolysin containing 5 mol of aminotyrosyl residues per mole of the enzyme, 5-NH_2-thermolysin; 0.17 s^{-1} and 0.74 mM for 10-NH_2-thermolysin; and 0.12 s^{-1} and 0.77 mM for 15-NH_2-thermolysin. Thus K_m remained essentially unaltered, while k_{cat} decreased with the increase in the degree of nitration and amination, which resulted in the decrease in k_{cat}/K_m shown in Figs. 1 and 2.

Effect of Nitration and Amination on the NaCl-Dependent Activation of Thermolysin—The activity of thermolysin in the FAGLA hydrolysis is pH-dependent with the optimum pH around 7, and the activities at pH 5.5 and 8.5 relative to that at the optimum pH are, respectively, 60 and 25% at 0 M NaCl and 15 and 15% at 4 M NaCl (*15*). The activity (k_{cat}/K_m) measured in the pH range of 5.5-8.5 decreased as the degree of nitration increased, but it was affected little by the pH (Fig. 4). The dependence of the activity on the number of nitrotyrosyl residues introduced into thermolysin was similar in the absence and in the presence of 4 M NaCl, although it was a little more sensitive to the degree of nitration at 4 M NaCl than 0 M NaCl:

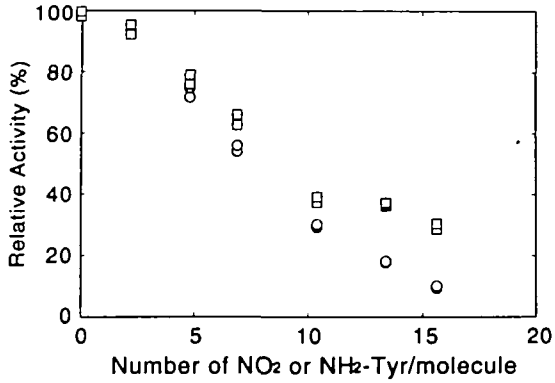

Fig. 2. **Dependence of the peptidase activity of the modified thermolysin on the number of tyrosyl residues nitrated or aminated.** Thermolysin was nitrated with tetranitromethane (TNM) as described under the "EXPERIMENTAL PROCEDURES," and the nitrated thermolysin was reduced with a 60-fold molar excess of $Na_2S_2O_4$ in 40 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ at 25°C. The contents of nitrotyrosyl and aminotyrosyl residues were determined spectrophotometrically. The peptidase activities of the nitrated thermolysin (○) and aminated thermolysin (□) were determined for the hydrolysis of 0.2 mM FAGLA in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 25°C. The relative activity shown on the ordinate is defined in the legend to Fig. 1.

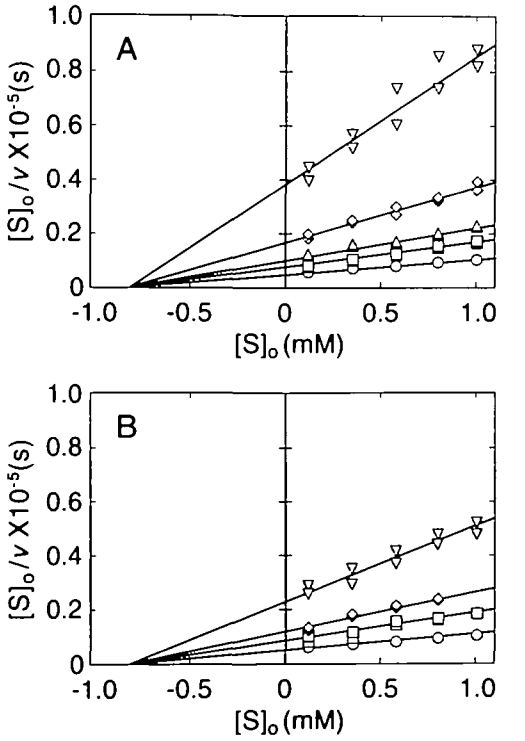

Fig. 3. **Hanes-Woolf plots for the hydrolysis of FA-Leu-Ala-NH_2 catalyzed by the nitrated and aminated thermolysins.** The reaction was carried out in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 0.1-1.0 mM FA-Leu-Ala-NH_2 and at 25°C. The enzyme concentrations in the reaction solution were 0.28-0.36 μM. (A) Nitrated thermolysins. The number of nitrotyrosyl residues per mole of thermolysin: 0 (○), 5 (□), 7 (△), 10 (◇), and 15 (▽). (B) Aminated thermolysins. The number of aminotyrosyl residues per mole of thermolysin: 0 (○), 5 (□), 10 (◇), and 15 (▽). $[S]_o$ is the initial substrate concentration, and v is the reaction velocity.

50% relative to the native enzyme activity was observed at 5 and 7 tyrosyl residues nitrated, respectively, at any pH. The ratio of the activity observed in the presence of 4 M NaCl to that in its absence was defined as the degree of activation. The relationship between the degree of activation in the pH range of 5.5-8.5 and the number of nitrotyrosyl residues introduced into thermolysin is shown in Fig. 5. The degree of activation at each pH examined decreased almost linearly with the increase in the degree of nitration. The extent of the decrease for the nitrated thermolysin with a certain number of nitrotyrosyl residues was pH-dependent, and was larger when pH of the reaction medium was higher. In the case of 14-NO_2-thermolysin, the degree of activation decreased to 80% at pH 8.5 and 40% at pH 5.5 compared with the native enzyme. When the degree of activation of the native enzyme was taken as 100%, the

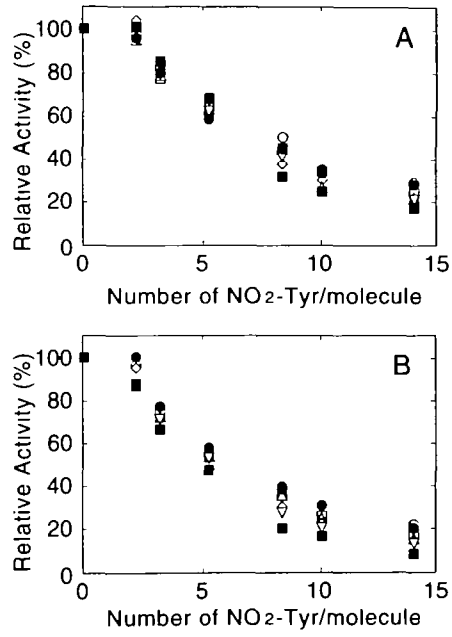

Fig. 4. **Effect of nitration on the peptidase activity of thermolysin at various pHs.** The reaction was carried out with 0.06-0.1 μM thermolysin and 0.25 mM FAGLA at 25°C in the absence (A) and presence (B) of 4 M NaCl. The buffers employed were 40 mM Mes-NaOH buffer at pH 5.5 (●), 6.0 (○), and 6.5 (□), and 40 mM Tris-HCl buffer at pH 7.0 (△), 7.5 (◇), 8.0 (▽), and 8.5 (■), and all buffers contained 10 mM $CaCl_2$. The percent ratio of the activity (k_{cat}/K_m) of the nitrated thermolysin to that of the native enzyme at each pH is indicated on the ordinate.

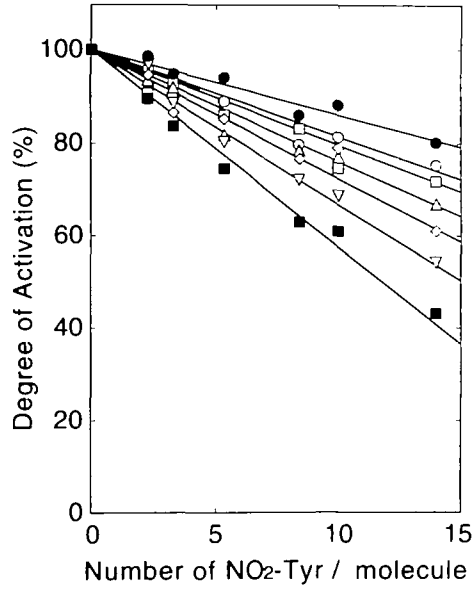

Fig. 5. **Effect of nitration on the degree of activation of thermolysin by 4 M NaCl at various pHs.** The degree of activation shown on the ordinate axis was evaluated as the percent ratio of the degree of activation by 4 M NaCl of the nitrated thermolysin to that of the native enzyme at each pH, based on the results given in Fig. 4. The pH values employed were 5.5 (●), 6.0 (○), 6.5 (□), 7.0 (△), 7.5 (◇), 8.0 (▽), and 8.5 (■). The lines were drawn by linear least-squares regression.

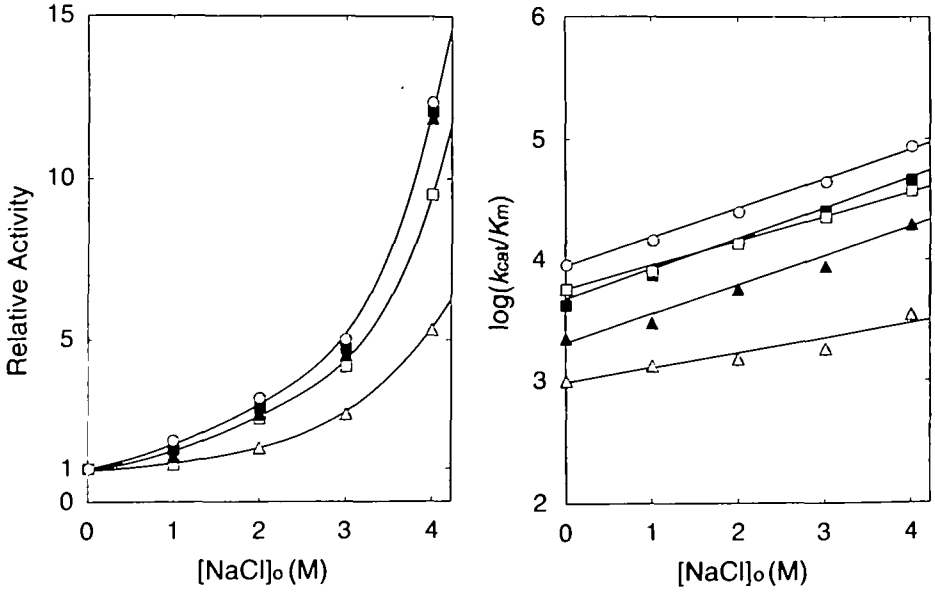

Fig. 6. **Effect of nitration and amination on the NaCl-dependent activation of thermolysin.** The reaction was carried out with 0.22 mM FAGLA and 0.3 μM native or modified thermolysin in 40 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ at 25°C. (A) The relative activity shown on the ordinate axis is defined as the ratio of the peptidase activity (k_{cat}/K_m) at a given NaCl concentration to that (2.2×10^4 $M^{-1} \cdot s^{-1}$) in the absence of NaCl. (B) The logarithmic relationship of the peptidase activity (k_{cat}/K_m) of thermolysins with NaCl concentration. The native thermolysin (○); the modified thermolysins containing 6 (□) and 15 (△) nitrotyrosyl residues; and containing 6 (■) and 15 (▲) aminotyrosyl residues. $[NaCl]_o$ is the initial NaCl concentration.

decrease in the degree of activation per nitrotyrosyl residue introduced into thermolysin was evaluated, from the slopes of the straight lines in Fig. 5 to be 1.4% at pH 5.5; 1.9% at pH 6.0; 2.1% at pH 6.5; 2.4% at pH 7.0; 2.9% at pH 7.5; 3.4% at pH 8.0; and 4.3% at pH 8.5. Accordingly, the degree of activation by 4 M NaCl at pH 7.0 was decreased from 15 to 10 by introducing 14 nitrotyrosyl residues into native thermolysin, and decreased further to 5 by raising the pH to 8.5. The pK_a value of a phenolic hydroxyl group of nitrotyrosine is reported to be 7 (*21*), and the average pK_a value in the 14-NO_2-thermolysin was determined to be 7.0 ± 0.2 by spectrophotometric titration at 428 nm (data not shown).

Figure 6 shows the NaCl-dependent activation of the native, nitrated, and aminated thermolysins in the hydrolysis of FAGLA at pH 8.0. The nitrated thermolysin showed the lower activation than the native enzyme. The degree of activation decreased with the increase in the degree of nitration, and the values at 4 M NaCl were 13, 10, and 5 for the native, 6-NO_2- and 15-NO_2-thermolysins, respectively. However, when the nitrotyrosines in the nitrated thermolysin were reduced to aminotyrosines, the degree of the activation was restored to that of the native enzyme. Plots of log (k_{cat}/K_m) *vs.* $[NaCl]_o$ for the modified thermolysin

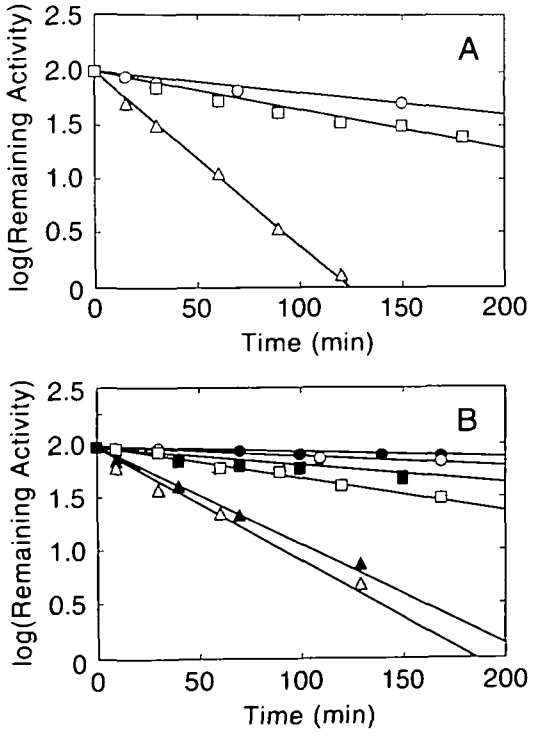

Fig. 7. **Semi-logarithmic plots for the thermal inactivation of the native and nitrated thermolysins.** Thermolysin (7 μM) was incubated in 40 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ at 60, 70, or 80°C for the times indicated, then at 25°C for 3 min prior to the start of the peptidase reaction. (A) The native thermolysin at 60°C (○), 70°C (□), and 80°C (△). (B) The nitrated thermolysins containing 7 (open symbols) and 14 (closed symbols) nitrotyrosyl residues at 60°C (○, ●), 70°C (□, ■), and 80°C (△, ▲). The peptidase activity was determined for the reaction of 0.2–0.3 μM thermolysin with 0.27 mM FAGLA in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$. The remaining activity represents the percent ratio of the (k_{cat}/K_m) value at a given incubation time to that at time zero. The lines were drawn by linear least-squares regression.

(*13*), being based on the data given in Fig. 6A, show good linearity, and the slopes were determined to be 0.25 M^{-1} for 6-NO_2-thermolysin, 0.18 M^{-1} for 15-NO_2-thermolysin, and 0.27 M^{-1} for both 6-NH_2- and 15-NH_2-thermolysins and for the native thermolysin (Fig. 6B). In other words, the degree of activation at x M NaCl was expressed as 1.9^x for the native, 6-NH_2- and 15-NH_2-thermolysins, 1.8^x for 6-NO_2-thermolysin, and 1.5^x for 15-NO_2-thermolysin.

Effect of Nitration on Thermal Stability—Figure 7 shows kinetic curves of the thermal inactivation of both native and nitrated thermolysins at 60, 70, and 80°C, in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 8.0. It is shown that the nitrated enzymes are seen to be more stable to heat treatment than the native enzyme, and the stability seems to increased with the degree of nitration. The calculated apparent first-order rate constants of the inactivation at 60, 70, and 80°C were respectively $(2.1 \pm 0.2) \times 10^{-3}$ min^{-1}, $(3.6 \pm 0.3) \times 10^{-3}$ min^{-1}, and $(15.8 \pm 0.5) \times 10^{-3}$ min^{-1} for the native thermolysin; $(1.1 \pm 0.2) \times 10^{-3}$ min^{-1}, $(3.3 \pm 0.2) \times 10^{-3}$ min^{-1}, and $(10.0 \pm 0.3) \times 10^{-3}$ min^{-1} for 7-NO_2-thermolysin; and $(0.72 \pm 0.10) \times 10^{-3}$ min^{-1}, $(1.8 \pm 0.2) \times 10^{-3}$ min^{-1}, and $(7.9 \pm 0.3) \times 10^{-3}$ min^{-1} for 14-NO_2-thermolysin. The activation energies for the thermal inactivation were determined from the Arrhenius plots to be 18 ± 3, 22 ± 4, and 24 ± 4 kcal/mol for the native, 7-NO_2-, and 14-NO_2-thermolysins, respectively.

DISCUSSION

Effect of Nitration and Amination on Hydrolytic Activity of Thermolysin—Nitration of tyrosyl residues brings about a pK_a shift of approximately three units from 10 to 7 for a phenolic hydroxyl group by introducing a substituent nitro group at the 3-position of the aromatic ring (*18, 19*). On the other hand, amination of the tyrosyl residue by reducing the nitro group to an amino group restores the pK_a to 10 (*21*). The change in the ionization state of tyrosyl residues by nitration and amination is expected to change the electrostatic state of the surface of the enzyme.

Thermolysin is progressively inactivated with the introduction of nitrated and aminated tyrosyl residues into the enzyme (Fig. 2), and thus the inactivation is considered to be brought about by sequential modification of multiple tyrosyl residues rather than modification of a specific tyrosyl residue. In the thermolysin-catalyzed hydrolysis of FA-Leu-Ala-NH_2 as substrate (*13*), k_{cat} decreases with the nitration and amination of the enzyme, whereas K_m is not altered (Fig. 3), indicating that the modifications do not affect formation of the enzyme-substrate complex. In terms of the decrease in k_{cat} by the nitration, the inactivation of thermolysin is likely to be similar to that of neutral protease from *Bacillus amylosaccharitcus*, of which the inactivation is associated in part with a charge effect of ionized phenolic oxygen (*23*). However, the progressive decrease in the activity of thermolysin is not significantly affected by pH in the range of 5.5–8.5 (Fig. 4A). Thus, it may be suggested that the ionization of the nitrated tyrosyl residues, which results from lowering the pK_a of the phenolic hydroxyl group, is not directly related to the progressive inactivation of thermolysin. The results shown in Fig. 3 indicate that the inactivation of thermolysin by nitration and amination corresponds to a non-competitive type of inhibition, in which a ternary complex of the

enzyme with the substrate and inhibitor may be formed (*24*). Thus, the effect of the introduction of nitro or amino groups into tyrosyl residues of thermolysin seems to be equivalent to that of the binding of non-competitive inhibitor to sites other than the active site of the enzyme.

Effect of Nitration and Amination on the NaCl-Dependent Activation of Thermolysin—We have examined the possibility that changes in the electrostatic properties of thermolysin due to nitration and amination of tyrosyl residues affect its NaCl-dependent activation. The degree of activation of the nitrated thermolysin is considerably affected by a change in pH of the medium in the range of 5.5-8.5 (Fig. 5), and it decreases with the increase in the number of nitro groups introduced into tyrosyl residues on the enzyme surface. Since the average pK_a value of the nitrotyrosyl residues of the nitrated thermolysin is 7.0 ± 0.2, a protonated form of nitrotyrosyl residue could be predominant at pH 5.5, and an ionized form at pH 8.5. The degree of activation decreases with the increase in the number of negative charges on the nitrotyrosyl residues accompanying an increase in pH (Fig. 5). Hence, the increase in the negative charge on the surface of thermolysin due to nitration appears to be a major cause of the large decrease in the degree of the NaCl-dependent activation, although the effect of introducing the nitro groups cannot be ignored. Interestingly, the decrease in the degree of activation by the nitration was totally restored to the level of the native enzyme when nitrotyrosyl residues were reduced to aminotyrosyls (Fig. 6A). The degree of activation is dependent on the degree of nitration, and the dependence decreases as the degree of nitration increases (Fig. 6). The degree of activation at x M NaCl of the nitrated thermolysins containing 6 and 15 nitrotyrosyl residues is expressed as 1.8^x and 1.5^x, respectively, while the value of the native enzyme is 1.9^x (*13*). The aminated thermolysin showed the same degree of activation (1.9^x) as the native one (Fig. 6B). Restoration of the degree of activation by amination may be explained most plausibly by the disappearance of the increased amount of negative charges on the tyrosyl residues when nitro groups are converted to amino groups.

We have reported previously that the degree of activation by NaCl shows bell-shaped pH dependence with the optimum pH around 7 (*15*). It seems that removing positive charges from amino groups decreases the degree of activation in the alkaline pH region (pH 7-9), and that removing negative charges from carboxyl groups decreases the degree in the acidic pH region (pH 5-7). The effect of the change in ionization state of tyrosyls on the degree of activation appears to correspond with the pH-effect in the alkaline pH region but not in the acidic region. The value of the isoelectric point (pI) of thermolysin of 5.1 (*27*) suggests that the salt-dependent activation is controlled by the local ionized groups on the surface of the enzyme rather than the gross value of the electrostatic charge.

Classification of Tyrosyl Residues by Reactivity with TNM—The states of the 28 total tyrosyl residues of thermolysin have been examined by pH-dependent ionization and nitration, and classified into five groups (*16*). Sixteen tyrosyl residues are exposed on the surface in solution and accessible to the solvent. They are classified into three independent groups according to their ionizability and reactivity to nitration: the most reactive 6-7 residues are in class I, another 6-7 are in class II, and the other 3 are in class III. The 12 buried residues are classified into two groups: 6 are ionized during incubation at pH 12.2-12.6 for 15 s-10 min, and the other 6 are not ionized during incubation at pH 12.6 for 10 min. The three classes of surface tyrosyl residues are each situated in different electrostatic and hydrophobic microenvironments. The microenvironments of classes II and III are negatively charged and/or hydrophobic. The pK_a value of 10.2 of class I tyrosyl residues is slightly higher than that (pK_a 9.7) of free tyrosine. This pK_a value, together with the lower rate constant of class I for nitration, suggests that these tyrosyl residues are not entirely free on the surface, but are slightly constrained. The higher pK_a values of classes II and III (11.4 and 11.8, respectively) may reflect microenvironments unfavorable to ionization. The states of tyrosyl residues on the surface of thermolysin were estimated on the basis of an X-ray crystallographic analysis (*8*). Five residues (Tyr-75, 110, 211, 251, and 296) are within 3-7 Å of positively charged groups and are expected to ionize more easily than the others. The accessibility to water of Tyr-24 and 27 is higher than 90%, and no negatively charged residues are present around them. Accordingly, they might be in class I. Tyr-66, 193, 217, and 274 are close to negatively charged groups (within 3-5 Å), and their ionization is considered to be fairly suppressed. They are candidates for class III. The remaining residues (Tyr-46, 151, 157, 179, 221, and 242) whose accessibility to water was calculated to be 40%, might belong to class II. It is noted that Tyr-157 is located in the active site. It was reported that when 17 tyrosyl residues were acetylated by *N*-acetylimidazole, the remaining caseinolytic activity was 73%; and when 3-4 more tyrosyl residues were modified by further acetylation, the remaining activity was 45% (*28*). The partially inactivated enzyme regained almost full activity upon treatment with hydroxylamine. The easily acetylated residues might be the same as those classified in classes I-III by nitration and pH-dependent ionization in our study (*16*). These modified tyrosyl residues are considered not to be crucially involved in the activity, although Tyr-157 is at the active site. It is interesting that the 3-4 tyrosyl residues acetylated after the first 17 residues cannot be nitrated or ionized at pH up to 12.2.

TNM reacts not only with tyrosine but also with histidine, methionine, cysteine, and tryptophan. Thermolysin has no cysteinyl residues, and it was confirmed by amino acid analysis that the residues other than tyrosyl residues were not modified with TNM. An essential lack of significant autolysis during the modification was also confirmed by gel filtration HPLC of the modified proteins on a TSKgel G3000SW column.

In conclusion, it has been demonstrated that a change in the ionization state on the surface of thermolysin affects the NaCl-dependent activation of thermolysin, suggesting that electrostatic interaction between the charged groups of the enzyme and ions in the medium might be an important factor in the activation. In addition, the slight increase in thermal stability due to nitration (Fig. 7) might also be associated with an increase in negative charges on the surface of the enzyme. The introduction of charged sites onto the surface of a protein may stabilize its structure by strengthening the interaction with water molecules around the protein (*25, 26*). Most recently, we have demonstrated that the solubility of thermolysin, which is considerably low

(~1 mg/ml) in a common buffer of low ionic strength, is greatly increased, to 80 mg/ml, by the addition of salts, and that the enzyme is cold-soluble (*29*). The interaction between ions of salts and charged groups on the surface of the enzyme could significantly affect not only its activity but also its solubility.

To elucidate in detail the relationship between the electrostatic properties of the surface of thermolysin and the salt activation, chemical modifications of other amino acid residues and site-directed mutagenesis producing a change in the charged state on the surface of the enzyme are in progress (*30*).

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