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Effects of pH, Temperature, and Alcohols on the Remarkable Activation of Thermolysin by Salts[1]

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The activity of thermolysin in the hydrolysis of *N*-[3-(2-furyl)acryloyl] (FA)-dipeptide amides and *N*-carbobenzoxyl-L-aspartyl-L-phenylalanine methyl ester is remarkably enhanced by high concentrations (1-5 M) of neutral salts. The activation is due to an increase in the molecular activity, k_{cat}, while the Michaelis constant, K_m, is not affected by the addition of NaCl. In the present study, the effect of NaCl on the thermolysin-catalyzed hydrolysis of FA-glycyl-L-leucine amide (FAGLA) has been examined by changing the pH and temperature, and by adding alcohols to the reaction mixture. The enzyme activity, expressed by k_{cat}/K_m, is pH-dependent, being controlled by two functional residues with pK_a values of 5.4 and 7.8 in the absence of NaCl. The acidic pK_a is shifted from 5.4 to 6.7 by the addition of 4 M NaCl, while the basic one is not changed. The degree of activation at a given concentration of NaCl is pH dependent in a bell-shaped manner with the optimum pH around 7. Although the activity increases in both the presence and absence of NaCl with increasing temperature from 5 to 35°C, the degree of activation decreases. Alcohols inhibit thermolysin, and the degree of activation decreases with increasing alcohol concentration. The degree of activation tends to increase with increasing dielectric constant of the medium, although it varies considerably depending on the species of alcohol. Electrostatic interactions on the surface and at the active site of thermolysin are suggested to play a significant role in the remarkable activation by salts.

Key words: electrostatic interaction, halophilicity, metalloproteinase, salt-activation, thermolysin.

Thermolysin [EC 3.4.24.27] is a thermostable neutral metallo-endopeptidase isolated from the culture broth of *Bacillus thermoproteolyticus* (*1, 2*). It requires essentially one zinc ion for enzyme activity and four calcium ions for the structural stability (*3-5*), and catalyzes specifically 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 the three-dimensional structure (*9*) are known. The reaction mechanism is controversial, and several models have been proposed (*10-15*). According to crystallographic and kinetic studies (*10-13*), a nucleophilic water molecule is initially co-ordinated to the active site zinc ion. It directly adds to the substrate carboxamide linkage, at the same time that the carbonyl oxygen enters the ligand sphere of the zinc ion. The carboxyl group of Glu 143 is considered to function first as a general base, deprotonating the zinc-co-ordinated water nucleophile, and subsequently as an acid, protonating the cleavable amide nitrogen during breakdown of the tetrahedral intermediate. Tyr 157 and His 231 are proposed to play a stabilizing role in the transition state of the tetrahedral intermediate. The pH-activity studies of thermolysin indicate that the catalysis involves two ionic groups with pK_a values of 5.0-5.6 and 7.5-8.3. The acidic pK_a (pK_{a_1}) is ascribed to Glu 143, and the alkaline pK_a (pK_{a_2}) is assigned to the imidazole group of His 231, while the pK_a of the zinc-co-ordinated water molecule is assumed to be greater than 9. On the other hand, it has been proposed from inhibitor-binding studies (*14, 15*) that substrate binding involves complete displacement of water bound to the active-site zinc ion (pK_a 5.3 in the free enzyme), and His 231 (pK_a 8.0 in the free enzyme) induces peptide bond hydration of the substrate. The relatively low pK_a value for zinc-bound water is explained in terms of the heightened Lewis acidity owing to the zinc ion.

Holmquist and Vallee (*16*) first reported that some neutral salts, such as NaCl and NaBr, significantly activated the thermolysin-catalyzed hydrolysis of *N*-[3-(2-furyl)-acryloyl](FA)-glycyl-L-leucine amide (FAGLA) and its ester analogue FA-Gly-OLeu-NH_2. We have reported the activation of thermolysin by high concentrations (1-5 M) of neutral salts in the hydrolysis and synthesis of *N*-carbobenzoxyl-L-aspartyl-L-phenylalanine methylester (ZAPM), a precursor of a synthetic sweetener, as well as in the hydrolysis of FAGLA (*17*). NaCl and NaBr are the most

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Abbreviations: FA, furylacryloyl; FAGLA, *N*-[3-(2-furyl) acryloyl]-glycyl-L-leucine amide; Tris, tris(hydroxymethyl)aminomethane; ZAPM, *N*-carbobenzoxyl-L-aspartyl-L-phenylalanine methylester.

effective salts for this activation, and the activity increases with increase in salt concentration in an exponential fashion. Thermolysin is activated 7-fold at 3.8 M NaCl and NaBr in the hydrolysis of ZAPM. Recently, we have studied the effect of salts on such activity by using a series of substrates, FA-dipeptide amides having various hydrophobic amino acids at the cleavable bond (*18*). Although the enzyme activity varies widely depending on the substrate, the degree of activation at a given concentration of NaCl is rather similar, and the activation is in the range of 11-17 times at 4 M NaCl. This indicates that the degree of activation is not very dependent on the amino acid side chains at the scissile bond of the substrates. The molecular activity, k_{cat}, and Michaelis constant, K_m, can be evaluated separately in the case of ZAPM, FA-Phe-Ala-NH_2, and FA-Leu-Ala-NH_2, and the activation has been demonstrated to be induced solely by increasing k_{cat}. The effectiveness of monovalent cations for increasing k_{cat} follows the order $Na^+ > K^+ > Li^+$. We have described a unique absorption difference spectrum that is observed on mixing thermolysin with NaCl and NaBr, suggesting a conformational change of the enzyme upon interaction with the salts (*19*). The cause of the salt activation is not known, and there remains a possibility that the activation correlates with the difference spectrum. The activity of thermolysin increases progressively with increasing salt concentration, and does not show any saturating behavior even at the saturated concentration of salts. In the present study, we sought to reveal the cause of the activation by examining the effects of pH, temperature, and addition of alcohols to the reaction medium, and we have established that the activation is controlled in a complex manner by electrostatic interactions on the surface and at the active site of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—A three-times-crystallized-and-lyophilized preparation of thermolysin (Lot T8BA51; 8360 PU/mg according to the suppliers) was purchased from Daiwa Kasei, Osaka. This preparation was used without further purification. The thermolysin solution was filtered through a Millipore filter, Type HA (pore size: 0.45 μm) before use. The concentration was determined spectrophotometrically using an absorbance value, A (1 mg/ml), at 277 nm of 1.83 (*17*), and a molecular mass of 34.6 kDa (*8*). FAGLA (Lot 370513) was obtained from the Peptide Institute, Osaka. The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient, $\varepsilon_{345} = 766\ M^{-1} \cdot cm^{-1}$ (*17*). All other chemicals were of reagent grade, purchased from Nacalai Tesque, Kyoto.

Hydrolysis of FAGLA—Hydrolysis of FAGLA by thermolysin was measured by following the decrease in absorbance at 345 nm with a Shimadzu spectrophotometer UV-2200. The amount of FAGLA hydrolyzed was estimated by using the molar absorption difference on hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1} \cdot cm^{-1}$ (*17*), in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ (standard buffer) with NaCl added up to 5 M (*17*). The molar absorption difference was confirmed not to be affected under the conditions used in this study at temperatures in the range of 5-35°C, and in the presence of alcohols in the range of 0-12%. Due to the limited solubility of FAGLA, it is difficult to obtain a FAGLA concentration sufficiently larger than the Michaelis constant, K_m, in order to estimate the kinetic parameters, k_{cat} and K_m, separately. Under the pseudo-first order condition, the reaction rate v is expressed by $(k_{cat}/K_m)\ [E]_o\ [S]_o$, where $[E]_o$ and $[S]_o$ are the initial concentrations of the enzyme and substrate, respectively, and the enzyme activity was evaluated using the specificity constant, k_{cat}/K_m.

Effect of pH on the salt activation of thermolysin-catalyzed hydrolysis of FAGLA was examined in 40 mM sodium acetate buffer at pH 4.5-5.7, 40 mM Tris-maleate buffer at pH 5.2-8.0, and 40 mM Tris-HCl buffer at pH 7.0-9.0, all of which contained 10 mM $CaCl_2$ and various concentrations (0-4 M) NaCl, at 25°C. The effect of temperature in the range of 5-35°C and addition of alcohols (methanol, ethanol, 1-propanol, 2-propanol, and *tert*-amyl alcohol) at concentrations of 0-12% (v/v) on the hydrolysis was examined in the standard buffer with NaCl added up to 5 M.

The ratio of the reaction rate v in the presence of x M NaCl to that in the absence of NaCl (v at x M NaCl/v at 0 M NaCl) was defined as the degree of activation at x M NaCl. The degree of activation was evaluated generally in this study at 4 M NaCl.

The dielectric constant (D) value of an alcohol-water mixture solution containing alcohol up to 12% (v/v) can be evaluated by the following equation: $D = f_w \cdot D_w + f_a \cdot D_a$, where f_a and f_w are the volume fractions of the alcohol and water, respectively (and thus $f_a + f_w = 1$), and D_a and D_w are the dielectric constants of the alcohol and water, respectively (*20*). As experimental determination of the D values of solutions containing high concentration of salts is virtually impossible, they were evaluated theoretically according to the above equation.

RESULTS

Effects of NaCl on the pH Dependence of FAGLA Hydrolysis—Figure 1A shows the pH dependence of the reaction rate v for the thermolysin-catalyzed hydrolysis of FAGLA in the presence of various concentrations of NaCl (0-4 M) at 25°C. A bell-shaped pH dependence with optimal pH around 7 was observed over the range of NaCl concentrations studied. The maximum value, v_{max}, of the reaction rate, at the optimum pH was shown to be enhanced in an exponential fashion, and the pH optimum shifted simultaneously to the alkaline side, with increase in the NaCl concentration. The relative activity, v/v_{max}, was plotted against pH (Fig. 1B). The bell-shaped pH profiles suggest the involvement of two ionizable groups in the thermolysin-catalyzed hydrolysis of FAGLA. The pH optimum and v_{max} values were as follows, respectively: 6.65 and $3.26 \times 10^4\ M^{-1} \cdot s^{-1}$ at 0 M NaCl; 6.9 and $6.84 \times 10^4\ M^{-1} \cdot s^{-1}$ at 1 M NaCl; 7.1 and $23.7 \times 10^4\ M^{-1} \cdot s^{-1}$ at 3 M NaCl; and 7.2 and $40 \times 10^4\ M^{-1} \cdot s^{-1}$ at 4 M NaCl. Plots of log v obtained at a given NaCl concentration against pH (Dixon plots) were fitted with three straight lines with slopes of +1, 0, and −1, and two pK_a values were determined at each NaCl concentration, suggesting that the reaction rate is controlled by at least two ionizable residues. The acidic pK_a (pK_{a_1}) value shifted from 5.4 to 6.7 with increasing NaCl concentration from 0 to 4 M, while the alkaline one (pK_{a_2}) was almost constant (7.9-7.7) over the NaCl concentration range examined (Fig. 1C). Figure 1D

shows that the degree of activation of thermolysin by 4 M NaCl in the hydrolysis of FAGLA is pH-dependent in a bell-shaped manner. The degree of activation was 12 at the pH optimum, pH 7.3, while it was only 2 and 4.5 at pH 5.7 and 9.0, respectively. The Dixon plot of the degree of activation at 4 M NaCl [log (degree of activation) *vs.* pH] was fitted by three straight lines with slopes of +1, 0, and −1, and two pK_a values of 6.7 and 8.0 were obtained. These values are similar to the pK_a values, 6.7 and 7.8, obtained for the activity of thermolysin at 4 M NaCl.

Effect of Temperature on the Salt Activation of Thermolysin—The reaction rate of the thermolysin-catalyzed hydrolysis of FAGLA increased 4.0 times with increasing temperature from 5 to 35°C in the absence of NaCl, and increased 2.4 times in the presence of 4 M NaCl (Fig. 2A). On the other hand, the degree of activation of thermolysin by 4 M NaCl decreased almost linearly with increase of temperature (Fig. 2B), from 18 at 5°C to 10 at 35°C.

Effect of Alcohols on the Salt Activation of Thermolysin—The reaction rate of the thermolysin-catalyzed hydrolysis of FAGLA decreased in a pseudo-exponential manner with increasing methanol concentration from 0 to 12% in the presence and absence of 4 M NaCl (Fig. 3A), and at 12% methanol, the reaction rates decreased to 6 and 16% of that obtained at 0% methanol, respectively. The degree of activation of thermolysin at 4 M NaCl decreased from 11 to 4 with increasing methanol concentration from 0 to 12%.

Other alcohols such as ethanol, 1-propanol, 2-propanol, and *tert*-amyl alcohol inhibited thermolysin in the absence and presence of NaCl in a similar manner to that observed with methanol (Fig. 3A). The respective concentrations of methanol, ethanol, 1-propanol, 2-propanol, and *tert*-amyl alcohol giving 50% of the activity obtained at 0% alcohol were 3, 1, 0.2 , 0.4, and 2% in the absence of NaCl. On the other hand, they were 1.4, 0.4, 0.1, 0.2, and 0.9% in the presence of 4 M NaCl, indicating that inhibition of ther-

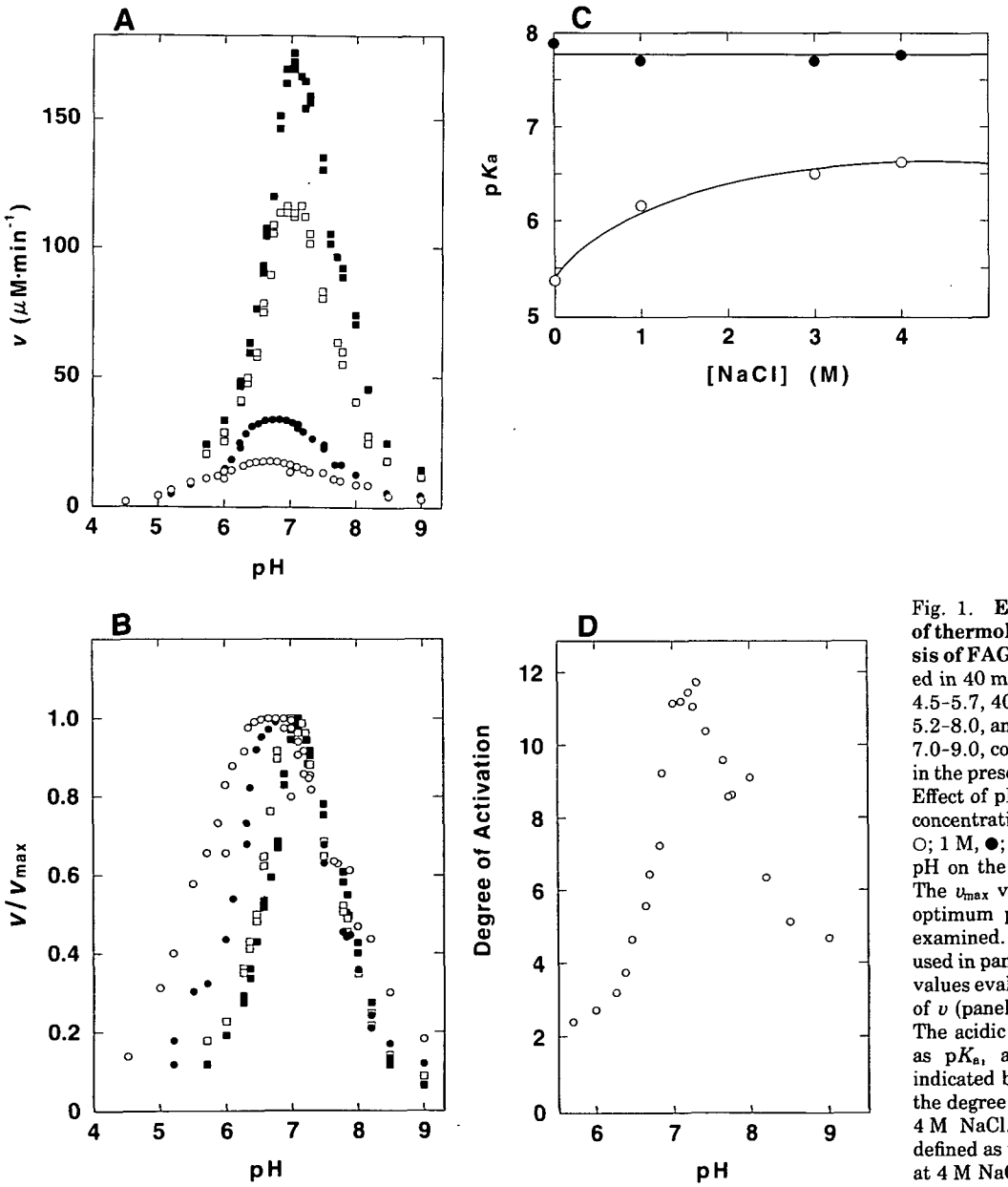

Fig. 1. **Effect of pH on the activation of thermolysin by NaCl in the hydrolysis of FAGLA.** The reaction was performed in 40 mM sodium acetate buffer at pH 4.5-5.7, 40 mM Tris-maleate buffer at pH 5.2-8.0, and 40 mM Tris-HCl buffer at pH 7.0-9.0, containing 10 mM $CaCl_2$, at 25°C, in the presence or absence of 4 M NaCl. A: Effect of pH on the reaction rate, v. NaCl concentration added to the buffers: 0 M, ○; 1 M, ●; 3 M, □; and 4 M, ■. B: Effect of pH on the relative reaction rate, v/v_{max}. The v_{max} value is the reaction rate at the optimum pH at the NaCl concentration examined. Symbols are the same as those used in panel A. C: Dependence of the pK_a values evaluated from the pH-dependence of v (panel A) on the NaCl concentration. The acidic and basic pK_as are designated as pK_{a_1} and pK_{a_2}, respectively, being indicated by ○ and ●. D: Effect of pH on the degree of activation of thermolysin by 4 M NaCl. The degree of activation is defined as the ratio of the reaction rate, v, at 4 M NaCl to that at 0 M NaCl.

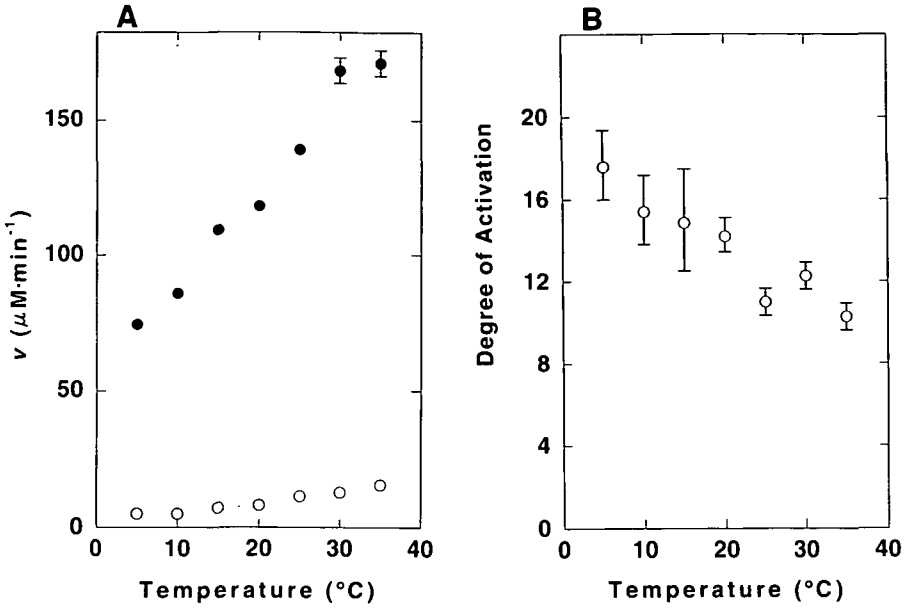

Fig. 2. **Effect of temperature on the activation of thermolysin by NaCl in the hydrolysis of FAGLA.** The reaction was performed in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5, at the temperature indicated, in the presence or absence of 4 M NaCl. A: Effect of temperature on the reaction rate, *v*. NaCl concentration added to the buffers: 0 M, ○; and 4 M, ●. B: Effect of temperature on the degree of activation at 4 M NaCl.

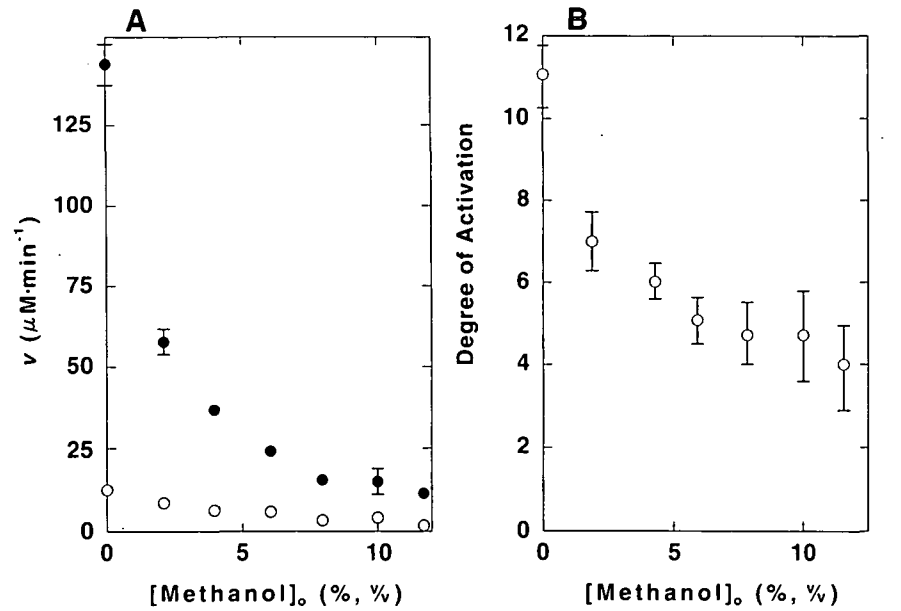

Fig. 3. **Effect of methanol on the activation of thermolysin by NaCl in the hydrolysis of FAGLA.** The reaction was performed in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5, at 25°C, in the presence or absence of 4 M NaCl. A: Effect of methanol on the reaction rate, *v*. NaCl concentration added to the buffers: 0 M, ○; and 4 M, ●. B: Effect of temperature on the degree of activation at 4 M NaCl.

molysin by the alcohols is doubled by adding 4 M NaCl. The order of inhibitory effect of the alcohols is 1-propanol > 2-propanol > ethanol > *tert*-amyl alcohol > methanol in either the presence or absence of 4 M NaCl. Although NaCl activates thermolysin even in the presence of alcohols, they greatly reduce the effect of NaCl.

The degree of activation of thermolysin by 4 M NaCl decreased in a pseudo-exponential manner with increasing alcohol concentration (Fig. 4A). It was 11 in the absence of alcohols, and was reduced to half at 4.7% methanol, 0.6% ethanol, 0.2% 1-propanol, 0.5% 2-propanol, or 1.1% *tert*-amyl alcohol. At 4% alcohol, the degrees of activation were 6, 4, 3, and 2 times with methanol, *tert*-amyl alcohol, ethanol, and 2-propanol, respectively, and no activation was observed with 1-propanol. The order of decreasing degree of activation by the alcohols is the same as that in the inhibition of thermolysin. The dependence of the degree of activation on the theoretically evaluated dielectric constant of the reaction medium (*20*) is shown in Fig. 4B. The degree of activation varies depending on the alcohol species at a certain value of the dielectric constant, suggesting that the degree is determined not only by the dielectric constant of the medium, but also by other characteristics of the alcohols.

In the absence of NaCl, the activities of thermolysin at 4 and 0.4% ethanol were 18 and 74% of that at 0% ethanol. When the ethanol was diluted from 4 to 0.4%, the activity increased from 18 to 70%. In the presence of 4 M NaCl, the activities at 4 and 0.4% ethanol were 5 and 42% of that at 0% ethanol, and after diluting the ethanol from 4 to 0.4%, the activity increased to 41%. Similar results were obtained with other alcohols, and the inhibition by alcohols appears to be fully reversible. Methanol showed mixed-type inhibition of thermolysin-catalyzed hydrolysis of

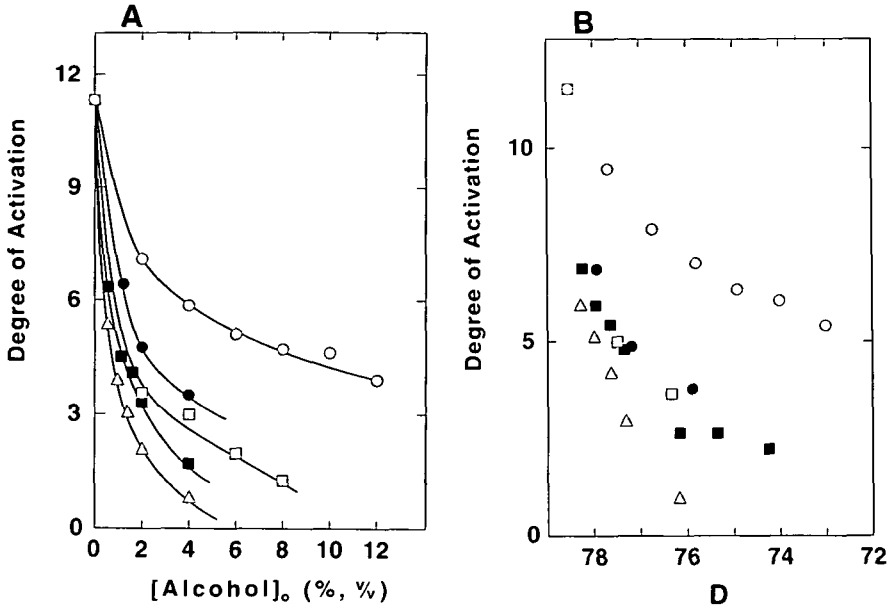

Fig. 4. **Effect of alcohols on the activation of thermolysin by NaCl in the hydrolysis of FAGLA.** The reaction was performed in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5, at 25°C in the presence or absence of 4 M NaCl. A: Effect of alcohols on the degree of activation. Alcohols: methanol, ○; ethanol, □; 1-propanol, △; 2-propanol, ■; and *tert*-amyl alcohol, ●. B: Dependence of the degree of activation on the theoretically evaluated dielectric constant, D, of the reaction medium. Symbols are the same as those used in panel A. The D value of alcohol-water mixture was evaluated by using the following equation: $D = f_w \cdot D_w + f_a \cdot D_a$, where f_a and f_w are volume fractions of the alcohol and water, respectively (and thus $f_a + f_w = 1$), and D_a and D_w are the dielectric constants of the alcohol and water, respectively.

FAGLA both in the absence and presence of 4 M NaCl (data not shown), although the inhibitor constants could not be evaluated precisely because the solubility of FAGLA is lower than the K_m values (*17*).

DISCUSSION

Causes of the Salt-Activation of Thermolysin—Addition of NaCl might modify the structure of thermolysin, *e.g.*, by shielding surface charges, promoting the binding of hydrated ions to the surface of thermolysin, and causing disintegration of the water structure. The shielding effect is generally saturated at 0.5-1.0 M NaCl. However, activation of thermolysin is observed with increasing [NaCl] up to 5 M in an exponential manner. Therefore, this effect is not a major factor in the activation, which might instead be caused by a conformational change of the enzyme and/or an environmental factor of the medium.

The Shift in pK_a Values of the Thermolysin-Catalyzed Hydrolysis of FAGLA by the Addition of NaCl—The pK_{a_1} value shifted from 5.4 to 6.7 with increase of [NaCl] from 0 to 4 M, while the pK_{a_2} value did not change much (Fig. 1C). The nature of the functional groups has been uncertain, and the pK_{a_1} has been suggested to be due to Glu 143 (*10, 11*) or to the water molecule co-ordinated to the active-site zinc ion (*14, 15*). The change in pK_{a_1} can be attributed to a change in electrostatic environment around the pK_{a_1} group, which destabilizes the ionized form of the group. The distance of the group from negative charge(s) may be reduced and/or that from positive charge(s) increased (*21*). If Glu 143 is presumed to contribute to pK_{a_1}, the value of 6.7 obtained with 4 M NaCl seems to be extraordinarily high for the carboxylate. Interestingly, Asp 325 of *Escherichia coli* lactose permease exhibits a pK_a of 8.5 (*22*), and Asp 96 and Asp 115 in bacteriorhodopsin exhibit anomalously high values (>9.5) (*23*). The electrostatic interactions of the pK_{a_1} group can be interpreted by two models: the point-charge model refers to the electrostatic interactions of the group with a specific residue in its vicinity, and the surface-charge model refers to those with non-specific residues dispersed on the surface of the enzyme.

The ionization state of the pK_{a_1} group may be affected by the interaction with ions of NaCl or by conformational changes induced by the high concentration of NaCl. The nearest neighbours of Glu 143 and the active-site zinc ion are Glu 166, Asp 150, Asp 170, His 146, His 142, His 231, and Arg 203 (*9*). The states of these residues are changed by the addition of NaCl, and are involved in the pK_{a_1} shift. According to the point-charge model (*24-26*), the increment in pK_a, ΔpK_a, for an ionizable group (the charge of which is $Z_1 e$) caused by the other ionizable group (the charge of which is $Z_2 e$) is given by the following formula

$$2.303\ RT\ \Delta pK_a = N\ Z_1\ Z_2\ e^2/(D\ r). \quad (1)$$

Using the numerical values $e = 4.80 \times 10^{-10}$ esu, $N = 6.02 \times 10^{23}$, $R = 8.31 \times 10^7$ erg·deg^{-1}·mol^{-1}, and $T = 298$ K, we have:

$$r = 2.43 \times 10^6 \cdot Z_1\ Z_2/(D \cdot \Delta pK_a)\ (\text{cm}). \quad (2)$$

Although there are only a few reports on the effective dielectric constants of protein surfaces, the values so far observed are in the range of 40-55 (*25-29*). These values were used for the effective dielectric constant around the pK_{a_1} group of thermolysin, and the distance between the group and the ionized group was calculated to be 4.1 ± 0.7 Å. His 146 is located in the neighbourhood of Glu 143 and the active-site zinc ion [the distance between them is 4-5 Å (*30*)], and it is plausible that the distance increases with the addition of NaCl, accompanied with the increase of pK_{a_1}. It is noteworthy that a change in the pK_a of His 57 of δ-chymotrypsin from 7.0 to 8.0 was observed on altering the overall surface charge by 28 units (namely, changing 14 lysyl residues to 14 negatively charged succinate-half-amides) (*31*). The observed change in the pK_a is in reasonable agreement with that expected from a modification involving an appreciable change in the net charge of the protein. A change in pK_{a_1} of thermolysin as large as 1.3 units (Fig. 1C) could be attributable to changes in the electrical charges on the protein surface. At present, it is

not known whether the point-charge or the surface-charge model is more effective to explain the shift of pK_{a_1}.

It has been reported that proteins isolated from halophilic bacteria are rich in acidic amino acids on the surface, and that their interaction with hydrated salt cations may enhance their stability (*32, 33*). Hydration of ions is dependent on the ionic species, and it is in the order of Hofmeister's series; $Li^+ > Na^+ > K^+$ (*34, 35*). The order of ions for the efficiency in the activation of thermolysin is $Na^+ > K^+ > Li^+$ (*17-19*). This difference suggests that the activation of thermolysin is not a result only of the size or hydration potential of ions, but rather depends on their interactions with charged groups on the enzyme.

pH Dependence of the Degree of Activation by Salts—The pK_{a_1} value increases in a hyperbolic manner to the saturated level from 5.4 to 6.7 with increase in [NaCl] from 0 to 4 M (Fig. 1C), whereas the activity of thermolysin increases in an exponential manner and saturating behavior is not observed (*17, 18*). Therefore, the salt-activation is likely not to be directly related to the pK_{a_1} shift. The degree of activation shows a bell-shaped pH-dependence controlled by two residues of pK_a 6.7 and 8.0 (Fig. 1D), and the pK_a values are the same as those obtained for the activity at 4 M NaCl (Fig. 1C), suggesting that the activation as well as the activity is controlled by the same groups. The isoelectric point (pI) of thermolysin is at pH 5.1 (*35*), where the activation is almost negligible (Fig. 1D). Carboxyl and amino groups on the surface of thermolysin may be fully ionized at around pH 7 where the degree of activation is maximum, and the pH dependence of the degree of activation (Fig. 1D) seems to correspond with the number of charges on the surface of thermolysin. Binding of hydrated ions to the charged groups may stabilize the enzyme structure and this may activate the enzyme (*33, 36*).

Effects of Temperature and Alcohols on the Degree of Activation by Salts—The concept of dielectric constant is not a precise one in a solution containing high salt concentrations as used in this study, though the dielectric constant in the medium generally decreases with increase in temperature and in alcohol concentration. Therefore, the results shown in Figs. 2B and 3B can be interpreted as indicating that the degree of activation increases with increasing dielectric constant of the medium. According to Coulomb's law, the smaller the dielectric constant of the medium is, the stronger the electrostatic force is. Thus, the electrostatic force between charged groups on the surface of thermolysin and that between charged groups and hydrated ions work to suppress the degree of activation. The relationship between the degree of activation and electrostatic interaction is probably opposite to that deduced from the pH dependence of the degree of activation. The electrostatic interaction is dependent not only on the dielectric constant of the medium, but also on the temperature and the alcohol content. This may be a cause of the discrepancy. The effect of alcohols on the decrease in the activity and the degree of activation follow the order of 1-propanol > 2-propanol > ethanol > *tert*-amyl alcohol > methanol (Fig. 4), which is not the same as that of the dielectric constant, the values of which at 25°C are 20.3, 19.9, 24.6, 11.7, and 32.6, respectively (*20*). In particular, the dielectric constant of *tert*-amyl alcohol is the smallest, though the effect of this alcohol is intermediate between those of methanol and ethanol. Although the dielectric constants of 1- and 2-propanols are almost the same, the activity and degree of activation with 1-propanol are obviously lower than those with the same concentration of 2-propanol. The activity and degree of activation with the branched alcohol are larger than those with the linear one of the same carbon number. Methanol shows mixed-type inhibition, indicating that it binds both active and inhibitory sites. Alcohols may affect the enzyme activity by lowering the dielectric constant and by binding directly to thermolysin.

Effect of Salts on Other Halophilic Enzymes—Nuclease H of *Micrococcus varians* (*37*) and a serine protease, halolysin, of an archaebacterium (*38*) are considered to be halophilic enzymes (*39*). The activation behavior of these enzymes by salts is exponential or bell-shaped depending on the substrate, and the effect of cations is in the order of $K^+ > Na^+ > Li^+$, being different from that for thermolysin (*18*). Aspartic proteases from retro viruses, as well as pepsin, increase the activity with increasing [NaCl] up to 5 M, and the activation is derived only from the decrease in the Michaelis constant (*40-42*). The cause of the activation of these proteases is probably different from that in the case of thermolysin (*43*). We are currently studying the effects of substrate structure on the salt-activation of thermolysin. The effects of charged groups of thermolysin on the activity have also been investigated by means of chemical modification (*44*) and site-directed mutagenesis. A series of such studies on thermolysin and other enzymes (*45, 46*) may provide an answer as to how the enzyme activity is controlled.

REFERENCES

1. Endo, S. (1962) Studies on protease produced by thermophilic bacteria. *J. Ferment. Technol.* **40**, 346-353
2. Matsubara, H. and Feder, J. (1971) in *The Enzymes* 3rd ed. (Boyer, P.D., ed.) Vol 3, pp. 721-795, Academic Press, New York
3. Latt, S.A., Holmquist, B., and Vallee, B.L. (1969) A zinc metalloenzyme. *Biochem. Biophys. Res. Commun.* **37**, 333-339
4. Feder, J., Garrett, L.R., and Wildi, B.S. (1971) Studies on the role of calcium in thermolysin. *Biochemistry* **10**, 4552-4555
5. Tajima, M., Urabe, I., Yutani, K., and Okada, H. (1976) Role of calcium ions in the thermostability of thermolysin and *Bacillus subtilis* var. *amylosaccharitícus* neutral protease. *Eur. J. Biochem.* **64**, 243-247
6. Morihara, K. and Tsuzuki, H. (1970) Thermolysin: Kinetic study with oligopeptides. *Eur. J. Biochem.* **15**, 374-380
7. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157-162
8. Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A., and Neurath, H. (1972) Amino-acid sequence of thermolysin. *Nature* **238**, 35-37
9. Holmes, M.A. and Matthews, B.W. (1982) Structure of thermolysin refined at 1.6 Å resolution. *J. Mol. Biol.* **160**, 623-639
10. Hangauer, D.G., Monzingo, A.F., and Matthews, B.W. (1984) An interactive computer graphics study of thermolysin-catalyzed peptide cleavage and inhibition by *N*-carboxymethyl dipeptides. *Biochemistry* **23**, 5730-5741
11. Matthews, B.W. (1988) Structural basis of the action of thermolysin and related zinc peptidases. *Acc. Chem. Res.* **21**, 333-340
12. Izquierdo, M.C. and Stein, R.L. (1990) Mechanistic studies of thermolysin. *J. Am. Chem. Soc.* **112**, 6054-6062
13. Izquierdo-Martin, M., and Stein, R.L. (1992) Mechanistic studies on the inhibition of thermolysin by a peptide hydroxamic acid. *J. Am. Chem. Soc.* **114**, 325-331
14. Mock, W.L. and Aksamawati, M. (1994) Binding to thermolysin

of phenolate-containing inhibitors necessitates a revised mechanism of catalysis. *Biochem. J.* **302**, 57-68
15. Mock, W.L. and Stanford, D.J. (1996) Arazoformyl dipeptide substrates for thermolysin. Confirmation of a reverse protonation catalytic mechanism. *Biochemistry* **35**, 7369-7377
16. Holmquist, B. and Vallee, B.L. (1976) Esterase activity of zinc neutral proteases. *Biochemistry* **15**, 101-107
17. Inouye, K. (1992) Effects of salts on thermolysin: Activation of hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, and a unique change in the absorption spectrum of thermolysin. *J. Biochem.* **112**, 335-340
18. Inouye, K., Lee, S.-B., and Tonomura, B. (1996) Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts. *Biochem. J.* **315**, 133-138
19. Inouye, K., Kuzuya, K., and Tonomura, B. (1994) A spectrophotometric study on the interaction of thermolysin with chloride and bromide ions, and the state of tryptophyl residue 115. *J. Biochem.* **116**, 530-535
20. Mellan, I. (1959) in *Source Book of Industrial Solvents* Vol. 3, pp. 199-200, Van Nostrand Reinhold, London
21. Inouye, K., Tonomura, B., and Hiromi, K. (1977) The states of tyrosyl and tryptophyl residues in a protein proteinase inhibitor (*Streptomyces subtilisin* inhibitor). *J. Biochem.* **82**, 1207-1215
22. Frillingos, S. and Kaback, H.R. (1996) Monoclonal antibody 4B1 alters the pK_a of a carboxylic acid at position 325 (Helix X) of the lactose permease of *Escherichia coli*. *Biochemistry* **35**, 10166-10171
23. Bashford, D. and Gerwert, K. (1992) Electrostatic calculations of the pK_a values of ionizable groups in bacteriorhodopsin. *J. Mol. Biol.* **224**, 473-486
24. Edsall, J.T. and Wyman, J. (1958) in *Biophysical Chemistry*, Vol. 1, pp. 450-463, Academic Press, New York
25. Inouye, K., Tonomura, B., and Hiromi, K. (1979) The interaction of a tyrosyl residue and carboxyl groups in the specific interaction between *Streptomyces* subtilisin inhibitor and subtilisin BPN'. *J. Biochem.* **85**, 1115-1126
26. Inouye, K., Tonomura, B., Hiromi, K., Fujiwara, K., and Tsuru, D. (1979) Further studies on the interaction between a protein proteinase inhibitor, *Streptomyces* subtilisin inhibitor, and thiol-subtilisin BPN'. *J. Biochem.* **85**, 1127-1134
27. Masuda-Momma, K., Shimakawa, T., Inouye, K., Hiromi, K., Kojima, S., Kumagai, I., Miura, K., and Tonomura, B. (1993) Identification of amino acid residues responsible for the changes of absorption and fluorescence spectra on the binding of subtilisin BPN' and *Streptomyces* subtilisin inhibitor. *J. Biochem.* **114**, 906-911
28. Sternberg, M.J.E., Hayes, F.R.F., Russell, A.J., Thomas, P.G., and Fersht, A.R. (1987) Prediction of electrostatic effects of engineering of protein charges. *Nature* **330**, 86-88
29. Russell, A.J., Thomas, P.G., and Fersht, A.R. (1987) Electrostatic effects on modification of charged groups in the active site cleft of subtilisin by protein engineering. *J. Mol. Biol.* **193**, 803-813
30. Monzingo, A.F. and Matthews, B.W. (1984) Binding of *N*-carboxymethyl dipeptide inhibitors to thermolysin determined by X-ray crystallography: A novel class of transition-state analogues for zinc peptidases. *Biochemistry* **23**, 5724-5729
31. Valenzuela, P. and Bender, M.L. (1971) Kinetic properties of succinylated and ethylenediamine-amidated δ-chymotrypsins. *Biochim. Biophys. Acta* **250**, 538-548
32. Zaccai, G., Cendrin, F., Haik, Y., Borochov, N., and Eisenberg, H. (1989) Stabilization of halophilic melate dehydrogenase. *J. Mol. Biol.* **208**, 491-500
33. Zaccai, G. and Eisenberg, H. (1990) Halophilic proteins and the influence of solvent on protein stabilization. *Trends Biochem. Sci.* **15**, 333-337
34. Pundak, S., Aloni, H., and Eisenberg, H. (1981) Structure and activity of malate dehydrogenase from the extreme halophilic bacteria of the dead sea. *Eur. J. Biochem.* **118**, 471-477
35. Inouye, K. (1991) Chromatographic behaviors of proteins and amino acids on a gel filtration matrix, TSK-GEL Toyopearl. *Agric. Biol. Chem.* **55**, 2129-2139
36. Madern, D., Pfister, C., and Zaccai, G. (1995) Mutation at a single acidic amino acid enhances the halophilic behaviour of malate dehydrogenase from *Haloarcula marismortui* in physiological salts. *Eur. J. Biochem.* **230**, 1088-1095
37. Kamekura, M. and Onishi, H. (1978) Halophilic nuclease from a moderately halophilic *Micrococcus varians*. *J. Bacteriol.* **119**, 339-344
38. Kamekura, M. and Seno, Y. (1990) A halophilic extracellular protease from a halophilic archaebacterium strain. *Biochem. Cell Biol.* **68**, 352-359
39. Inouye, K. (1994) Halophilic enzymes (in Japanese). *Seikagaku* **66**, 446-450
40. Tropea, J.E., Nashed, N.T., Louis, J.M., Sayer, J.M., and Jerina, D.M. (1992) Effect of salt on the kinetic parameters of retroviral and mammalian aspartic acid proteases. *Bioorg. Chem.* **20**, 67-76
41. Wondrak, E.M., Louis, J.M., and Oroszlzn, S. (1991) The effect of salt on the Michaelis Menten constant of the HIV-1 protease correlates with the Hofmeister series. *FEBS Lett.* **280**, 344-346
42. Szeltner, Z. and Polgar, L. (1996) Conformational stability and catalytic activity of HIV-1 protease and both enhanced at high salt concentration. *J. Biol. Chem.* **271**, 5458-5463
43. Yang, J.J., Artis, D.R., and Van Wart, H.E. (1994) Differential effect of halide anions on the hydrolysis of different dansyl substrates by thermolysin. *Biochemistry* **33**, 6516-6523
44. Lee, S.-B., Inouye, K., and Tonomura, B. (1997) The states of tyrosyl residues in thermolysin as examined by nitration and pH-dependent ionization. *J. Biochem.* **121**, 231-237
45. Inouye, K., Osaki, A., and Tonomura, B. (1994) Dissociation of dimer of bovine erythrocyte Cu,Zn-superoxide dismutase and activity of the monomer subunit: Effects of urea, temperature, and enzyme concentration. *J. Biochem.* **115**, 507-515
46. Inouye, K., Izawa, S., Saito, A., and Tonomura, B. (1995) Effects of alcohols on the hydrolysis of colominic acid catalyzed by *Streptococcus* neuraminidase. *J. Biochem.* **117**, 629-634