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Effect of Salts on the Solubility of Thermolysin: A Remarkable Increase in the Solubility as Well as the Activity by the Addition of Salts without Aggregation or Dispersion of Thermolysin[1]

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Thermolysin is remarkably activated in the presence of high concentrations (1-5 M) of neutral salts [Inouye, K. (1992) *J. Biochem.* 112, 335-340]. The activity is enhanced 13-15 times with 4 M NaCl at pH 7.0 and 25°C. In this study, the effect of neutral salts on the solubility of thermolysin has been examined. Although the solubility was only 1.0-1.2 mg/ml in 40 mM Tris-HCl buffer, pH 7.5, in the temperature range between 0 and 60°C, it was increased greatly by the addition of salts. With NaCl, the solubility showed a bell-shaped behavior with increasing NaCl concentration, and the maximum solubility (10 mg/ml) was at 2.0-2.5 M NaCl. With LiCl and NaI, it increased progressively to 20-50 mg/ml with increasing salt concentration up to 5 M. The solubility observed in the presence of salts decreased with increasing temperature from 0 to 60°C, and also with the order of chaotropic anion effect. The molecular weight of thermolysin was estimated to be $33.0(\pm 2.5)\times 10^3$ in the presence of 0-3 M NaCl, suggesting that thermolysin exists as a monomer in the presence or absence of 3 M NaCl. The possibility that aggregation and/or dispersion of thermolysin might be related to the remarkable activation by salt was ruled out.

Key words: halophilicity, metalloproteinase, salt-activation, solubility, thermolysin.

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 involving hydrophobic amino acid residues. The amino acid sequence (*6, 7*) and three-dimensional structure (*8*) are available, and the kinetic mechanism of the reaction has been proposed (*9, 10*).

We have previously reported remarkable 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 (*11*), and in the hydrolysis of *N*-[3-(2-furyl)acryloyl] (FA)-dipeptide amides with different amino acids at the scissile bond as well (*12*). The activation is brought about most effectively by NaCl and NaBr, and the activity increases in an exponential fashion with increasing salt concentration. The molecular activity, k_{cat}, and Michaelis constant, K_m, can be evaluated separately in the cases of ZAPM, FA-L-Leu-L-Ala amide, and FA-L-Phe-L-Ala amide, and the activation has been demonstrated to be induced solely by an increase in k_{cat} (*11, 12*). 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, 13*). The degree of activation shows a bell-shaped pH dependence with the optimum pH around 7 and it decreases significantly with increase of the temperature and with increasing alcohol concentration added to the reaction medium (*14*). Accordingly, the salt-dependent activation might be related to the electrostatic interaction of thermolysin with ions in the medium. The states of tyrosyl residues of thermolysin have been estimated by means of nitration and pH-dependent ionization, and the degree of activation of thermolysin by NaCl is demonstrated to be dependent on the ionization states of tyrosyl residues (*15*). Recently, we have demonstrated that the thermal stability of thermolysin is also increased by adding high concentration of NaCl without accompanying detectable conformational change (data to be published). Therefore, the effects of NaCl on the activity and stability are considered to be independent. It is possible that the activation of thermolysin could be derived from aggregation or dispersion of thermolysin in the presence of high concentrations of salts. However, in the present paper, it is demonstrated that the solubility of thermolysin is rather low in common buffers of low ionic strength, but increases markedly by adding salts without either aggregation or dispersion of thermolysin. The increase in the solubility, as well as the activity and stability, in the presence of salts could be beneficial for the industrial application of ther-

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

molysin, *e.g.* in thermolysin-assisted peptide synthesis.

MATERIALS AND METHODS

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*). FAGLA (Lot 370513) was obtained from the Peptide Institute (Osaka), and the concentration was determined spectrophotometrically using a molar absorption coefficient, $\varepsilon_{345}=766\ M^{-1}\cdot m^{-1}$ (*11*). All other reagents were of reagent grade, purchased from Nacalai Tesque (Kyoto). All spectrophotometric measurements were carried out with a Shimadzu UV-2200 spectrophotometer.

Hydrolysis of FAGLA—Hydrolysis of FAGLA by thermolysin was performed in the standard buffer containing 0-5 M neutral salts. The enzyme solution (0.1 ml) was added to the substrate solution (2.0 ml) in a cuvette to give the final concentrations of [thermolysin] = 98.0 nM and [FAGLA] = 0.12-12.0 mM, and the hydrolysis was measured by following the decrease in absorbance at 345 and 322 nm (*11*). The molar absorption differences $\Delta\varepsilon_{345}$ and $\Delta\varepsilon_{322}$ on the hydrolysis were -4.93×10^2 and $-2.05\times10^3\ M^{-1}\cdot cm^{-1}$, respectively, in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5 (standard buffer) at 25°C. These values were confirmed not to be changed by the addition of salts up to 5 M. The reaction was carried out at substrate concentrations lower than the respective Michaelis constant K_m values (pseudo-first order conditions) because of the low solubility of FAGLA. The first-order rate constant for FAGLA hydrolysis equals $(k_{cat}/K_m)[E]_o$, where $[E]_o$ is the total enzyme concentration, allowing us to estimate the specificity constant k_{cat}/K_m.

Solubility of Thermolysin—An excess amount of lyophilized thermolysin powder was mixed at 25°C with the standard buffer containing 0-5 M neutral salt and incubated for 1 h at a temperature between 0 and 60°C with gentle stirring. The mixture was then filtered with a Millipore membrane filter, Type HA (pore size: 0.45 μm), at the same temperature. The concentration of thermolysin in the filtrate was determined spectrophotometrically as described above, and was defined as the solubility of thermolysin.

Estimation of the Molecular Weight of Thermolysin in the Presence of NaCl—The molecular weight of thermolysin in the presence of 0-3 M NaCl was estimated with a Tosoh LS-8000 low-angle laser light scattering photometer (Tokyo), after elution from a high-performance liquid chromatography (HPLC) column in the standard buffer at 25°C (*16, 17*). A hundred microliters of the thermolysin solution (1.00 mg/ml) was injected using a CCPM solvent-delivery system into a column system, in which a TSK SW guard column, and a TSKgel G3000SW column and a TSKgel G2000SW column [size of both columns: 7.5 mm (inner diameter) × 60 cm] were connected in tandem. The flow-rate was 0.3 ml/min, and the elution was monitored first by a low-angle laser light scattering photometer, and then by a differential refractometer (TSK RI-8020). Calibration lines were obtained with bovine serum albumin (66.0 kDa), ovalbumin (42.5 kDa), and β-lactoglobulin (36.8 kDa) as standard proteins.

RESULTS AND DISCUSSION

Effect of Salts on the Hydrolysis of FAGLA—The effect of neutral salts on the reaction rate of thermolysin-catalyzed hydrolysis of FAGLA was examined. Figure 1 shows dependence on the salt concentration of the relative activity for the hydrolysis of FAGLA in the standard buffer at 25°C. The k_{cat}/K_m value at 0 M salt was $2.2\times10^4\ M^{-1}\cdot s^{-1}$. The activity increased in an apparently exponential fashion with increasing concentration of NaCl, NaBr, KCl, and KBr, and it seemed to increase further with increasing salt concentration as long as the solubility of the salts permitted. As has been demonstrated in the activation of thermolysin-catalyzed hydrolysis by NaCl (*12*), the $\log(k_{cat}/K_m)$ values increased linearly with an increase in the concentration of NaBr, KCl, and KBr as well (data not shown). The activity (k_{cat}/K_m) at x M salt is expressed as

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

where x and 0 refer to the salt concentrations of x and 0 M, respectively, and a is the slope of the straight line in the plot of $\log(k_{cat}/K_m)$ against the salt concentration. Equation 1 can be converted to

$$(k_{cat}/K_m)_x/(k_{cat}/K_m)_0=(10^a)^x=\alpha^x \qquad (2)$$

where 10^a is expressed as α. Equation 2 indicates that the degree of activation at x M salt, $(k_{cat}/K_m)_x/(k_{cat}/K_m)_0$, is

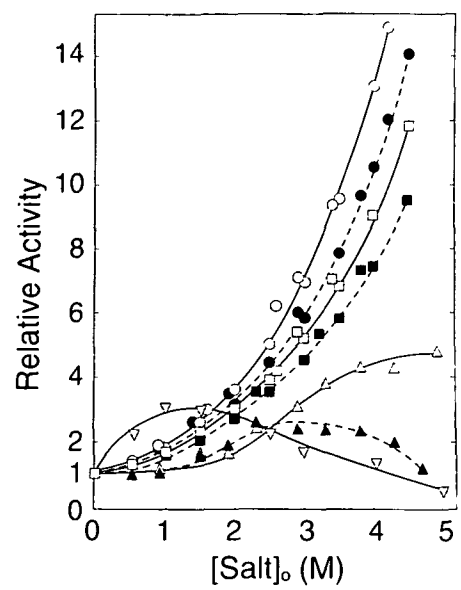

Fig. 1. **Effect of salts on the thermolysin activity in the hydrolysis of FAGLA.** The reaction was carried out in the standard buffer (40 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.5) containing various salts as indicated. The final concentration of thermolysin was 98.0 nM. The relative k_{cat}/K_m value obtained in the presence of salt to that at 0 M salt was plotted on the ordinate. The k_{cat}/K_m value at 0 M salt is $2.2\times10^4\ M^{-1}\cdot s^{-1}$. Salts examined are NaCl, ○; NaBr, ●; KCl, ⁻; KBr, ■; LiCl, △; LiBr, ▲; and NaSCN, ▽.

equal to $(10^a)^x$. In the case of NaCl, the value was already reported to be 1.90^x (*12*). Based on the data in Fig. 1, the degrees of activation at x M salt were calculated to be 1.80^x, 1.73^x, and 1.65^x, for NaBr, KCl, and KBr, respectively. On the other hand, the dependence of the thermolysin activity on [LiCl] showed a saturating tendency at concentrations higher than 4 M. The thermolysin activity showed a bell-shaped behavior depending on [LiBr] and [NaSCN] with the maximal activity at around 3 and 1 M, respectively, and the activity was even suppressed at 5 M. Difference of the activation behavior by lithium salts from that by sodium and potassium salts could be a clue to the activation mechanism of thermolysin.

It is noteworthy that the effectiveness of monovalent cations on the activation of thermolysin was in the order of $Na^+ > K^+ > Li^+$ and that of anions was $Cl^- > Br^-$ (Fig. 1). The α values for chloride salts (NaCl and KCl) are 1.05-1.06 times larger than those of the corresponding bromide salts (NaBr and KBr), and similarly, the α values for sodium salts (NaCl and NaBr) are 1.09-1.10 times larger than those of the corresponding potassium salts (KCl and KBr), suggesting that the effect of salts on the activation of thermolysin may be multiplier effects of the respective ions.

As will be mentioned below, NaSCN was the most effective in increasing the solubility of thermolysin (see Fig. 4). The dependence of the thermolysin activity on [NaSCN] was bell-shaped (Fig. 1). The maximum activity observed at around 1 M NaSCN was only three times as high as the activity observed in the absence of NaSCN, although the solubility of thermolysin was 73 mg/ml at 1.0 M NaSCN, being 61 times higher than that in the absence of the salt. There seems to be no correlation between the effects of salts on the activity and solubility of thermolysin.

Effects of Salts on the Solubility of Thermolysin—The dependence of the solubility of thermolysin on the concentration of various salts such as NaCl, KCl, LiCl, NaBr, and NaI at 0 and 37°C is shown in Fig. 2. Values of the solubility in the standard buffer were 1.2 and 1.0 mg/ml at 0 and 37°C, respectively. Considering the isoelectric point, pI = 5.1, of thermolysin (*14*), thermolysin is sparingly soluble as compared with common water-soluble proteins such as albumin. All salts examined, however, increased the solubility. The dependence of the solubility on [NaCl] and [KCl] showed a bell-shaped behavior with the maximum solubility at 2-2.5 M salts. In the case of LiCl, the solubility increased progressively with increasing salt concentration up to 5 M. When 3 M NaBr or NaI was added, the solubility reached as much as 40 mg/ml. With all salts examined, the effects were larger at 0 than at 37°C. The solubility of thermolysin was shown to depend greatly on the species and concentration of the salt added.

The temperature-dependence of the solubility of thermolysin was examined between 0 and 60°C (Fig. 3). The solubility in the absence of salt was not substantially dependent on temperature, and was 1.0-1.2 mg/ml. On the other hand, it decreased with increasing temperature in the presence of NaCl. It was 9.4 mg/ml at 0°C and decreased to 5.5 mg/ml at 60°C at 3.0 M NaCl, and was 5.0 mg/ml 0°C and decreased to 1.8-2.0 mg/ml at 37-60°C at 4.5 M NaCl. In the presence of NaCl, the solubility is larger at lower temperature than higher one, suggesting that thermolysin is a so-called cold-soluble protein.

The effects of 1.0 M sodium salts on the solubility of thermolysin were compared (Fig. 4). Salts were placed in the order of chaotropic ion effect of their anions. Chaotropic ion effect can be defined as the ability of ions to break the intermolecular network of water molecules and to increase chaos in a solution. The solubility of thermolysin increased

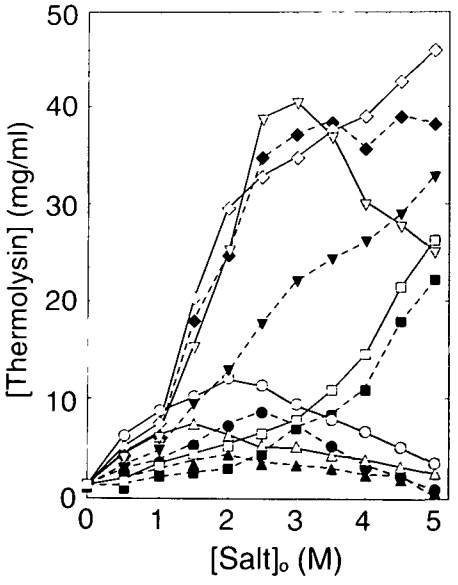

Fig. 2. **Effect of salts on the solubility of thermolysin.** An excessive amount of thermolysin lyophilized powder was mixed with the standard buffer containing salts of the concentration indicated at 0°C (open symbols) and 37°C (closed symbols) for 1 h, and the mixture was filtered with a Millipore membrane filter type HA. The concentration of thermolysin in the filtrate was regarded as the solubility, which is plotted on the ordinate. Salts examined are NaCl, ○ and ●; KCl, △ and ▲; LiCl, □ and ■; NaBr, ▽ and ▼; and NaI, ◇ and ◆.

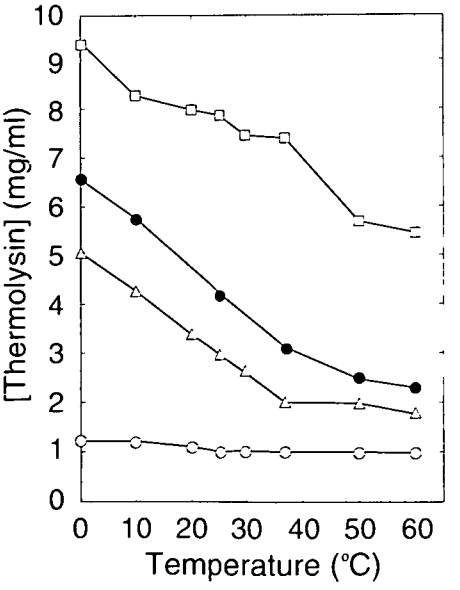

Fig. 3. **Temperature-dependence of the solubility of thermolysin.** The solubility was determined in the presence of NaCl by the method described in the legend to Fig. 2. The NaCl concentrations were 0 M, ○; 3.0 M, □; 4.0 M, ●; and 4.5 M, △.

almost in parallel with the order of chaotropic ion effect. Higher solubility at 0°C in comparison with that at 37°C might be explained by the fact that the chaotropic ion effect is enhanced at lower temperature. With NaSCN at 0°C, the solubility amounted to 73 mg/ml, equal to that of albumin, which has the highest solubility under physiological conditions. NaSCN is known to decrease the conformational stability of macromolecules and to have a salting-in effect, like KSCN (*18*). SCN^- ions might bind to thermolysin and increase the net charge of the protein; this might increase the electrostatic free energy of the protein, and the resulting repulsive forces would increase the solubility of the protein in aqueous solutions, as has been discussed for albumin (*18*). Addition of 1.0 M NaSCN increased the activity of thermolysin in the FAGLA hydrolysis by 3 times compared to that in its absence. Above 1.0 M, the activity gradually decreased and at 5 M NaSCN it was lower by half than that obtained in the absence of the salt. NaSCN, which led to a marked increase in the solubility of thermolysin, inhibited the activity of the enzyme at higher concentration (Fig. 1). It should be noted that the effectiveness of cations on the activation of thermolysin is in the order of $Na^+ > K^+ > Li^+$, which is different from that of the ionic radius of the monovalent cations, $K^+ > Na^+ > Li^+$, and that of Hofmeister's series, $K^+ < Na^+ < Li^+$, suggesting that the activation of thermolysin is induced by specific interaction between the ions and the enzyme.

The enthalpy of hydration of each anion is plotted in Fig. 4 in an attempt to find a quantitative relation between the hydration of ions and their effects on the solubility (*19*). However, as the order of chaotropic ion effect is not explained by the enthalpy of hydration of anions, there seems to be no relation between the solubility and the hydration of ions. According to Melander and Horváth (*20*), the solubility of proteins should depend on the hydrophobic surface properties of the proteins. They analyzed the salt-dependence of the protein solubility by evaluating the dual effect of salt on the electrostatic and hydrophobic interactions, and found that protein solubility in aqueous salt solutions could be explained in terms of the surface tension of the solvent, as there was a good correlation between the molal surface tension increments of salts and the anionic lyotropic series. The solvent-accessible surface area of thermolysin was evaluated by using an empirical equation suggested from the data of 46 monomeric proteins (*21, 22*). The fractions of the solvent-accessible surface area contributed by non-polar, polar, and charged groups are estimated to be 0.55, 0.31, and 0.14, respectively, and these values show little deviation from the values obtained for other proteins (*22*), and can be applied commonly to proteins. Thus, no characteristic point was found on the solvent-accessible surface area of thermolysin. As far as we know, a protein proteinase inhibitor, *Streptomyces* subtilisin inhibitor is a protein of low solubility (*23*). The solubility is 1-2 mg/ml at room temperature. This protein is a typical cold-soluble one, and the solubility increases to 5 mg/ml at 0°C. The inhibitor is a homodimer of a compact subunit of 11.5 kDa. Although there is no characteristic similarity between the structures of the inhibitor and thermolysin, it should be noted that thermolysin is composed of two compact domains of almost the same size. Therefore, it can be regarded as similar to an intramolecular dimer. The values of isoelectric point (pI) of *Streptomyces* subtilisin inhibitor and thermolysin are 4.3 and 5.1, respectively (*24*). The compact dimeric structure and low pI value might be related to the low solubility and cold-soluble property of the proteins.

Estimation of the Molecular Weight of Thermolysin in the Presence of NaCl—Thermolysin is known to be a monomeric protein in a dilute buffer solution such as the standard buffer, and there has been no evidence reported so far regarding its oligomerization. On the other hand, the solubility in the buffer is low for a water-soluble protein

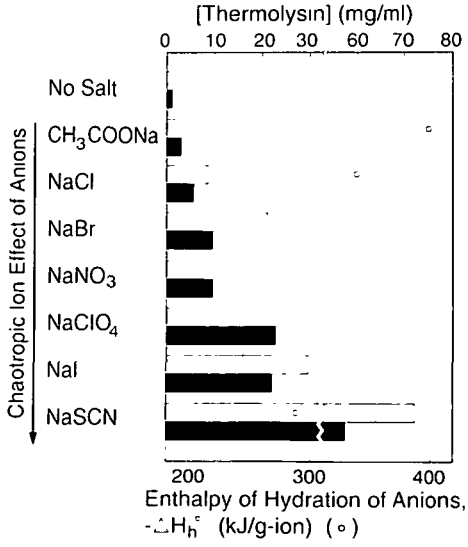

Fig. 4. **Dependence of the solubility of thermolysin on the presence of 1.0 M sodium salts.** The solubility was determined at 0°C (open bars) and 37°C (closed bars) by the method described in the legend to Fig. 2. Salts were placed in the order of chaotropic anion effect. Values of enthalpy of hydration of anions, $-H_h°$ (shown by open circles) were taken from Ref. *19*.

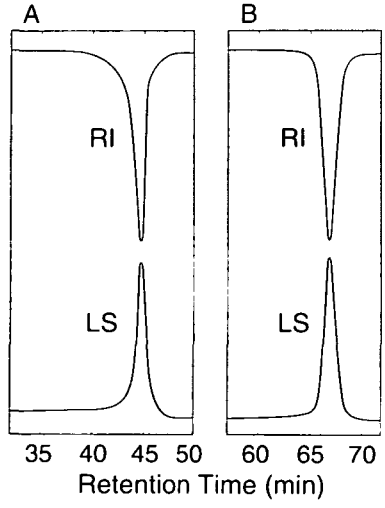

Fig. 5. **Estimation of molecular weight of thermolysin by a low-angle laser light scattering photometer.** Chromatograms of thermolysin by HPLC using a TSKgel G3000SW + G2000SW column system were obtained using refractive index (RI) and laser light scattering (LS) detectors. Thermolysin solutions of 1.00 mg/ml in the standard buffer containing 0 and 3 M NaCl at 25°C were injected into the column system. A: [NaCl] = 0 M, and B: [NaCl] = 3.0 M.

TABLE I. **Estimation of the molecular weight of thermolysin by a low angle laser light scattering photometer.**

NaCl (M)	Peak position (min)	h(LS)/h(RI)	Molecular weight
0	44.2	1.73	30,500
0.5	39.8	1.76	35,500
1.0	39.6	1.70	33,300
2.0	42.2	1.63	33,400
3.0	67.2	1.60	33,900

A thermolysin solution of 1.00 mg/ml in 40 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5 (standard buffer) containing NaCl at the concentration indicated was applied to the measurement at 25°C. The molecular weight of thermolysin calculated from the amino acid and metal composition is 34,600 (*6*).

(Figs. 2-4), raising the possibility that thermolysin could be in an aggregated-and-dispersed form in such a concentrated salt solution as 1-5 M NaCl. In order to clarify this point, the molecular weight of thermolysin was measured in the presence of 0-3 M NaCl by using a low-angle laser light scattering photometer (Fig. 5, Table I). When RI stands for detection by refractive index and LS, detection by laser light scattering; h is the peak height of HPLC; M, molecular weight; and i indicates molecular species i, the following relation holds:

$$h(\mathrm{LS})_i/h(\mathrm{RI})_i = K\,M_i \qquad (3)$$

where K is a mechanical constant. The value of $h(\mathrm{LS})_i/h(\mathrm{RI})_i$ for the standard proteins observed in the presence of various concentrations of NaCl was plotted against the molecular weight, M_i (data not shown). The plot was linear through the origin. The constant K at each NaCl concentration was evaluated, and introduced into Eq. 3 to obtain the molecular weight of thermolysin. At any NaCl concentration, the molecular weight of thermolysin was in the range of $30.0\text{-}34.0\times10^3$, and the average was estimated to be $33.0(\pm2.5)\times10^3$. Because the molecular weight of thermolysin calculated from the amino acid and metal composition is 34.6×10^3 (*6*), it is clear that thermolysin exists in a monomer form in the presence of 0-3 M NaCl. The effect of NaCl on the solubility of thermolysin thus has nothing to do with aggregation or dispersion of the enzyme molecules.

Neutral salts were shown to increase not only the solubility of thermolysin but also its activity. Addition of these salts enables us to make an enzyme solution of high concentration with enhanced activity. Such a solution could reduce the reaction-time of the enzyme reaction, which would be beneficial especially in industrial applications of the enzyme, *e.g.*, enzymatic synthesis of ZAPM, a precursor of aspartame. By using thermolysin solution of high concentration prepared by adding a high concentration of salts, a high enzyme activity can be attained in the reaction vessel. NaCl and NaBr are the most effective in the activation of thermolysin. The activity for FAGLA hydrolysis is enhanced 13 times at 25°C and 10 times at 37°C by adding 4 M NaCl, and 10 times at 25°C with 4 M NaBr (Fig. 1). The respective values of thermolysin solubility at 4 M NaCl and NaBr are 4.2 and 34.2 mg/ml at 25°C (Figs. 2 and 3). The solubility in the absence of salts is 1.0 mg/ml. Salt activation of thermolysin has been shown to be brought about only by increase in the molecular activity, k_{cat}, while the Michaelis constant, K_m, is not altered. The total activity of thermolysin, v, is given by the Michaelis-Menten equation: $v=\{k_{cat}\cdot[E]_o\cdot[S]_o\}/\{K_m+[S]_o\}$, which can be expressed as $(k_{cat}/K_m)\cdot[E]_o\cdot[S]_o$ under pseudo-first order conditions at the initial substrate concentration $[S]_o$. Accordingly, when we use maximally solubilized thermolysin solution with 4 M NaCl and NaBr, the total activity, v, in the reaction mixture could be enhanced 55 and 360 times, respectively, compared with that in the absence of salts. In the case of 1 M NaSCN, the solubility is 88 mg/ml at 25°C, and the activity enhancement is 3.2 times. Thus, the total activity could be enhanced 280 times. Industrial application of thermolysin has been limited because of its sparing solubility. This limitation can be overcome by adding a high concentration of salts. This would enable us to perform the reaction in a much reduced time, minimizing side reactions and decomposition of the products and substrates, and reducing operating costs as well.

REFERENCES

1. Endo, S. (1962) Studies on protease produced by thermophilic bacteria (in Japanese). *J. Ferment. Technol.* **40**, 346-353
2. Matsubara, H. and Feder, J. (1971) Other bacterial, mold, and yeast proteases 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) Thermolysin: 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. *amylosaccharitiicus* neutral protease. *Eur. J. Biochem.* **64**, 243-247
6. Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A., and Neurath, H. (1972) Amino-acid sequence of thermolysin. *Nature* **238**, 35-37
7. O'Donohue, M.J., Roques, B.P., and Beaumont, A. (1994) Cloning and expression in *Bacillus subtilis* of the *npr* gene from *Bacillus thermoproteolyticus* Rokko coding for the thermostable metalloprotease thermolysin. *Biochem. J.* **300**, 599-603
8. Holmes, M.A. and Matthews, B.W. (1982) Structure of thermolysin refined at 1.6 Å resolution. *J. Mol. Biol.* **160**, 623-639
9. 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
10. 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
11. 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
12. 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
13. 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
14. Inouye, K., Lee, S.-B., Nambu, K., and Tonomura, B. (1997) Effects of pH, temperature, and alcohols on the remarkable activation of thermolysin by salts. *J. Biochem.* **122**, 358-364
15. 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
16. Takagi, T. (1982) Confirmation of molecular weight of *Aspergillus oryzae* α-amylase using the low angle laser light scattering technique in combination with high pressure silica gel chromato-

graphy. *J. Biochem.* **89**, 363-368
17. Arakawa, T., Langley, K.E., Kameyama, K., and Takagi, T. (1997) Molecular weights of glycosylated and nonglycosylated forms of recombinant human stem cell factor determined by low-angle laser light scattering. *Anal. Biochem.* **203**, 53-57
18. Arakawa, T. and Timasheff, S.N. (1982) Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry* **21**, 6545-6552
19. Ohtaki, H., Tanaka, M., and Funahashi, S. (1977) *Youeki Hannou no Kagaku* (in Japanese), pp. 215-216, Gakkai Shuppan Center, Tokyo
20. Melander, W. and Horváth, C. (1977) Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the lyotropic series. *Arch. Biochem. Biophys.* **183**, 200-215
21. Chothia, C. (1975) Structural invariants in protein folding. *Nature* **254**, 304-308
22. Miller, S., Janin, J., Lesk, A.M., and Chothia, C. (1987) Interior and surface of monomeric proteins. *J. Mol. Biol.* **196**, 641-656
23. Inouye, K., Tonomura, B., Hiromi, K., Kotaka, T., Inagaki, H., Sato, S., and Murao, S. (1977) The determination of molecular weights of *Streptomyces* subtilisin inhibitor and the complex of *Streptomyces* subtilisin inhibitor and subtilisin BPN′ by sedimentation equilibrium. *J. Biochem.* **84**, 843-853
24. Inouye, K. (1991) Chromatographic behaviors of proteins and amino acids on a gel filtration matrix, TSK-GEL Toyopearl. *Agric. Biol. Chem.* **55**, 2129-2139