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Inhibitory Effects of Alcohols on Thermolysin Activity as Examined Using a Fluorescent Substrate[1]

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Alcohols inhibit the thermolysin-catalyzed hydrolysis of *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu-NH_2 and decrease the NaCl-induced activation of thermolysin in a concentration-dependent manner [K. Inouye *et al.* (1997) *J. Biochem.* 122, 358–364]. In this study, the inhibitory effects of alcohols on thermolysin activity were examined in detail using 10 different alcohols and a fluorescent substrate, (7-methoxycoumarin-4-yl) acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2. The inhibition by all alcohols examined is completely reversible, and thermolysin activity is recovered by dilution. The inhibitor constants (K_i) are in the range of 35–430 mM, and the order of the inhibitory effect is 1-pentanol, 1-propanol, 2-butanol, 2-methyl-1-propanol > 1-butanol > 2-propanol > ethanol, *tert*-amyl alcohol ≫ *tert*-butyl alcohol ≫ methanol. Linear and secondary alcohols whose mains chains consist of more than 3 carbons inhibit thermolysin effectively. Thermolysin activity is decreased by decreasing the dielectric constant, *D*, of the reaction medium containing the alcohol, and the decrease depending on the *D* value was almost the same manner for all alcohols except methanol, *tert*-butyl alcohol, and *tert*-amyl alcohol. Alcohols may inhibit thermolysin activity both by binding to the active site, most possibly to the S1′ subsite, of thermolysin and by altering the electrostatic and hydrophobic environment around the thermolysin molecule.

Key words: alcohol, dielectric constant, inhibitor, metalloproteinase, thermolysin.

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (*1, 2*). It requires 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 (*6, 7*). The amino acid sequence (*8, 9*) and three-dimensional structure (*10*) are known, and the reaction mechanism has been proposed (*11, 12*).

We previously reported the remarkable activation as well as stabilization of thermolysin by high concentrations (1–4 M) of neutral salts (*13–15*). The peptidase activity of thermolysin increases in an exponential fashion with increasing salt concentration, and the degree of activation at x M NaCl is expressed by 1.9^x (*7*). The activation is brought about solely by an increase in k_{cat}, and the Michaelis constant is not altered (*7, 13*), although a report appeared later that suggested that the activation mechanism might be different depending on the substrate examined (*16*). In the case of *N*-[3-(2-furyl)acryloyl] (FA)-glycyl-L-leucine amide (FAGLA) as a substrate, the degree of activation induced by NaCl shows a bell-shaped pH dependence with an optimum pH around 7, and decreases significantly with increasing temperature. Alcohols inhibit the thermolysin-catalyzed hydrolysis of FAGLA, and the activity decreases to 30% in the presence of 5% (v/v) methanol (*17*). The degree of activation by salt defined as (k_{cat}/K_m at 4 M NaCl)/(k_{cat}/K_m at 0 M NaCl) is 12 in the absence of alcohols, but decreases with increasing alcohol concentration in the reaction medium reaching 5 in the presence of 5% (v/v) methanol (*17*). It is obvious that alcohols attenuate the activating effect of salts. Thermolysin is known to be a robust enzyme, and is resistant to denaturation thermally and by denaturants. To our knowledge, however, the sensitivity of thermolysin to alcohols as observed in the inhibition of FAGLA hydrolysis is one of the highest, and is of the same level reported for *Streptococcus* neuraminidase which is inhibited to 30% by 5% methanol, ethanol or 2-propanol (*18*). Considering that α-chymotrypsin and subtilisins are activated 4–5 times by 30% methanol (H. Nagai (1999) Master's Thesis, Kyoto University), the inhibition of thermolysin by alcohols as well as neuraminidase could be a key to their structure-activity relationship.

The objective of this study was to evaluate the inhibitory effects of alcohols on the peptidase activity of thermolysin

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Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; MMP, matrix metalloproteinase; MOCAc-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-leucyl-glycyl-L-leucyl-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-alanyl-L-arginine amide; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-leucyl-Gly; L(Dpa)AR, L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-alanyl-L-arginine amide; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

in detail and its mechanism. FAGLA has been used widely as a suitable substrate for thermolysin (*3–7, 13, 16*). The hydrolysis of FAGLA is measured by following the decrease in absorbance at 345 nm, and the amount of FAGLA hydrolyzed is estimated using the molar absorption difference on hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\cdot cm^{-1}$ (*13*). A difficulty in using FAGLA is that the value of the molar absorption difference ($\Delta\varepsilon_{345}$) must be confirmed or determined at the conditions used. In order to avoid this difficulty in the presence of various concentrations of various alcohols, a fluorescent substrate, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR] was applied in this study. This substrate has been commonly used for studies on matrix metalloproteinases (MMPs) and the peptide bond between glycine and leucine residues is known cleaved by MMPs (*19–21*). In the present study, we describe the possibility of using MOCAc-PLGL(Dpa)AR as a substrate for thermolysin. The thermolysin-catalyzed hydrolysis of this substrate is strongly inhibited by increasing alcohol concentrations, and the structure of the alcohol molecule and dielectric constant of the reaction medium play important roles in the inhibition. We will also discuss the size and shape of the alcohol binding site(s).

MATERIALS AND METHODS

Materials—A three times crystallized and lyophilized preparation of thermolysin (Lot T5CB491; 8,360 PU/mg according to the supplier) was purchased from Daiwa Kasei (Osaka). The preparation was used without further purification. The thermolysin solution was filtered through a Millipore membrane 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 (*13*) and a molecular mass of 34.6 kDa (*8*).

A fluorescent substrate, MOCAc-PLGL(Dpa)AR (Lot 490418, 480429), and MOCAc-L-prolyl-L-leucyl-glycine (MOCAc-PLG, Lot 471218), were purchased from the Peptide Institute (Osaka), and their concentrations were determined using the molar absorption coefficients $\Delta\varepsilon_{410} = 7.5\ M^{-1}\cdot cm^{-1}$ and $\Delta\varepsilon_{324} = 12.9\ M^{-1}\cdot cm^{-1}$, respectively (*19, 22, 23*). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto).

The HPLC apparatus consisted of a solvent-delivery system CCPM-II, a UV monitoring system UV-8020, a computer-control system PX-8020, a degasser SD-8020, a column oven CO-8020 and an integrator Chromatocorder 21, and was purchased from Tosoh (Tokyo).

HPLC of the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The hydrolysis of MOCAc-PLGL(Dpa)AR by thermolysin was detected by HPLC to confirm the cleavage site following the method developed for MMP (*20*). The reaction was initiated by adding 30 μl of the substrate solution (670 μM) dissolved in DMSO to 970 μl of the thermolysin solution (19.1 nM) in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ at pH 7.5. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR in the reaction solution were 18.6 nM and 20 μM, respectively. The reaction was stopped by mixing 100 μl of the reaction solution with 400 μl of 1% trifluoroacetic acid (TFA). The mixture (100 μl) was applied to HPLC on a TSKgel ODS-80Ts column [4.6 mm (inner diameter) × 150 mm](Tosoh) equilibrated with 0.1% TFA (*20*). A linear gradient was generated from 20 to 70% acetonitrile from 5 min for 15 min at a flow-rate of 1 ml/min. The absorption at 335 nm was measured at 25°C.

Fluorometric Analysis of the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was performed by mixing 1,242 μl of the thermolysin solution in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ with 8 μl of MOCAc-PLGL(Dpa)AR (234 or 117 nM) dissolved in DMSO at pH 7.5, 25°C. The hydrolysis of MOCAc-PLGL(Dpa)AR was measured by following the increase in fluorescence intensity at 393 nm with excitation at 328 nm using a JASCO FP-777 fluorescence spectrophotometer (Tokyo). The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 10 nM and 1.5 μM (or 0.75 μM), respectively. The reaction was carried out under pseudo-first order conditions, where the substrate concentration is much lower than the K_m value, in order to avoid absorptive quenching effects (*21, 22*). The initial reaction rate, v, of the production of MOCAc-PLG was evaluated by comparing the fluorescence intensity with that of an authentic MOCAc-PLG (0.44 μM) solution. Activity is expressed by the specificity constant (k_{cat}/K_m), which was determined by the method of Sakoda and Hiromi (*24, 25*).

Effects of Alcohols on the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—A fluorometric analysis of the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was performed in the presence or absence of alcohols (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, *tert*-butyl alcohol, 1-pentanol, and *tert*-amyl alcohol). The effect of 1,4-dioxane was also examined. The reaction was carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.6% DMSO at 25°C. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 10 nM and 1.5 μM (or 0.75 μM), respectively. The inhibitory effects of the alcohols were evaluated by Dixon plot to determine the inhibitor constant, K_i (*22, 25*).

Evaluation of the Dielectric Constant of the Reaction Mixture—The dielectric constant (D) of the reaction mixture was determined by the following equation (*17*):

$$D = f_W \cdot D_W + f_a \cdot D_a + f_D \cdot D_D \quad (1)$$

where f_W, f_a, and f_D are the volume fractions of water, alcohol, and DMSO, respectively (and thus $f_W + f_a + f_D = 1$), and D_W, D_a, and D_D are their respective dielectric constants (*26*).

Dilution of the Alcohol—The effect of dilution after incubating thermolysin with an alcohol was examined at final concentrations of thermolysin and MOCAc-PLGL(Dpa)AR of 10 nM and 1.5 μM, respectively. In the case of methanol, thermolysin (100 nM) in 50 mM Tris-HCl plus 10 mM $CaCl_2$ buffer containing 3.0% methanol was incubated at 25°C for 15 min, and then diluted 10 times with buffer without methanol. The thermolysin activity was measured immediately after the dilution by fluorometric analysis. For comparison, a thermolysin solution (100 nM) containing 0.3% methanol was incubated at 25°C for 15 min, diluted 10 times with buffer containing 0.3% methanol, and the enzyme activity was measured. *Tert*-butyl alcohol and *tert*-amyl alcohol were examined in the same way. In the case of other alcohols (ethanol, 1-propanol, 2-propanol, 1-butanol,

2-butanol, 2-methyl-1-propanol, and 1-pentanol), the alcohol concentration of the reaction mixture was 1.0% and after dilution was 0.1%.

RESULTS AND DISCUSSION

MOCAc-PLGL(Dpa)AR as a Substrate for Thermolysin—There has been no report so far in which MOCAc-PLGL(Dpa)AR was used as a substrate for thermolysin (*26, 27*). It was examined whether MOCAc-PLGL(Dpa)AR can be a thermolysin substrate. When the mixture of thermolysin and MOCAc-PLGL(Dpa)AR was applied to HPLC, two peaks, in addition to the peak of MOCAc-PLGL(Dpa)AR at 16.6 min, appeared at 6.2 and 13.4 min (Fig. 1). Their heights increased with incubation time of the mixture, while the height of the MOCAc-PLGL(Dpa)AR peak decreased, suggesting that the two newly observed peaks are products formed by the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. Using authentic compounds, the products eluting at 6.2 and 13.4 min were identified as L(Dpa)AR and MOCAc-PLG, respectively. Thus it has been confirmed that thermolysin cleaves the peptide bond between glycine and leucine in MOCAc-PLGL(Dpa)AR, in the same manner as it cleaves the Gly-Leu bond in FAGLA. The dependence of the reaction rate (v) on substrate concentration ($[S]_0$) was examined, and it was shown that the K_m value of thermolysin for MOCAc-PLGL(Dpa)AR is beyond the maximum solubility (*ca.* 90 μM) of the substrate under the conditions used. Thus the K_m value of thermolysin for MOCAc-PLGL(Dpa)AR is much larger than that of matrilysin, which has been determined to be 45 μM (*20*). Therefore, the following reaction was carried out under pseudo-first order conditions where the substrate concentration is much lower than the K_m value, to avoid its absorptive quenching effects (*21, 22*).

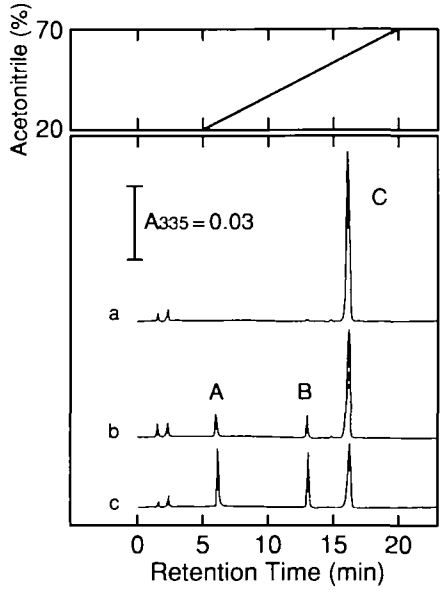

Fig. 1. **HPLC analysis of the products of MOCAc-PLGL-(Dpa)AR hydrolysis catalyzed by thermolysin.** The reaction was carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 3% DMSO at 25°C. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 18.6 nM and 20 μM, respectively. The reaction times were a, 0 min; b, 3 min; and c, 10 min. Separation and determination of the products were performed by HPLC with a TSKgel ODS-80Ts column. MOCAc-PLGL(Dpa)AR, MOCAc-PLG and L(Dpa)AR eluted at peaks C (16.6 min), B (13.4 min), and A (6.2 min), respectively.

Effect of Alcohol on the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The specificity constant (k_{cat}/K_m) of the hydrolysis of MOCAc-PLGL(Dpa)AR by thermolysin decreased with increasing concentrations of all alcohols examined (Fig. 2). From the Dixon plot for determining the inhibitor constant, K_i of thermolysin for 1-propanol (Fig. 3), K_i was determined to be 0.28% (v/v) or 38 mM. The two lines obtained at different substrate concentrations crossed apparently at a point in the second quadrant, suggesting that the mode of inhibition was judged to be competitive. For all alcohols examined other than 1-propanol, the linear lines also crossed at a point in the second quadrant, slightly above the $[I]_0$ axis (data not shown), and the mode of inhibition was judged barely to be competitive. However, it should be noted that competitive inhibition is hardly discriminated from non-competitive inhibition when the v value is much smaller than the V_{max} value, or when the K_i value is much smaller than a substrate constant (K_s) that is close to the K_m (*22, 28*). The reaction in the present study was performed under conditions where the substrate concentration was much lower than the K_m value, and thus

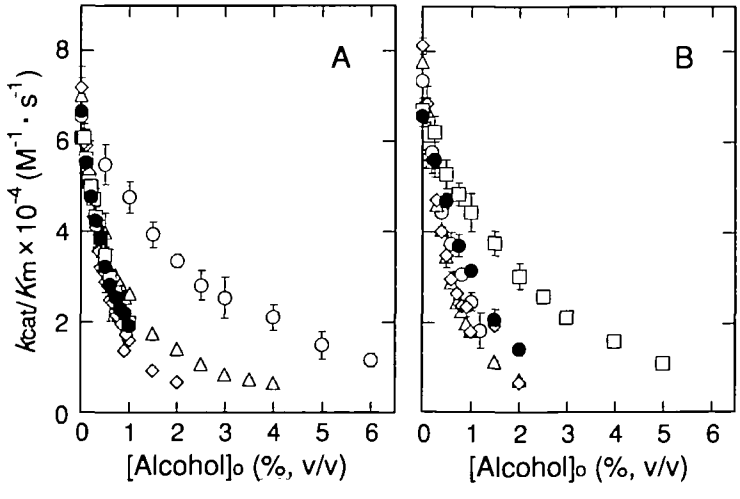

Fig. 2. **Dependence of the thermolysin activity on alcohol concentration.** The thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.6% DMSO at 25°C, and the specific constant, k_{cat}/K_m, was plotted against the alcohol concentration of the reaction mixture. Panel A: Effect of linear alcohols on thermolysin activity. Alcohols: methanol, ○; ethanol, △; 1-propanol, ◇; 1-butanol, □; and 1-pentanol, ●. Panel B: Effect of branched alcohols on thermolysin activity. Alcohols: 2-propanol, ○; 2-butanol, △; 2-methyl-1-propanol, ◇; *tert*-butyl alcohol, □; and *tert*-amyl alcohol, ●.

it is difficult to define the mode of inhibition. Consequently, the type of inhibition by the alcohols examined was considered to be competitive, which is consistent with the results of X-ray crystallography (*29*, see below), al-though the possibility of non-competitive inhibition cannot be excluded. Alcohols might bind to the active site of thermolysin and inhibit the enzyme competitively. Since thermolysin cleaves favorably peptides containing a hydropho-bic residue such as leucine or isoluecine at the P1′ position, and even at the P1 position (*6, 7*), it is reasonable that small alcohol molecules could fit into the S1′ and S1 pockets.

The K_i values of the alcohols examined ranged from 0.28 to 1.8% (v/v), or from 38 to 430 mM (Table I). In the case of linear alcohols, the order of inhibitory effect (when compared in molar concentration) is 1-propanol, 1-pentanol > 1-butanol > ethanol ≫ methanol. As the number of carbon atoms increases from 1 to 3, the inhibitory effect as evaluated by K_i is enhanced more than 10 times. When the number of carbons increases from 3 to 5,however, the inhibitory effect does not change much. This indicates that the length of 1-propanol is sufficient to give maximum inhibition. In the case of branched alcohols, 2-butanol and 2-methyl-1-propanol are the most effective inhibitors. When alcohols with the same number of carbons are compared, the inhibitory effect of 1-propanol is obviously stronger than that of 2-propanol, while 1-butanol is a little weaker than 2-butanol and 2-methyl-1-propanol. The inhibitory effect of bulky alcohols, such as *tert*-butyl alcohol and *tert*-amyl alcohol, is much weaker compared to other alcohols with the same number of carbons. The results suggest that there might be inhibitory binding site(s) for alcohols on the surface of thermolysin, and that the size of the site(s) could be large enough to accommodate 1-propanol (3 carbons) sufficiently to inhibit the thermolysin activity. As for branched alcohols, secondary alcohols are stronger inhibitors than linear alcohols except for 2-propanol. Tertiary alcohols seem to be too bulky to fit into the inhibitory binding site(s). Considering the inhibitory activities shown in Table I, it is suggested that the inhibitory binding site could be a pocket accommodating most suitably a carbohydrate moiety with a linear three-carbon chain and a branched methyl chain,

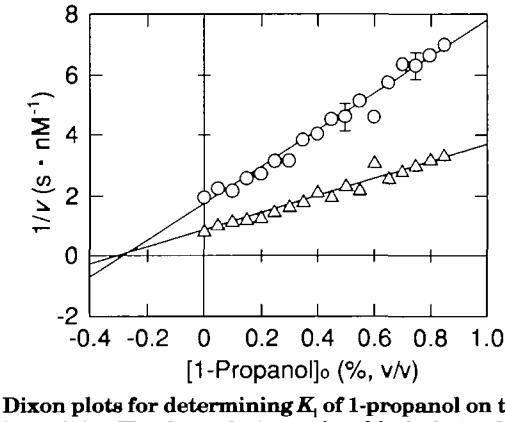

Fig. 3. **Dixon plots for determining K_i of 1-propanol on the thermolysin activity.** The thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.6% DMSO in the absence and presence of 1-propanol at 25°C. The initial concentration of thermolysin was 10 nM. The initial concentrations of MOCAc-PLGL(Dpa)AR were 0.75 μM, ○, and 1.5 μM, △. The 1-propanol concentration giving 50% of the activity observed in the absence of 1-propanol (IC_{50}) was 0.37%. The inhibitor constant, K_i, of 1-propanol was determined to be 0.28 ± 0.04% (v/v) or 38 ± 5 mM according to the value of the intersection point of the lines obtained at different substrate concentrations.

TABLE I. **The inhibitory effect of alcohols.** Numbers in parentheses indicate the number of carbons. The K_i values in volume percent (%, v/v) were evaluated from the Dixon plot to determine K_i (Fig. 3). The K_i values in molar concentration (mM) were calculated from the K_i values of volume percent and the molecular weight. Based on the results shown in Fig. 4, the alcohols could be classified into two groups. Group I: ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, and 1-pentanol. Group II: methanol, *tert*-butyl alcohol, and *tert*-amyl alcohol. The K_i values are shown with the standard deviations (SD).

Alcohol	Molecular weight	Inhibitor constant (K_i)	
		% (v/v)	mM
Methanol (1)	32.04	1.7 ± 0.2	430 ± 60
Ethanol (2)	46.07	0.60 ± 0.07	100 ± 10
1-Propanol (3)	60.10	0.28 ± 0.04	38 ± 5
2-Propanol (3)	60.10	0.50 ± 0.06	65 ± 8
1-Butanol (4)	74.12	0.46 ± 0.06	50 ± 6
2-Butanol (4)	74.12	0.37 ± 0.05	40 ± 5
2-Methyl-1-propanol (4)	74.12	0.38 ± 0.03	41 ± 3
tert-Butyl alcohol (4)	74.12	1.8 ± 0.3	190 ± 30
1-Pentanol (5)	88.15	0.38 ± 0.05	35 ± 4
tert-Amyl alcohol (5)	88.15	1.2 ± 0.2	110 ± 20

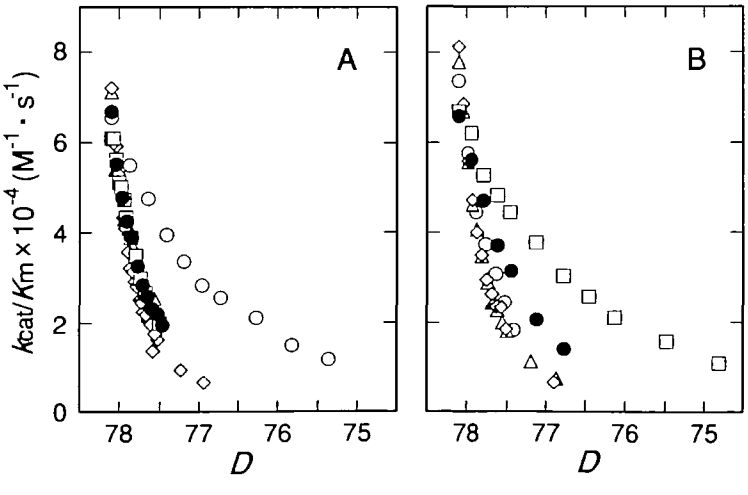

Fig. 4. **Dependence of the thermolysin activity on the theoretically evaluated dielectric constant, *D*, of the reaction mixture.** The *D* value of reaction mixture was evaluated from the following equation: $D = f_W \cdot D_W + f_a \cdot D_a + f_D \cdot D_D$, where f_W, f_a, and f_D are the volume fractions of water, the alcohol and the DMSO, respectively (and thus $f_W + f_a + f_D = 1$), and D_W, D_a, and D_D are their respective dielectric constants (*16*). The conditions for the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR are the same as those described in the legend to Fig. 3. Panel A: Effect of linear alcohols on thermolysin activity. Alcohols: methanol, ○; ethanol, △; 1-propanol, ◇; 1-butanol, □; and 1-pentanol, ●. Panel B: Effect of branched alcohols on thermolysin activity. Alcohols: 2-propanol, ○; 2-butanol, △; 2-methyl-1-propanol, ◇; *tert*-butyl alcohol, □; and *tert*-amyl alcohol, ●.

that is, similar to the side chains of leucine and isoleucine.

Effect of Dielectric Constant on the Reaction Medium—The specificity constants, k_{cat}/K_m, of thermolysin measured in the presence of various concentrations of each alcohol were plotted against the theoretically calculated dielectric constant, *D*, of the reaction medium (Fig. 4). The thermolysin activity and the *D* value of the reaction medium are in good correlation, and the lower the *D* value, the lower the activity. In the case of linear and branched alcohols, except for methanol, *tert*-butyl alcohol, and *tert*-amyl alcohol, the same *D* values give the same k_{cat}/K_m values. It is evident that the dielectric constant of the reaction medium is one of the important factors for the activity of thermolysin, and that a decrease in the dielectric constant suppresses the activity. It is interesting that the alcohols with weak inhibitory effects (methanol, *tert*-butyl alcohol and *tert*-amyl alcohol) showed a different behavior in the relationship between *D* value and thermolysin activity, suggesting that not only the *D* value but also the size and shape of the alcohol affects the inhibitory effects against thermolysin. Here, the alcohols examined can be classified into 2 groups: Group I comprises ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, and 1-pentanol; and Group II comprises methanol, *tert*-butyl alcohol, and *tert*-amyl alcohol.

In order to examine the effect of the *D* value, the effects of 1,4-dioxane (*D*: 2.1) and methanol (*D*: 32.6) were compared. The relative activity in the presence of 2.5% dioxane was 25% of that in the absence of the co-solvent (data not shown), and similar to that in the presence of 4.0% methanol. The *D* values of the buffer solutions containing 2.5% dioxane and 4.0% methanol were almost the same (*D*: 76.3), and no activity was observed at 4.0% dioxane. Accordingly, it has been shown that a co-solvent with a smaller *D* value inhibits thermolysin strongly at a lower concentration.

Effect of Dilution of Alcohol on the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—After incubation of thermolysin with an alcohol at 25°C for 15 min, the alcohol was diluted, and the effect of dilution was examined (Table II). The thermolysin activities in the presence of 0.3 and 3.0% methanol were 86 and 32%, respectively, of that obtained in the absence of methanol. When 3.0% methanol was diluted to 0.3%, the activity recovered to 87%. The effect of dilution of other alcohols was also examined, and the activities were shown to recover completely. Thus the inhibition of the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR by alcohols is reversible for the alcohol concentrations up to 3.0% for methanol, *tert*-butyl alcohol and *tert*-amyl alcohol, and up to 1.0% for ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol, and 1-pentanol.

Inhibitory Mechanism of Alcohols on the Thermolysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—According to the evidence that thermolysin activity is inhibited by alcohols, it appears that electrostatic and hydrophobic in-

TABLE II. **Effect of dilution of the alcohol on thermolysin activity.** The activity was measured at 10 nM thermolysin and 1.5 μM MOCAc-PLGL(Dpa)AR in 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ and 0.6% DMSO after dilution of the alcohol. The activity in absence of alcohol was taken as 100% relative activity. The reversibility is the percentage of activity recovered upon dilution (3.0→0.3% or 1.0→0.1%) of an alcohol compared to the activity at 0.3 or 0.1% of the alcohol. The activity measurements were carried out five times under each condition, and the relative activity is shown with the standard deviation (SD).

	Relative activity (%)			Reversibility (%)
	0.3%	3.0%	3.0→0.3%	
Methanol	86 ± 4	42 ± 1	87 ± 2	101
tert-Butyl alcohol	92 ± 2	41 ± 2	96 ± 3	105
tert-Amyl alcohol	76 ± 5	13 ± 1	79 ± 4	104
	Relative activity (%)			Reversibility (%)
	0.1%	1.0%	1.0→0.1%	
Ethanol	94 ± 5	46 ± 3	96 ± 1	101
1-Propanol	87 ± 3	28 ± 1	90 ± 2	103
2-Propanol	89 ± 4	41 ± 2	91 ± 2	102
1-Butanol	86 ± 5	34 ± 1	93 ± 4	108
2-Butanol	85 ± 2	29 ± 2	88 ± 8	103
2-Methyl-1-propanol	87 ± 5	26 ± 1	89 ± 1	103
1-Pentanol	88 ± 2	32 ± 2	92 ± 2	105

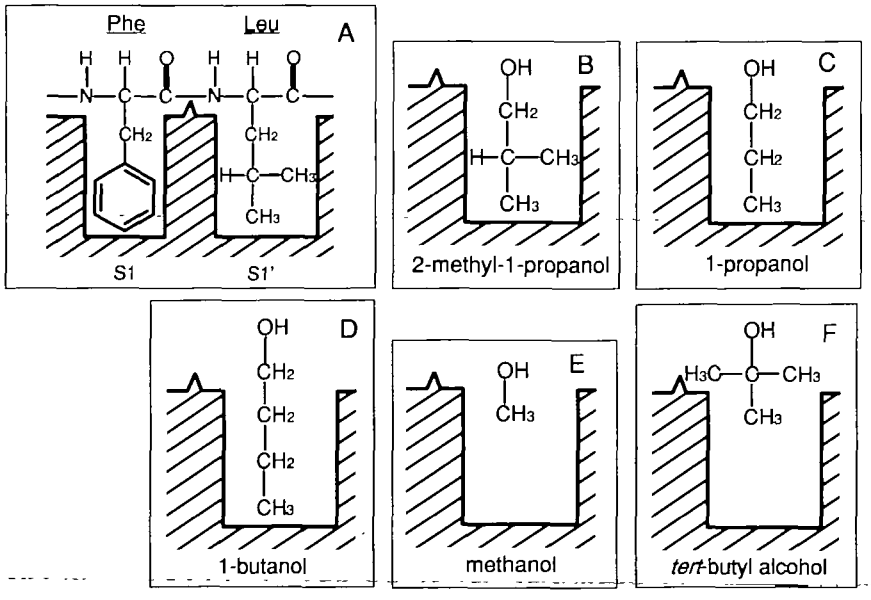

Fig. 5. **Schematic view of the binding of alcohols to the subsites of thermolysin.** Panel A: The S1 and S1′ subsites bind the side-chains of phenylalanine and leucine, respectively, most favorably (7). Panels B–F: Speculative binding modes of 2-methyl-1-propanol (B), 1-propanol (C), 1-pentanol (D), methanol (E), and *tert*-butyl alcohol (F). Methanol is too small and *tert*-butyl alcohol is too bulky to fit the S1′ subsite favorably. 2-Methyl-1-propanol is considered to bind the subsite as the side-chain of leucine does.

teractions between thermolysin and the substrate could play significant roles in the inhibition. The observation that alcohols in Group I show the same relationship between the inhibitory effect and the D value (Fig. 4) suggests that these alcohols inhibit thermolysin by the same mechanism, probably by interrupting the interaction of the substrate with the active site of the enzyme. It is noteworthy that alcohols in Group II show a different behavior from alcohols in Group I in the relationship between the inhibitory effect and the D value, suggesting that alcohols in Group II might inhibit thermolysin by different principles from those in Group I. The K_i values for alcohols (Table I) can be interpreted reasonably by assuming an alcohol-binding pocket that accommodates 1-propanol, 2-butanol, 2-methyl-1-propanol, and 1-pentanol. The seven alcohols in Group I may inhibit thermolysin by interacting favorably with the active-site pocket and also by changing the electrostatic and hydrophobic interactions on the surface of thermolysin and the bulk medium as the result of reducing the D value of the medium. On the other hand, methanol is supposed to be too small and *tert*-butyl alcohol and *tert*-amyl alcohol too bulky to bind the pocket. These three alcohols in Group II might interact with the active site weakly and inhibit thermolysin mainly by reducing the D value.

The transition-state theory for reactions between ions gives us an expression for the dependence of the rate constant (k) on the D value (*30*). It is predicted that a linear plot of log k against $1/D$ will have a negative slope if the charges of the ions are the same sign and a positive slope if the charges are of the opposite sign. The large decrease in k_{cat}/K_m, which is the second-order rate constant for the binding between thermolysin [pI = 5.1 (*31*)] and the substrate [MOCAc-PLGL(DPa)AR] to form the transition-state complex, accompanying a small decrease in the D value (D: 78.2 to 74.8) suggests that the enzyme reaction is controlled by the interaction between ions of opposite sign with large effective charges and at a short distance. The effect of the dielectric constant of the medium on thermolysin activity has to be studied more precisely using various substrates from the viewpoint of a collision between thermolysin and the substrate and stabilization of the transition state.

According to an X-ray crystallographic study conducted using thermolysin crystals soaked in 2–100% 2-propanol (*29*), only minor changes in the protein conformation were observed, and an increasing number of 2-propanol-binding sites were identified as the alcohol concentration increased. At 5% 2-propanol, the alcohol binds preferentially at the S1′ subsite [the nomenclature used for subsites (S) of the active site and for naming the amino acid residues (P) are those of Schechter and Berger (*32*)], and occupies all four of the main subsites in the active site when the alcohol concentration is very high. The S1′ subsite binds leucine and phenylalanine with the same affinity, and the S1 and S1′ subsites bind phenylalanine with almost the same affinity (*7*). On the other hand, the binding affinity of the S1 subsite for leucine is one-tenth that for phenylalanine, although the hydrophobicity parameters of leucine and phenylalanine are very similar (π values are 1.81 and 1.95, respectively), suggesting that the S1 subsite prefers the phenylalanine side chain to the leucine side chain (*7*). Therefore, it is reasonable to speculate that alcohols in Group I (such as 1-propanol, 2-butanol, 2-methyl-1-propanol, and 1-pentanol) bind primarily to the S1′ subsite and secondarily to the S1 subsite (Fig. 5). The hydrophobic interaction of the P1′ residue of the substrate at the S1′ pocket is considered to be a major factor, and that at the S1 subsite with the P1 residue to be a secondary factor for determining the substrate specificity of thermolysin. The alcohol molecule bound at the S1′ subsite interrupts the binding of the leucine residue at the P1′ position of MOCAc-PLGL(Dpa)AR and FAGLA. On the other hand, we have observed that the relative activity of thermolysin in the hydrolysis of *N*-carbobenzoxy (*Z*)-aspartyl-phenylalanine methyl ester and its synthesis from *Z*-aspartic acid and phenylalanine methyl ester is enhanced up to 130% with increasing methanol concentration up to 20% (v/v), after which the activity decreases to 10% with a further increase in methanol concentration to 70% (v/v) (K. Inouye, unpublished results). Recently, it has been reported that the K_i value of an inhibitor of thermolysin decreases by up to an order of magnitude when the ethanol concentration in the medium is increased over the range 0–9% (v/v) (*33*). This antihydrophobic effect of ethanol correlates with the amount of hydrophobic surface area sequestered from the solvent upon association of the inhibitor and thermolysin. The results are consistent with the Lum-Chandler-Weeks explanation for the size-dependence of the hydrophobic effect (*34*), and it is not necessary to consider the specific interaction of ethanol with thermolysin. These results suggest that the effects of alcohols on the thermolysin activity might be dependent on the structure and nature of the substrate examined. Alcoholic solvents have been reported to induce spectral changes and pK_a shifts in the functional residues of thermolysin (*35*). A study to examine extensively the effects of alcohols on thermolysin activity using various substrates and the thermolysin structure is currently underway.

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