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Effects of Neutral Salts and Alcohols on the Activity of *Streptomyces caespitosus* Neutral Protease

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Streptomyces caespitosus **neutral protease (ScNP) is one of the smallest metalloproteinase with a molecular mass of 14 kDa. Effects of solvent composition on ScNP activity were examined using a peptide substrate. The k_{cat}/K_m values of ScNP exhibited bell-shaped pH-dependence with the optimal pH of 6.4–7.0 and the pK_a values of 5.0 ± 0.1 and 8.3 ± 0.1. ScNP activity increased in an exponential fashion with increasing [NaCl]. The relative k_{cat}/K_m value at 3.6 M NaCl to that at 0 M NaCl was 3.7, and the degree of the activation at x M NaCl was expressed as 1.2^x ($x < 2.0$) and 1.4^x ($x > 2.0$). On the other hand, ScNP activity decreased with increasing concentrations of LiCl, KCl, NaBr, LiBr, KBr and $NaClO_4$. Alcohols inhibited ScNP activity with the IC_{50} values, the concentration required for decreasing the activity at 50% of the maximum, of 0.77–6.54 M. The order of the inhibitory potency was 1-butanol, 2-methyl-1-propanol, 2-methyl-2-butanol > 2-methyl-2-propanol, 2-butanol, 1-propanol > 2-propanol ≫ ethanol ≫ methanol. The activities recovered completely by the dilution of alcohols, suggesting that the ScNP inhibition by alcohols is reversible. These characteristics of ScNP are compared with those of human matrix metalloproteinase 7 and thermolysin.**

Key words: alcohol, metalloproteinase, pH-activity profile, salt effect, *Streptomyces caespitosus* neutral protease.

Abbreviations: FAAFA, *N*-[3-(2-furyl)acryloyl]-Ala-L-Phe-NH2; FAGLA, *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu-NH2; FALAA, *N*-[3-(2-furyl)acryloyl]-L- Leu-L-Ala-NH2; MOCAc-PLGL(Dpa)-AR, *N*-(7-methyoxycoumarin-4-yl) acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropioyl]-L-Ala-L-Arg-NH2; ScNP, *Streptomyces caespitosus* neutral protease.

Streptomyces caespitosus neutral protease (ScNP) [EC 3.4.24.77] is a zinc metalloproteinase produced in the culture broth of *S. caespitosus* (*1*). ScNP is one of the smallest metalloproteinases with a molecular mass of 14 kDa (*2*), and catalyses specifically the hydrolysis of peptide bonds with aromatic amino acid residues at the P1' position (*3*). X-ray structural analysis reveals that ScNP contains one zinc ion and one calcium ion (*4*, *5*). In the catalytic mechanism of ScNP, Glu84 is thought to enhance the nucleophilicity of the zinc-bound water and assigned to be the residue responsible for pK_{e1} (*4*). However, site-directed mutagenesis study on ScNP has not been reported.

The majority of zinc metalloproteinase exhibits the consensus sequence HEXXH in their active site and includes five subfamilies (astacin, serratia, matrixin, snake venom and thermolysin) based on topological feature. All subfamilies except for the thermolysin, one which have the consensus sequence HEXXHXXGXXH and a methionine-containing turn (Met-turn) are grouped in the metzincin family, in which three histidine residues chelate a catalytic zinc ion (*6*). Because ScNP has a novel zinc-binding sequence H^{83}EXXHXXGXXD^{93} and Met-turn, it was first classified in the *Streptomyces* small neutral protease family (*3*). Today, metzincins encompasses zinc metalloproteinases having the consensus sequence HEXXHXXGXX(H/D) and a Met-turn (*7*), and thus ScNP belongs to the metzincin family. On the other hand, it is interesting to note that some zinc metalloproteinases have different zinc-binding sequence from the common HEXXH (for example, HEXXXH in dipeptidyl peptidase III) (*8*).

We have evaluated the effects of solvent conditions on thermolysin [EC 3.4.24.27], and human matrix metalloproteinase 7 (matrilysin or MMP-7) [EC 3.4.24.23] (*9–17*). Thermolysin is a thermostable neutral metalloproteinase from *Bacillus thermoproteolyticus* and belongs to the thermolysin subfamily (*18–20*). Thermolysin activity was enhanced 13–15 times with 4 M NaCl in the hydrolysis of a neutral substrate *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu-NH_2 (FAGLA) and 6–7 times with 3.8 M NaCl in the hydrolysis of *N*-carbobenzoxy-L-Asp-L-Phe (ZDFM), a negatively charged substrate and a precursor of a synthetic sweetener, aspartame at pH 7.0, at 25°C (*9–12*). Importantly, the orders of ions for the efficiency in the activation are $Na^+ > K^+ > Li^+$, which is different from the Hofmeister's series corresponding to the degree of hydration of ions: $Li^+ > Na^+ > K^+$ (*9*, *10*, *13*). Alcohols inhibit thermolysin activity with the inhibitory potency depending on alcohol species (*14*). X-ray crystallographic analysis revealed that 2-propanol binds the S1′ subsite of thermolysin (*15*). Matrilysin is one of the

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smallest matrix metalloproteinases (MMPs) and belongs to the metzincin family. It is also activated by NaCl and inhibited by alcohols, although the magnitudes of the effects of NaCl and alcohols on matrilysin activity are considerably smaller than those on thermolysin (*16*, *17*). These results suggest that not only the dielectric constant of the reaction medium but also the interaction between ions and particular residues of thermolysin and matrilysin are involved in the effects of neutral salts and alcohols, although such residues have not yet been identified. We have proposed that neutral salts and alcohols are suitable probes for exploring the active-site geometry of enzymes and estimating the catalytic mechanism.

The aim of this study is to examine the effects of solvent conditions on ScNP activity. Recently, we have established an improved purification procedure for ScNP from culture supernatants of *S. caespitosus* (*21*). The pure preparation of ScNP obtained by this procedure does not contain any detectable pigment and is suitable for the spectrophotometric measurement of its catalytic activity. (*21*). In this study, we describe the effects of pH, neutral salts and alcohols on ScNP activity in the hydrolysis of a spectrophotometric substrate *N*-[3-(2-furyl)acryloyl]-Ala-L-Phe-NH_2 (FAAFA). We also discuss these characteristics of ScNP by comparing them with those of matrilysin and thermolysin.

MATERIALS AND METHODS

Materials—ScNP was purified from the dark brown lyophilized powder of the crude filtrate of *S. caespitosus* by the procedure we recently established (*21*). Briefly, the procedure comprises sequential ammonium sulphate fractionation and column chromatography procedures with anion exchange chromatography followed by hydrophobic-interaction chromatography and gel filtration. Purified ScNP revealed a single band with a molecular mass of 14 kDa by SDS–PAGE under reduced conditions and did not contain any detectable pigment. The concentration of ScNP was determined according to the method of Lowry *et al*. (*22*), using BSA as the standard. Furylacryloyl dipeptide amides FAAFA (Lot 106H0785), FAGLA (Lot 460519) and *N*-[3-(2-furyl)acryloyl]-L-Leu-L-Ala-NH_2 (FALAA) (Lot 57H5800) were purchased from Wako Pure Chemical (Osaka, Japan), Peptide Institute (Osaka), and Sigma (St Louis, MO, USA), respectively. Their concentrations were determined spectrophotometrically using the molar absorption coefficient, $\varepsilon_{345} = 766\,M^{-1}\,cm^{-1}$ (*23*). All spectrophotometric measurements were done with a Shimadzu UV-240 spectrophotometer (Kyoto, Japan).

Hydrolysis of Furylacryloyl Dipeptide Amides by ScNP, Matrilysin and Thermolysin—The reaction was initiated by mixing 1,880 μl of 50 mM MES buffer at pH 6.5 containing 1 mM $CaCl_2$ (buffer A), 100 μl of each of FAAFA, FAGLA and FALAA dissolved in DMSO (FAAFA) or buffer A (FAGLA and FALAA), and 20 μl of 16–33 μM ScNP in a cuvette. The reaction was then measured following the decrease in absorbance at 345 nm (*9*, *10*). The amount of FA-dipeptide amides hydrolysed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\,M^{-1}\,cm^{-1}$, at 25°C (*9*, *23*). Because the reaction was carried out under the conditions of the pseudo-first-order reaction due to sparing solubility of the substrates in comparison with the Michaelis constant K_m, the kinetic parameters, K_m and molecular activity k_{cat}, cannot be determined separately, and thus the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m.

pH-Dependence of ScNP Activity in the Hydrolysis of FAAFA—For the pH-dependence analysis, the reaction was carried out in 20 mM acetate-NaOH buffer at pH 4.2–5.8, 20 mM MES buffer at pH 5.0–7.0, 20 mM HEPES buffer at pH 6.5–8.3 and 20 mM TAPS buffer at pH 7.5–9.5 for each of which containing 1 mM $CaCl_2$. The kinetic parameters, the intrinsic k_{cat}/K_m, $[(k_{cat}/K_m)_o]$, and the proton dissociation constants (K_{e1} and K_{e2}) for the pH-dependence of the activity were calculated from Eq. 1 by a non-linear least squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT, USA).

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{(k_{cat}/K_m)_o}{1+([H]/K_{e1}) + (K_{e2}/[H])} \quad (1)$$

In this equation, $(k_{cat}/K_m)_{obs}$ and [H] mean the k_{cat}/K_m value observed and the proton concentration, respectively, at the specified pH.

Effects of Neutral Salts and Alcohols on the ScNP-catalysed Hydrolysis of FAAFA—A spectrophotometric analysis of the ScNP-catalysed hydrolysis of FAAFA was performed in the presence or absence of neutral salts (NaCl, KCl, NaBr, KBr, LiCl, LiBr, $NaNO_3$ and $NaClO_4$) or alcohols (methanol, ethanol, 1-propanol, 2-propanol, 2-methyl-2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol and 2-methyl-2-butanol) at 25°C. The reaction was initiated by mixing 1,780 or 1,880 μl of buffer A, 100 or 200 μl of FAAFA dissolved in DMSO and 20 μl of ScNP in a cuvette to give the final concentrations of 156 nM ScNP and 530–570 μM FAAFA in 50 mM MES buffer at pH 6.5 containing 1 mM $CaCl_2$, 5 or 10% DMSO, and either 0–4 M neutral salts or 0–40% alcohols. The reaction was measured and the enzyme activity was analysed as described earlier.

Effect of Dilution after Incubating ScNP with Alcohol—The effect of dilution after incubating ScNP with an alcohol on the ScNP-catalysed hydrolysis of FAAFA was examined as follows. In the case of methanol, ScNP (1.64 μM) in buffer A containing 20% methanol was incubated at 4°C for 1 h, and then diluted 10 times with buffer A without methanol. For comparison, ScNP (1.64 μM) in buffer A containing 2% methanol was incubated at 4°C for 1 h, and then diluted 10 times with buffer A containing 2% methanol. Ethanol, 1-propanol, 2-propanol and 2-methyl-2-butanol were examined in the same way. In the case of 1-butanol, 2-butanol, 2-methyl-1-propanol and 2-methyl-2-butanol, the alcohol concentration of the mixture at the 1-h incubation was 5% and then diluted to 0.5%. The reaction was initiated immediately after the dilution by mixing 1,900 μl of the above mixture and 100 μl of FAAFA dissolved in DMSO. The concentrations of ScNP and FAAFA in the reaction mixture were 156 nM and 690 μM, respectively.

RESULTS AND DISCUSSION

FAAFA as a Substrate for ScNP—To find a suitable spectrophotometric substrate for ScNP, we first evaluated three furylacryloyl dipeptide amides, FAAFA, FAGLA and FALAA. We also measured the hydrolysis of these three substrates by matrilysin and thermolysin for comparison. ScNP hydrolysed FAAFA but did not hydrolyse FAGLA or FALAA at all (Table 1), which is in agreement with the previous report that ScNP catalyses specifically the hydrolysis of peptide bonds with aromatic amino acid residues at the P1′ position (*3*). Matrilysin did not hydrolyse FAAFA, FAGLA or FALAA at all. This is explained by the previously reported observation that matrilysin, like other MMPs, has a large substrate-binding site consisting of eight subsites and prefers proline at the P3 position (*24*). Thermolysin hydrolysed all three substrates, which is in agreement with the previous report that thermolysin catalyses specifically the hydrolysis of peptide bonds with bulky hydrophobic amino acids such as phenylalanine or leucine at the P1′ position (*10*, *25*, *26*). We also evaluated an *N*-(7-methyoxycoumarin-4-yl)acetyl-L-Pro- L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L- Arg-NH_2 [MOCAc-PLGL(Dpa)-AR], a fluorescent substrate in which the peptide bond between glycine and leucine residues is cleaved by matrilysin and thermolysin, but ScNP did not hydrolyse this at all (data not shown). We thus decided to use FAAFA for subsequent analysis.

pH-Dependence of the ScNP-catalysed Hydrolysis of FAAFA—It was reported that the appropriate pH region for ScNP activity was 4.5–9.0 using oxidized insulin B-chain as the substrate and HPLC for detection (*3*). We examined the pH-dependence of k_{cat}/K_m of the ScNP-catalysed hydrolysis of FAAFA at 25°C (Fig. 1). The plot showed a bell-shaped curve with the optimal pH of 6.4–7.0. We therefore decided on pH 6.5 as an optimum pH for subsequent analysis. According to Eq. 1, the acidic pK_a (pK_{e1}) and alkaline pK_a (pK_{e2}) values were determined to be 5.0 ± 0.1 and 8.3 ± 0.1, respectively. It should be noted that the experimental data has a shoulder at pH values around 8.5 and thus the theoretical curve based on Eq. 1 does not fit them well at pH values above 8.5. This suggests that there might be a possibility that the activity is controlled additionally by a third ionizable group with a pK_a value around 9.

Table 2 compares pK_{e1} and pK_{e2} values of ScNP with those of matrilysin and thermolysin. The pH-dependence of ScNP activity is similar to that of thermolysin (*27*) compared with matrilysin (*28*). In thermolysin, His231 has been proposed to be the ionizable residue responsible for pK_{e2} although whether it works as an acid (*29*) or a base (*30*) is a matter of controversy. In ScNP, His94, the next residue to the zinc-binding consensus sequence $H^{83}EXXHXXGXXD^{93}$, locates at the surface, but the distance between NE2 of His94 and the catalytic Zn^{2+} ion is as far as 10.8 Å (Protein Data Bank number 1C7K) (*5*). Hence, His94 is declined as the candidate. On the other hand, Tyr95 sticks out to the Zn^{2+} ion, and the distance between OH of Tyr95 and the Zn^{2+} ion is 5.0 Å. Whether Tyr95 is the ionizable residue responsible for pK_{e2} or stabilize the substrate by hydrogen bonds, like Tyr157 of thermolysin (*31*), is not known at present.

Table 1. **Substrate specificity of ScNP, matrilysin, and thermolysin.**

Substrate	ScNP	Matrilysin	Thermolysin
		$(k_{cat}/K_m) \times 10^3$ ($M^{-1}s^{-1}$)	
FAAFA	3.6 ± 0.2 [a]	<0.1	93 ± 1
FAGLA	<0.1	<0.1	30 ± 1
FALAA	<0.1	<0.1	1.2 ± 0.1

[a]The *k*cat/*K*m values are shown with the standard deviation (SD).

Effects of Neutral Salts on ScNP Activity—Figure 2 shows dependence on salt concentration of the relative activity for the hydrolysis of FAAFA at pH 6.5, at 25°C. ScNP activity increased in an exponential fashion with increasing concentration of NaCl. The activity was enhanced 3.7 times by the addition of 3.6 M NaCl, and the degree of the activation at x M NaCl was expressed as 1.2^x ($x < 2.0$) and 1.4^x ($x > 2.0$). On the other hand, the activity decreased with increasing concentrations of LiCl, KCl, NaBr, LiBr, KBr and $NaClO_4$. The order of the inhibitory potency was $NaClO_4$, LiBr > KBr > LiCl, KCl, NaBr. In the case of $NaNO_3$, the relative ScNP activity first decreased to 0.83 at 1.3 M and then increased to 1.1 at 3.6 M. The results presented in this study are much different from those in thermolysin (*13*). Thermolysin activity markedly increased with increasing concentration of NaCl, NaBr, KCl and KBr with the degrees of the activation at x M salt in the range of 1.6^x to 1.9^x, and slightly increased with increasing concentration of LiCl and LiBr (*13*). Dependence of matrilysin activity on salt species has not yet been reported.

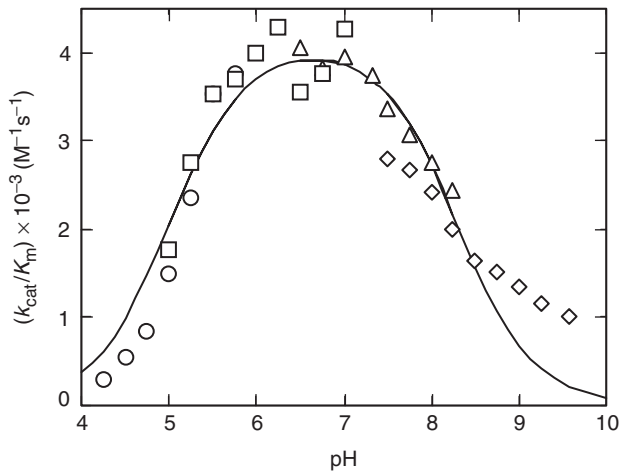

Fig. 1. **Effects of pH on the ScNP-catalysed hydrolysis of FAAFA.** The reaction was carried out with 330 nM ScNP and 540 μM FAAFA in 20 mM acetate-NaOH buffer at pH 4.2–5.8 (open circle), 20 mM MES buffer at pH 5.0–7.0 (open square), 20 mM HEPES buffer at pH 6.5–8.3 (open triangle) and 20 mM TAPS buffer at pH 7.5–9.5 (open diamond) for each of which containing 1 mM $CaCl_2$, at 25°C. Solid line represents a curve expressed by Eq. 1 with the $(k_{cat}/K_m)_o$ value of $4.1 \times 10^3\ M^{-1}s^{-1}$ and the pK_{e1} and pK_{e2} values of 5.0 and 8.3, respectively, which was drawn to fit the experimental data.

We have already proposed that the interaction between respective ions and particular residues is involved in thermolysin activation (*9*, *10*, *13*). However, it is puzzling why NaCl solely enhances ScNP activity. There is an interesting feature that the inhibitory potency of NaBr is less than those of LiBr and KBr and that of KCl is less than that of KBr. This suggests that Na^+ and Cl^- enhance ScNP activity while K^+, Li^+ and Br^- inhibit it. According to X-ray crystallographic analysis of bacterial K^+ channel, K^+ ion (ion diameter is 1.33 Å) is accommodated by particular four carbonyl oxygen atoms of the bacterial K^+ channel precisely enough to compensate the energy required for the dehydration of the K^+ ion (*32*). On the other hand, a Na^+ ion (ion diameter is 0.95 Å) is too small to be accommodated precisely enough to compensate that of the Na^+ ion. We speculate similar mechanism such as narrow selectivity for particular ions of ion channels might be involved in the NaCl-induced activation of ScNP. As to inhibition of ScNP by salts other than NaCl, it is not known whether the conformational change of ScNP or the interaction between respective ions and particular residues is the main mechanism at present.

Table 3 summarizes the degree of the NaCl-induced activation of ScNP, matrilysin and thermolysin, showing that ScNP is relatively similar to matrilysin compared with thermolysin in the magnitude of the activation. However, the NaCl concentrations at which the equation expressing the degree of the activation in the presence of x M NaCl changes are 2.0 M for ScNP but 0.5 M for matrilysin and thermolysin. This suggests a possibility that any conformational change of ScNP might occur at the NaCl concentration around 2.0 M. It has been reported that the activation by NaCl is due to an increase in k_{cat} but not a decrease in K_m in thermolysin namely due to stabilization of the transition state (*9*) and is due to a decrease in K_m but not an increase in k_{cat} in matrilysin namely due to stabilization of the ES complex (*12*). In the case of ScNP, k_{cat} and K_m could not be evaluated separately because FAAFA is sparingly soluble.

Effects of Alcohols on ScNP Activity—We examined the effects of alcohols on ScNP activity. The k_{cat}/K_m of the

Table 2. **pK_e values of ScNP, matrilysin, and thermolysin.**

Substrate	ScNP[a]		Matrilysin[b]		Thermolysin[c]	
	pK_{e1}	pK_{e2}	pK_{e1}	pK_{e2}	pK_{e1}	pK_{e2}
FAAFA	5.0 ± 0.1[d]	8.3 ± 0.1	n.d.[e]		n.d.	
MOCAc-PLGL(Dpa)-AR	n.d.		4.0±0.0	9.8 ± 0.0	n.d.	
FAGLA	n.d.		n.d.		5.3 ± 0.1	8.2±0.1

[a]The pK_e values were determined based on the results shown in Fig. 1 and Eq. 1. [b]Ref. (*28*). [c]Ref. (*27*). [d]The pK_e values are shown with SD. [e]Not detected.

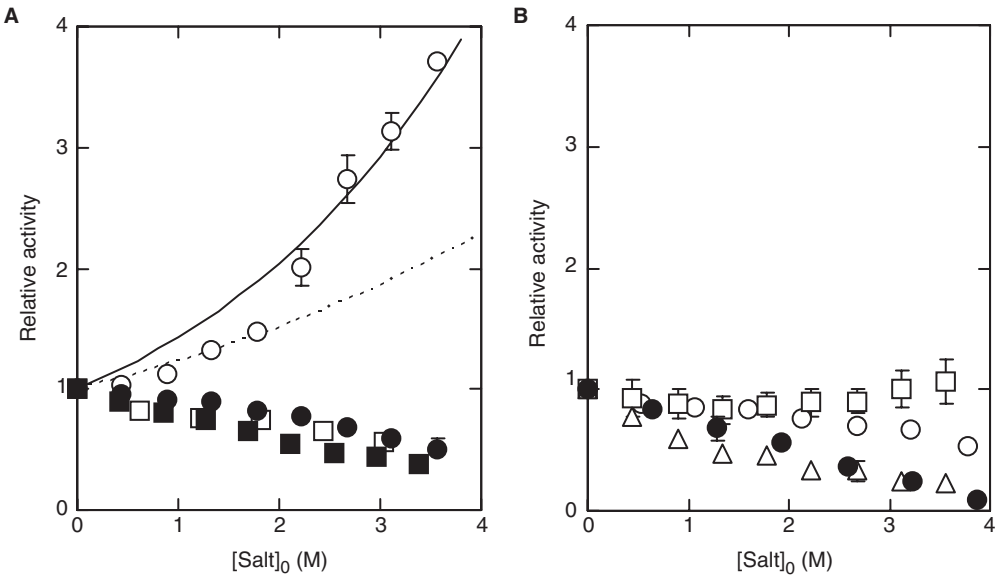

Fig. 2. **Effects of neutral salts on the ScNP activity in the hydrolysis of FAAFA.** The reaction was carried out in 50 mM MES buffer at pH 6.5 containing 1 mM $CaCl_2$, 10% DMSO and various concentrations of salts indicated. The concentrations of ScNP and FAAFA are 156 nM and 550 μM, respectively. The relative activity is defined as the ratio of the k_{cat}/K_m value at x M salt to that without salt (3,150 $M^{-1}s^{-1}$). Error bars indicate SD values. Panel A: Salts examined are NaCl (open circle), KCl (open square), NaBr (filled circle) and KBr (filled square). Solid and broken lines represent a curve expressed by $y = 1.4^x$ (y is the relative activity at x M NaCl) and that expressed by $y = 1.2^x$, respectively, which were drawn to fit the experimental data with NaCl. Panel B: Salts examined are LiCl (open circle), LiBr (filled circle), $NaNO_3$ (open square) and $NaClO_4$ (open triangle).

ScNP-catalysed hydrolysis of FAAFA decreased with increasing concentrations of all alcohols examined (Fig. 3). Linear lines were not given in the plot [(reciprocal of the velocity) versus (alcohol concentration)] (data not shown). Therefore, instead of obtaining K_i values, IC_{50} values (the concentrations of alcohols required for decreasing the activity at 50% of the maximum activity) were determined and compared with the K_i values of matrilysin and thermolysin previously reported (*14*, *17*) (Table 4). There is a striking similarity in the values between ScNP and matrilysin. The IC_{50} values for ScNP and the K_i values for matrilysin are in the range of 0.77–6.54 M and 0.66–4.80 M, respectively, while the K_i values for thermolysin are 10–20 times less (38–430 mM). The orders of inhibitory potency for ScNP and matrilysin are almost the same: 1-butanol, 2-methyl-1-propanol, 2-methyl-2-butanol > 2-methyl-2-propanol, 2-butanol, 1-propanol > 2-propanol ≫ethanol ≫ methanol. When a methyl group is introduced to 1-propanol and 2-butanol to form 2-methyl-1-propanol and 2-methyl-2-butanol, respectively, the inhibitory potencies increase by 2.1 and 1.5 times for ScNP and 1.7 and 1.2 times for matrilysin, but decrease by 0.92 and 0.36 times for thermolysin.

In the following, we discuss a possible conformational change of ScNP caused by alcohols. In order to confirm whether the irreversible inactivation of ScNP does not happen by alcohols, we examined the effects of dilution

Table 3. **Degree of the activation of ScNP, matrilysin, and thermolysin by NaCl.**

Substrate	ScNP	Matrilysin	Thermolysin
FAAFA[a]	1.2^{xb} (x < 2.0)	n.d.[c]	n.d.
	1.4^{x} (x > 2.0)		
MOCAc-PLGL (Dpa)-AR[d]	n.d.	2.1^{x} (x < 0.5)	4.7^{x} (x < 0.5)
		1.4^{x} (x > 0.5)	2.3^{x} (x > 0.5)
FAGLA[e]	n.d.	n.d.	1.9^{x}
ZDFM[f]	n.d.	n.d.	1.2^{x} (x < 0.5)
			1.8^{x} (x > 0.5)

[a]The degree of the activation by NaCl were determined based on the results shown in Fig. 2. [b]Degree of activation at x M NaCl at pH 6.5. [c]Not detected. [d]Ref. (12). [e]Ref. (11). [f]Ref. (9).

Table 4. **Inhibitory effects of alcohols on the activities of ScNP, matrilysin, and thermolysin.**

Alcohol	ScNP	Matrilysin[a]	Thermolysin[b]
	IC_{50} (M)[c]	K_i (M)[d]	K_i (M)[d]
Methanol (*1*)[e]	6.54 ± 0.15[f]	4.80 ± 0.12	0.430 ± 0.060
Ethanol (2)	4.48 ± 0.21	2.68 ± 0.09	0.100 ± 0.010
1-Propanol (3)	1.83 ± 0.05	1.14 ± 0.07	0.038 ± 0.005
2-Propanol (3)	2.56 ± 0.11	1.46 ± 0.06	0.065 ± 0.008
1-Butanol (4)	0.77 ± 0.06	0.78 ± 0.03	0.050 ± 0.006
2-Butanol (4)	1.60 ± 0.07	0.82 ± 0.03	0.040 ± 0.005
2-Methyl-1-propanol (4)	0.89 ± 0.03	0.66 ± 0.03	0.041 ± 0.003
2-Methyl-2-propanol (4)	1.39 ± 0.08	1.17 ± 0.04	0.190 ± 0.003
2-Methyl-2-butanol (5)	1.07 ± 0.02	0.67 ± 0.02	0.110 ± 0.020

[a]Ref. (*17*). MOCAc-PLGL(Dpa)-AR was used as a substrate. [b]Ref. (*14*). MOCAc-PLGL(Dpa)-AR was used as a substrate. [c]The concentration of alcohols required for decreasing the activity at 50% of the maximum, which is obtained based on the results shown in Fig. 3. [d]Inhibitor constant. [e]Numbers in parentheses indicate the number of carbon atoms. [f]The IC_{50} and K_i values in molar concentration (M) are calculated from those in volume percent and molecular weights of alcohols and are shown with SD.

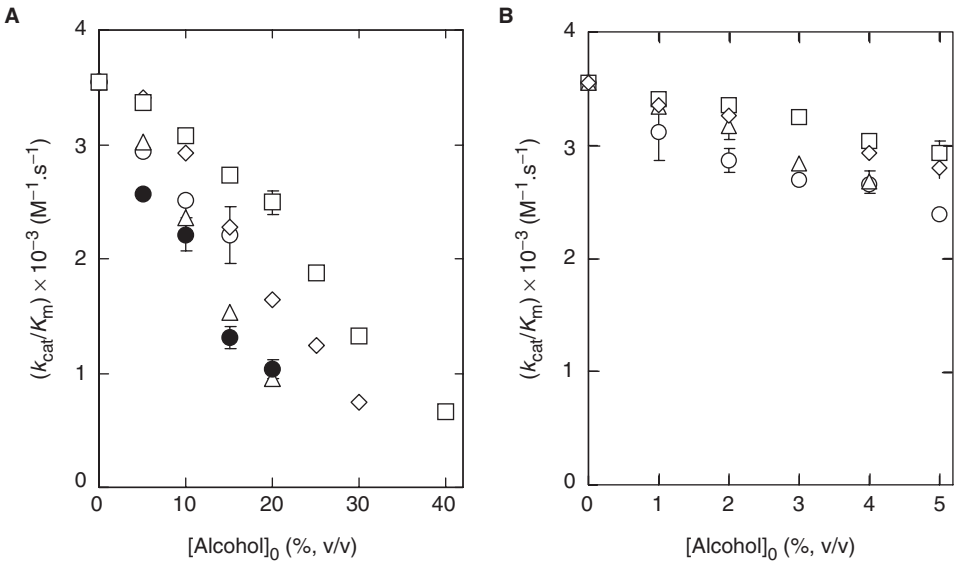

Fig. 3. **Effects of alcohols on the ScNP activity in the hydrolysis of FAAFA**. The reaction was carried out in 50 mM MES buffer at pH 6.5 containing 1 mM $CaCl_2$, 5% DMSO and various alcohols indicated. The concentration of ScNP and FAAFA are 156 nM ScNP and 550 μM, respectively. Error bars indicate SD values. Panel A: Alcohols examined are methanol (open circle), ethanol (open square), 1-propanol (open triangle), 2-propanol (open diamond) and 2-methyl-2-butanol (filled circle). Panel B: Alcohols examined are 1-butanol (open circle), 2-butanol (open square), 2-methyl-1-propanol (open triangle) and 2-methyl-2-propanol (open diamond).

Table 5. **Effects of dilution of alcohols on ScNP activity.**

Alcohol	Relative activity (%)			Reversibility (%)
	20%	2%	20→2%	
Methanol	62 ± 8	96 ± 5	96 ± 2	99
Ethanol	70 ± 3	96 ± 4	100 ± 3	104
1-Propanol	27 ± 0	93 ± 3	96 ± 2	104
2-Propanol	46 ± 0	95 ± 1	95 ± 3	100
2-Methyl-1-propanol	39 ± 3	92 ± 4	92 ± 4	99
	5%	0.5%	5→0.5%	
1-Butanol	83 ± 2	95 ± 3	96 ± 6	101
2-Butanol	69 ± 0	98 ± 1	100 ± 9	102
2-Methyl-2-propanol	39 ± 3	97 ± 2	107 ± 3	110
2-Methyl-2-butanol	79 ± 2	98 ± 2	106 ± 4	108

The reaction was carried out in 50 mM MES buffer at pH 6,5 containing 1 mM $CaCl_2$, 5% DMSO, and various alcohols indicated. The concentration of ScNP and FAAFA are 156 nM and 550 μM, respectively. The activity in the absence of alcohol was taken as 100% relative activity. The reversibility is the percentage of activity recovered upon dilution (20→2% or 5→0.5%) of an alcohol compared to the activity at 20 or 5% of the alcohol. The relative activity is shown with SD.

after incubating ScNP with an alcohol at 4°C for 1 h on ScNP activity (Table 5). In the case of methanol, ScNP activities in the presence of 20% and 2% methanol were 62% and 96%, respectively, of that obtained in the absence of methanol. When 20% methanol was diluted to 2%, the activity recovered to 96%. The activities recovered completely by the dilution of other alcohols, suggesting that the inhibition of the ScNP-catalysed hydrolysis of FAAFA by alcohols is reversible for the alcohol concentrations up to 20% for methanol, ethanol, 1-propanol, 2-propanol and 2-methyl-1-propanol, and up to 5% for 1-butanol, 2-butanol, 2-methyl-2-propanol and 2-methyl-2-butanol. These results suggest that no irreversible inactivation occurred under the conditions in which the inhibitory study was performed. These results also suggest that the alcohol effects on ScNP activity mainly result from specific interaction between alcohols and ScNP, although involvement of reversible conformational change of ScNP by alcohols cannot be denied.

These observations suggest that ScNP and matrilysin might have similar inhibitory binding sites for alcohol and that their size might be large enough to accommodate the length of 1-butanol (4-carbon chain) and the bulk of tertiary alcohols. Figure 4 shows the sequence alignment of ScNP and matrilysin. Only 22 out of 134 amino acids residues of ScNP are identical to those of matrilysin. However, there is a significant homology at the active site including the zinc-binding consensus sequence and a conserved methionine residue at the Met-turn. In addition, their topological structures (arrangement of α-elices and β-sheets) are identical. Figure 5 shows X-ray crystallographic structures of ScNP, matrilysin, and thermolysin. ScNP and matrilysin are virtually identical. Specifically, the active site lies in the shallow cleft between the β-sheet and the central α-helix, and the Met-turn is located beneath the catalytic zinc ion in these two enzymes. In contrast, in thermolysin, the central α-helix connects the β-rich N-terminal domain and the α-rich C-terminal domain and is located at the bottom of the active-site deep cleft. By the X-ray structural analysis of thermolysin crystals soaked in 2-propnol, 2-propanol was shown to bind

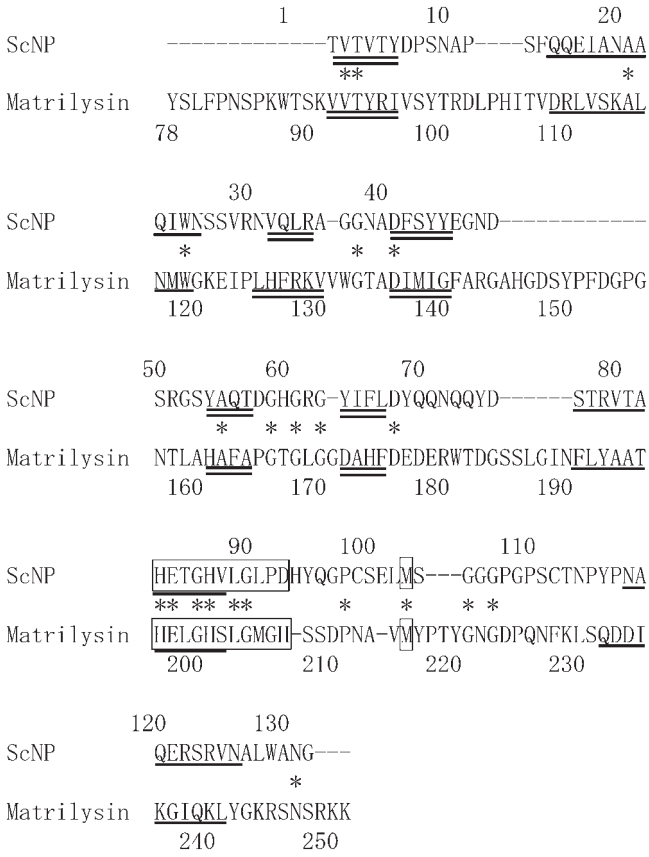

Fig. 4. **A sequence alignment of ScNP and matrilysin.** Homology search was performed using the search program DDBJ CLUSTALW and revised based on the data of X-ray crystallographic analysis (*31*, *34*). Amino acid numberings of matrilysin are according to the previous papers (*28*, *35*). The consensus zinc-binding sequence HEXXHXXGXX(H/D) in the active site and the conserved methionine residue at the Met-turn are boxed. α-helix is underlined, and β-sheet is double underlined. Asterisks show homologous amino acid residues.

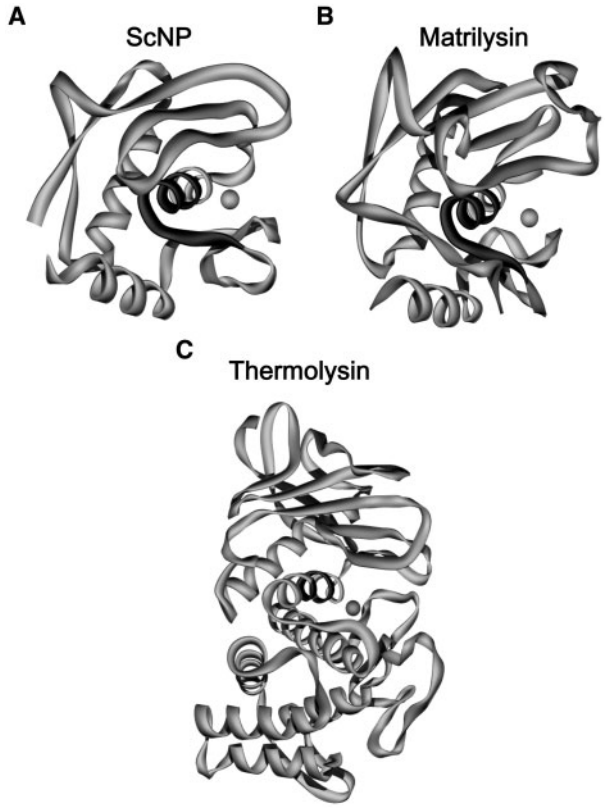

Fig. 5. **Overall protein structures of ScNP, matrilysin and thermolysin.** The protein structure of ScNP (A), matrilysin (B) and thermolysin (C) are based on the Protein Data Bank number 1C7K (*5*), 1MMQ (*34*) and 8TLN (*36*), respectively. Peptide chain is represented by a ribbon model. The consensus zinc-binding sequences HEXXHXXGXX(H/D) in ScNP and matrilysin and HEXXH in thermolysin are shown as filled ribbon. The catalytic zinc ion is shown as a sphere.

exclusively the S1′ subsite (*15*). In relation to this, we have proposed that alcohols with a three or four linear carbon chain (such as 1-propanol, 1-butanol, 1-butanol and 2-methyl-1-propanol) also bind the S1′ subsite because their K_i values are as small as that of 2-propanol (Table 5) (*14*). Therefore, it is anticipated that alcohols interact with particular residues at the active sites of ScNP and matrilysin, and affect their activity, like alcohols for thermolysin (*14*, *15*) and the inhibitors thiorphan and R-94138 for matrilysin (*33*).

In conclusion, the pH-dependence of ScNP activity is similar to that of thermolysin while the effects of neutral salts on ScNP activity are different from those on thermolysin activity. A striking similarity between ScNP and matrilysin was demonstrated in the alcohol-induced inhibition of enzyme activity, suggesting that ScNP and matrilysin might have similar inhibitory binding sites for alcohol. Further investigations are required to assign the ionizable residues of ScNP controlling the activity and to explore the mechanisms of the activation and inhibition of ScNP by neutral salts and alcohols.

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