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Identification and Characterization of an Antibacterial Peptide of the 26-kDa Protease of *Sarcophaga peregrina* with Antibacterial Activity[1]

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Previously, we purified a serine protease with a molecular mass of 26 kDa that exhibits potent antibacterial activity from a pupal extract of *Sarcophaga peregrina* (flesh fly). We divided this protease into 12 peptides and examined their antibacterial activity. A peptide corresponding to residues 155 to 174 (peptide 9) was found to exhibit antibacterial activity comparable to that of the 26-kDa protease. When *Escherichia coli* was treated with peptide 9, the permeability of both the outer and inner membranes increased, and substrates for β-lactamase and β-galactosidase entered the cells, but β-galactosidase did not leak out of the cells under these conditions. It was suggested that residues 6 to 18 of peptide 9 form an amphiphilic α-helix under hydrophobic conditions with an N-terminal basic loop and then interact with acidic phospholipids in the bacterial membranes.

Key words: amphiphilic α-helix, antibacterial peptide, bacterial membrane, insect, serine protease.

In holometabolous insects, extensive tissue replacements take place during metamorphosis (*1–5*). This is true for the digestive tract. In *Sarcophaga peregrina*, the larval mid-gut is embedded in the yellow body, a temporary organ formed by primordial cells derived from imaginal islands on the larval mid-gut, and disintegrates *in situ*. Then the yellow body itself extends to form the adult mid-gut with the development of adult structures

Previously, we purified and characterized a novel serine protease with a molecular mass of 26 kDa (26-kDa protease) from *Sarcophaga* pupae (*6*). As the 26-kDa protease gene was only activated transiently at the pupal stage and the resulting protease was detected exclusively in the yellow body, coinciding with the timing of mid-gut decomposition, this protease was suggested to participate in the disintegration of the larval mid-gut in the yellow body. Interestingly, this protease was found to cross-react immunologically with antibodies against sarcotoxin IA, an antibacterial protein of *Sarcophaga* (*7–10*). In fact, the 26-kDa protease showed antibacterial activity (*11*). Possibly, the 26-kDa protease has two functions: i.e. in decomposition of the larval mid-gut and killing of normal flora released into the yellow body when the larval mid-gut disintegrates. We demonstrated that the protease activity is not related to the antibacterial activity of the 26-kDa protease (*11*).

Several serine proteases and proteins structurally related to these proteases have been reported to have bactericidal activity and they were assumed to play roles in the host defense mechanisms against various organisms (*12–14*). These serine proteases are named serprocidins, and cathepsin G (*15*), elastase (*16*), protease-3 (*17*), and azurocidin (*18, 19*) are members of the serprocidin family. Therefore, the 26-kDa protease is the first serprocidin found in an insect. So far, very little is known about the bactericidal mechanism of serprocidins.

This paper reports the identification and characterization of an antibacterial peptide derived from the 26-kDa protease. This peptide consists of 20 amino acid residues and its antibacterial activity is almost the same as that of the intact 26-kDa protease.

MATERIALS AND METHODS

26-kDa Protease and Peptides—The 26-kDa protease was purified to homogeneity from *Sarcophaga* pupae, as described previously (*6*). The peptides used were synthesized using the solid-phase method and purified to homogeneity by HPLC on a reverse-phase column.

Antimicrobial Assaying—*Staphylococcus aureus* (Cowan strain) was grown in tryptic soy broth at 37°C. Bacteria at the logarithmic phase were collected and suspended at a density of 1×10^4 CFU/ml in 10 mM phosphate buffer, pH 5.5. The bacterial suspension (180 μl) and peptide samples dissolved in the same buffer (20 μl) were mixed and incubated for 1 h at 37°C. Then, 100 μl of each reaction mixture was plated onto a nutrient agar plate and incubated overnight at 37°C, and then the resulting bacterial colonies

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Abbreviations: CD, circular dichroism; CFU, colony-forming unit; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; OD, optical density; ONPG, *o*-nitrophenol-β-D-galactoside; PADAC, 7-[(thienyl-2-acetamido)-3-(2-(4-*N,N*-dimethylaminophenylazo)-pyridinium-methyl)-3-cephem-4-carboxylic acid]; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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were counted. The concentration of each peptide giving 90% growth inhibition (ED_{90}) was determined.

The minimum inhibitory concentrations of peptide 9 for various Gram-positive and Gram-negative bacteria, and fungi were examined by serial agar dilution method using Nutrient agar containing 1% glucose for fungi and Mueller-Hinton agar (Difco) for bacteria (*20*). The MIC was determined after an incubation for 42 h at 27°C against fungi, and incubations for 18 or 42 h at 37°C against bacteria, respectively.

Permeability Assaying of Bacterial Membranes—The membrane permeability of *E. coli* (ML-35p) cells was assayed spectrophotometrically by the method of Lehrer *et al.* (*21, 22*) with slight modifications. Outer membrane and inner membrane permeability was monitored as the penetration of substrates for β-lactamase and β-galactosidase, respectively, from outside of the cells. The former is a periplasmic enzyme, whereas the latter is a cytoplasmic one. To assay the penetration of the substrates of these enzymes into bacteria on treatment with peptide 9, we employed the following method. First, 135 μl of 10 mM sodium phosphate buffer containing each substrate (50 μM PADAC for β-lactamase or 2.5 mM ONPG for β-galactosidase) and peptide 9 (15 μM) was preheated at 37°C, and then 15 μl of an *E. coli* suspension (logarithmic-phase bacteria, 10^8 CFU/ml in 10 mM sodium phosphate buffer) was added, and the increase in the optical density at 415 nm was monitored with time. As a positive control for the enzymes, a reaction mixture was sonicated to disrupt the bacteria.

Preparation of Liposomes Containing Glucose and Assaying of Their Release of Glucose—Liposomes were prepared by the method of Kinsky *et al* (*23*) with slight modifications (*24*). All lipids were purchased from Sigma. An aliquot containing 2 μmol of phospholipids was dried completely in a rotary evaporator under reduced pressure. Then 0.2 ml of 0.3 M glucose was added and the lipids were dispersed with the aid of a vortex mixer to obtain multilamellar liposomes containing glucose. The liposomes were washed 3 times with 10 mM phosphate buffer (pH 7.4) containing 130 mM NaCl by centrifugation and finally suspended in 0.2 ml of the same buffer. To determine the sensitivity of liposomes to the peptides, various concentrations of the peptides dissolved in 10 μl of 10 mM phosphate buffer (pH 7.4) containing 130 mM NaCl were added to 10 μl of the liposome suspension. The mixtures were incubated at room temperature for 1 h and then the volume was increased by adding 20 μl of the same buffer. After centrifugation, the supernatant was collected and the amount of released glucose was determined with a Glucose B-Test kit (Wako Chemical, Osaka). Liposomes lysed with 10% Triton X-100 were used as a control for 100% trapped glucose.

Physicochemical Analysis—NMR spectra were recorded with a Bruker DRX-600 and Jeol α-500 spectrometer operating at 500 MHz. For NMR measurements, peptide 9 was dissolved in CD_3OH at an approximate concentration of 1 mM. CD spectra were recorded with a Jasco J-600 spectropolarimeter at 25°C.

RESULTS

Fragmentation of the 26-kDa Protease into 12 Peptides and Their Antibacterial Activity—As reported previously, the 26-kDa protease showed antibacterial activity and this activity was independent of its protease activity (*11*). Therefore, to determine whether this protease contains an internal antibacterial domain, we divided the 26-kDa protease into 12 peptides that represent almost the entire length of the 26-kDa protease, and synthesized them as amidated peptides and assayed their antibacterial activity. Each peptide consisted of about 20 amino acid residues. As summarized in Table I, peptides 9, 11, and 12 showed higher antibacterial activity than the others All these sequences were located in the C-terminal half of the 26-kDa protease. ED_{90} of peptide 9 was 0.015 μM, which is comparable to that of the 26-kDa protease, which was 0.039 μM.

Characterization of Peptide 9—It is not certain whether the sequence of peptide 9, which corresponds to residues 155–174 of the 26-kDa protease, is responsible for the antibacterial activity of this enzyme. However, as this peptide itself seemed to be valuable in terms of peptide antibiotics, we investigated its mode of action. We found that the antibacterial action of peptide 9 is bactericidal, and either the N-terminal part (RNTRYKNKIFDVM) or C-terminal part (LCAGLVK) alone had no appreciable antibacterial activity, it being less than 1/100 of that of peptide 9. The antibacterial activity of peptide 9 did not change with the pH of the assay medium, namely, almost the same activity was observed at pH 5.5, 7.4, and 8.2.

We examined the antibacterial specificity of peptide 9 by assessing the inhibition of the growth of various bacteria in the presence of this peptide. As summarized in Table II, peptide 9 was effective against both the Gram-positive and Gram-negative bacteria tested, although the MIC values ranged from 0.66 to 10.5 μM. We examined the effect of peptide 9 on the growth of three *Candida* strains. Two of the fungal strains were resistant to this peptide, but the

TABLE I. **Antibacterial activity of the peptides derived from the 26-kDa protease.**

			ED_{90} (μM)
Peptide 1	(1–20)	I V G G T Q V R Q N K Y P W T A Q L V K	2.9
Peptide 2	(21–41)	G R H Y P R L F C G G S L I N D R Y V L T	3 1
Peptide 3	(42–61)	A S H C V H N N R D Q I T V R L L Q L D	2 0
Peptide 4	(62–78)	R S S R D P G I T R Q V S K V I M	3 6
Peptide 5	(79–97)	H P Q Y D P V H I T N D V A L L R L D	>45
Peptide 6	(98–112)	T P V P F N D K I R P V C L P	>45
Peptide 7	(114–133)	K N H N F D N K D A I V A G W G L I K E	4.5
Peptide 8	(134–153)	G G V T S N Y L Q E V T V P I I T N Q Q	>45
Peptide 9	(155–174)	R N T R Y K N K I F D V M L C A G L V K	0 015
Peptide 10	(175–194)	Q G G K D A C Q G D S G G P L I V N E G	>45
Peptide 11	(195–214)	R Y K L A G V V S F G F G C A Q A N A P	0 77
Peptide 12	(215–234)	G V Y A R V S K F L D W I H N N S R D G	0.13
26-kDa protease	(1–239)		0 039

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MIC value for *Candida pseudotropicalis* was determined to be 21 μM. Therefore, we concluded that peptide 9 is an antibacterial peptide with broad antibacterial specificity and that it is also effective for a certain fungus.

Secondary Structure of Peptide 9—It is known that sarcotoxin IA takes an α-helix conformation and interacts with bacterial membrane (*9, 24*). To deduce the secondary structure of peptide 9, we obtained its CD and NMR spectra. From its CD spectra, peptide 9 was shown to take an α-helical conformation in methanol or a 800 mM SDS solution, suggesting that a part of peptide 9 is an α-helix in the bacterial membrane (Fig. 1). To identify the α-helical region in peptide 9, we recorded TOCSY and NOESY NMR spectra of peptide 9 in methanol and on the basis of the spectra, the site specific assignments for the proton signals from peptide 9 were established. From the analyses of NOE data, we concluded that the main chain from Lys 6 to Leu 18 takes an α-helical conformation under the condition used for the present experiments. A wheel projection of residues 6–18 is shown in Fig. 2. These residues formed an amphiphilic helix with hydrophilic and hydrophobic faces, suggesting that peptide 9 is able to interact with the bacterial membrane.

TABLE II **Antimicrobial specificity of peptide 9.**

Microorganism	MIC value (μM)
Staphylococcus aureus (Cowan)	0 66
Staphylococcus aureus (Smith)	2 6
MRSA (MS16526)	10 5
Bacillus subtilis (PCI219)	2 6
Corynebacterium bovis (1810)	1 32
Escherichia coli (NIH J)	5 27
Salmonella typhi (T-63)	0 63
Shigella flexneri 40 (JS11811)	10 5
Pseudomonas aeruginosa (A3)	10 5
Klebsiella pneumoniae (PCI102)	2.6
Candida pseudotropicalis	21 1
Candida albicans 3147	>42 2
Candida albicans QC strain	>42 2

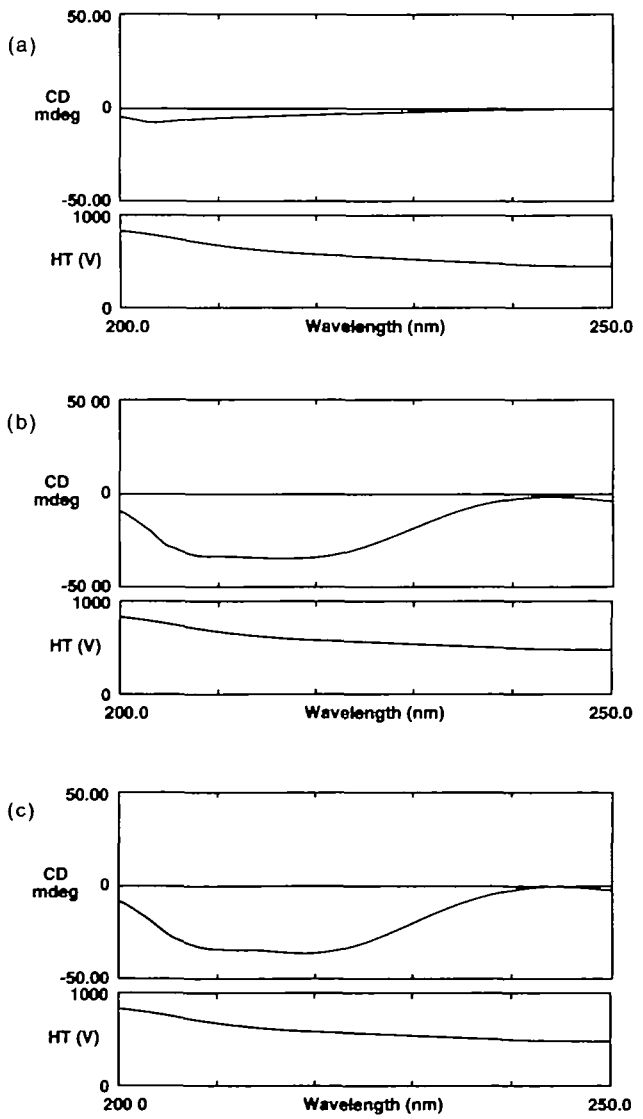

Fig 1. **CD spectra of peptide 9.** CD spectra of peptide 9 in (a) H_2O, (b) methanol, and (c) 800 mM SDS were recorded at 25°C

Change in the Permeability of Bacterial Membrane on Interaction with Peptide 9—As peptide 9 was suggested to interact with bacterial membrane, we supposed that the primary target of peptide 9 is the bacterial membrane. To confirm the functional damage to the bacterial membrane caused by peptide 9, we examined changes in the permeability of the bacterial membrane. For this, we measured the β-lactamase (periplasmic enzyme) and β-galactosidase (cytoplasmic enzyme) activities in *E. coli* (ML-35p) cells to see if the outer and inner membranes had become permeable to the substrates for these enzymes on treatment with peptide 9. *E. coli* (ML-35p) expresses these enzymes constitutively (*21, 22*).

Appreciable hydrolysis of PADAC (β-lactamase substrate) and ONPG (β-galactosidase substrate) was detected with time in the peptide 9-treated bacteria, but not in the control bacteria (Fig. 3), suggesting that the outer membrane becomes permeable to both substrates and the inner membrane becomes permeable at least to ONPG under these conditions. As expected, peptide 9 did not interfere with the activities of these enzymes in bacterial lysates. It should be noted that β-galactosidase became detectable after a lag period of about 10 min, whereas the lag period

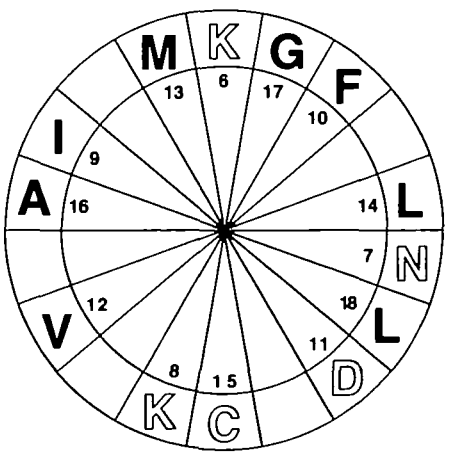

Fig. 2. **Wheel projection of the amino acid residues in the α-helical segment of peptide 9.** Open and closed characters indicate polar and non-polar residues, respectively. Residue numbers, from the amino terminal residue, are shown inside the wheel

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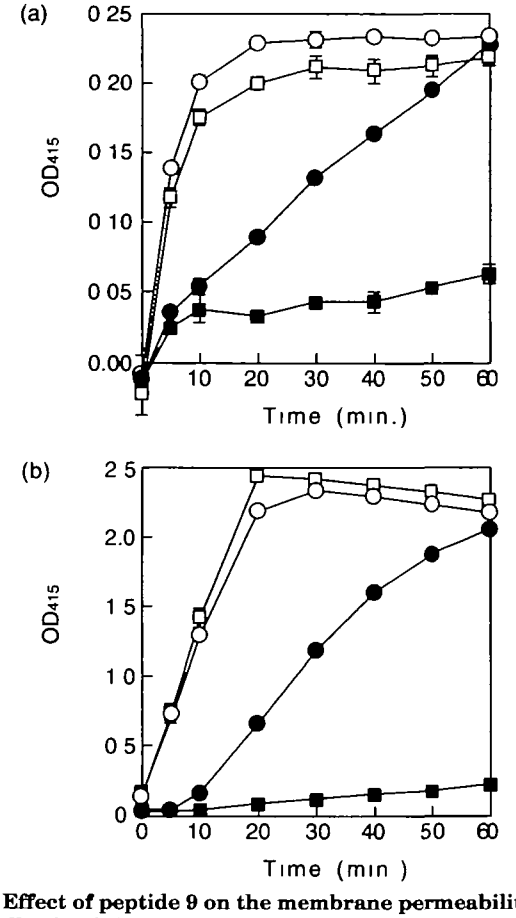

Fig 3 **Effect of peptide 9 on the membrane permeability of *E. coli* cells.** *E coli* (ML-35p) cells were incubated with peptide 9 (15 μM) and PADAC or ONPG, and then the increase in the optical density at 415 nm due to hydrolysis of each substrate was monitored. As controls, the β-lactamase and β-galactosidase activities of sonicated bacteria were assayed in the presence or absence of peptide 9 (a) Hydrolysis of PADAC by β-lactamase representing increased outer membrane permeability to PADAC; (b) hydrolysis of ONPG by β-galactosidase representing increased outer and inner membrane permeability to ONPG (■) Intact bacteria; (●) bacteria treated with peptide 9; (□) sonicated bacteria, (○) sonicated bacteria with peptide 9

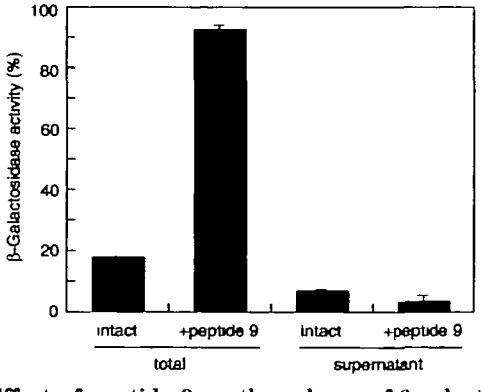

Fig 4. **Effect of peptide 9 on the release of β-galactosidase from the bacterial cytoplasm.** *E. coli* (ML-35p) cells were incubated in the presence or absence of peptide 9 (15 μM) for 1 h, and then ONPG was added and β-galactosidase activity was determined. At the same time, the bacterial suspension was centrifuged and β-galactosidase activity in the supernatant was determined. Enzyme activity is shown relative to that of sonicated bacteria.

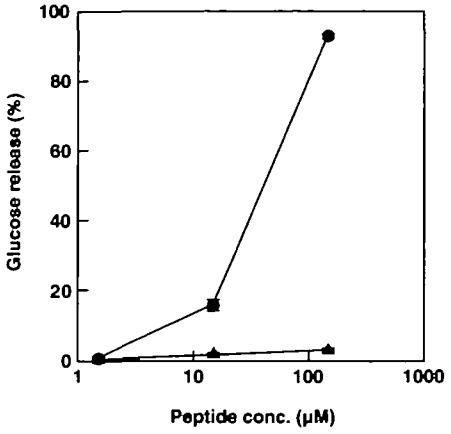

Fig 5 **Release of glucose from liposomes in the presence of peptide 9.** Liposomes containing glucose were prepared with phosphatidylethanolamine/phosphatidylglycerol/cardiolipin (7 2 1) Peptide 9 (●) or the C-terminal 7 residues (LCAGLVK) of peptide 9 (▲) were incubated with liposomes for 1h at room temperature. Then the amount of glucose in the supernatant was determined to calculate the percentage release of glucose from the liposomes.

for β-lactamase, if any, was much shorter. Possibly, it takes about 10 min for peptide 9 to disorganize the inner membrane, but disorganization of the outer membrane occurs almost instantaneously.

There remained a possibility that β-galactosidase leaked out of the cells when *E. coli* was treated with peptide 9. To examine this possibility, we centrifuged the cells after treatment with peptide 9 and then measured the β-galactosidase activity in the resulting supernatant. As shown in Fig. 4, β-galactosidase activity in the supernatant was negligible compared with that in the peptide 9–treated bacterial suspension. Thus, we concluded that macromolecules in the cytoplasm, including β-galactosidase itself, did not leak out of the cells when *E. coli* was treated with peptide 9.

As peptide 9 was shown to disorganize the bacterial inner membrane, we examined the direct interaction between peptide 9 and phospholipid vesicles consisting of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in a molar ratio of 7:2:1, which resembles the phospholipid composition of *E. coli*. As shown in Fig. 5, glucose entrapped in the vesicles was released when the vesicles were treated with increasing amounts of peptide 9, but a fragment of peptide 9, LCAGLVK, that was used as a negative control did not cause glucose release. Similar results were obtained with phospholipid vesicles consisting of phosphatidylglycerol and cardiolipin in a molar ratio of 3:1, which resembles the phospholipid composition of *S. aureus* (data not shown). These results suggest that peptide 9 directly interacts with bacterial membranes, resulting in their disorganization.

DISCUSSION

We arbitrarily divided the 26-kDa protease into 12 peptides and found that one of them, peptide 9, exhibited potent antibacterial activity comparable to that of the original enzyme. It is totally unknown whether this peptide is in fact the antibacterial domain of the 26-kDa protease. As reported before, the amino acid identity between the 26-

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kDa protease and bovine trypsin is 40% (*6*), but that between peptide 9 and the corresponding region of trypsin is much less, being only 30%. Moreover, this region is on the surface of the trypsin molecule in a tertiary structure model of it (*25*) Thus, it is likely that the sequence of peptide 9 is unique to the 26-kDa protease and this region potentially interacts with bacteria when the 26-kDa protease has been added to the bacterial suspension.

We demonstrated that the membrane permeability increased when *E. coli* was treated with peptide 9, and substrates for β-lactamase and β-galactosidase, PADAC and ONPG, diffused into the cells, passing through the outer and inner membranes. On the other hand, cytoplasmic β-galactosidase did not leak into the medium under the same conditions. An explanation for this is that peptide 9 forms channels with a pore size allowing the passage of PADAC and ONPG, but not β-galactosidase, through the bacterial membranes. However, intrinsic peptide 9 in the 26-kDa protease may not be able to form channels like synthetic peptide 9. Therefore, if we assume that the antibacterial mechanisms of peptide 9 and the 26-kDa protease are the same, channel formation is not realistic.

As residues 6 to 18 of peptide 9 form an amphiphilic α-helix, and two Arg residues exist in the N-terminal loop consisting of 5 residues, it is possible that positively charged N-terminal loops bind to acidic phospholipids, and that the hydrophobic faces of the α-helices interact with bacterial membranes, resulting in their perturbation. Possibly, an increase in permeability of the bacterial membranes is induced as a result of interaction between peptide 9 and acidic phospholipids in the membranes. In fact, the permeability of phospholipid vesicles increased when peptide 9 interacted with them Consistent with this fact, we previously demonstrated that the 26-kDa protease selectively binds to acidic phospholipids, unlike bovine trypsin (*11*).

Although several synthetic peptides based on the sequences of serprocidins have been reported to have antimicrobial activity (*26–30*), no sequence exhibiting homology to that of peptide 9 is present among them. Moreover, the antibacterial domains of serprocidins seem to differ from each other. Therefore, even in the case of closely related antibacterial proteins, the antibacterial domains can not be deduced from their sequences. The 26-kDa protease contained two more antibacterial peptides (peptides 11 and 12) in its C-terminal region, although their antibacterial activities were less than that of peptide 9. There remains a possibility that these peptides also participate in the antibacterial activity of the 26-kDa protease.

From a chemotherapeutic viewpoint peptide 9 is interesting, because it shows antibacterial activity against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, which often cause opportunistic infections. Moreover, no hemolytic activity was detected for peptide 9 and it was effective even in the presence of 180 mM NaCl (data not shown). Peptide 9 may provide a clue as to novel peptide antibiotics.

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