Characterization of the antimicrobial peptide derived from sapecin B, an antibacterial protein of *Sarcophaga peregrina* (flesh fly)

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Sapecin B, an antibacterial protein of *Sarcophaga peregrina*, was divided into four peptides. A hendecapeptide derived from its helix region was found to have comparable antibacterial activity with that of the complete protein. This peptide had a much wider spectrum of antimicrobial activity than that of sapecin B, exhibiting activity on not only *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative), but also some

yeasts, including *Candida albicans*. The peptide was shown to bind to liposomes containing acidic phospholipids and cause release of entrapped glucose, suggesting that its primary site of action is the bacterial membrane. Its antimicrobial activity could be increased by substituting various amino acid residues for hydrophobic and/or basic ones.

INTRODUCTION

Sapecin is a potent antibacterial protein originally isolated from the culture medium of an embryonic cell line, NIH-Sape-4, derived from *Sarcophaga peregrina* (flesh fly) [1,2]. It consists of 40 amino acid residues and has bactericidal activity against various Gram-positive and Gram-negative bacteria, although in general its activity on the latter is lower. It has been shown to have strong affinity for cardiolipin, an acidic phospholipid in bacterial membranes [3]. Its homologues have been found in other dipteran insects [4,5]. So sapecin seems to constitute a protein family that are the main constituents of the humoral immunity system in many insects [6].

From ¹H-n.m.r. studies, Bontems et al. and other workers have pointed out that charybdotoxin, a toxin acting on K⁺ channels isolated from the venom of a scorpion, and sapecin share a common structural motif of an α -helix and β -sheet linked by three intramolecular disulphide bonds [7–9]. Recently, we isolated two sapecin homologues from a culture medium of NIH-Sape-4 from which we also isolated sapecin, and found that the molecular mass and amino acid sequence of one of them, named sapecin B, were very similar to those of charybdotoxin [10].

To determine the active site, if any, of sapecin B, essential for its antibacterial activity, we divided sapecin B into four regions according to the structural model of charybdotoxin [9] and synthesized four amidated peptides corresponding to these regions. We found that one of these peptides, consisting of 11 amino acid residues forming the α -helix region of sapecin B, showed similar antibacterial activity to that of the intact sapecin B. This α -helical region of sapecin B is probably responsible for the antibacterial activity, while other regions participate in determination of the antibacterial specificity of sapecin B, as this peptide showed a significantly different spectrum of antibacterial activity from that of sapecin B.

MATERIALS AND METHODS

Sapecin B and amidated peptides

Sapecin B was purified from the culture medium of NIH-Sape-

4 cells as described previously [10]. Amidated peptides were synthesized using the solid-phase method in a peptide synthesizer (Applied Biosystems, model 430A). Each peptide was purified to homogeneity by h.p.l.c. using a reverse-phase column of Synchropak RP-R (C₁₈) and its amino acid sequence was confirmed using a protein sequencer (Applied Biosystems, model 477A).

Assay of antimicrobial activity

Antimicrobial activity against bacteria was assayed as described previously using *Staphylococcus aureus* ATCC 65388P and *Escherichia coli* K-12 594 as indicator bacteria [1]. Bacterial growth, in the presence and absence of the test sample, was assessed by measuring the A_{650} of the medium after incubation for 180 min at 37 °C. Antifungal activity against various yeasts was assayed in essentially the same way as antibacterial activity. Yeasts were suspended at a density of 2×10^5 cells/ml in Sabouraud medium in the presence and absence of the test sample and incubated for 24 h at 30 °C; the A_{650} value was then measured. For assay of candidacidal activity, *Candida albicans* ATCC 36232 cells were suspended in Sabouraud medium at a density of 2×10^5 cells/ml in the presence and absence of the test sample and incubated for 1 h at 37 °C. Then the *Candida* suspension was diluted and plated on Sabouraud agar medium, and after incubation for 2 days at 37 °C, numbers of colonies were counted.

Identification of free thiol groups in the 7R–17K fragment of sapecin B

For determination of whether the two cysteine residues in the 7R–17K fragment form a disulphide bridge, the reactivities of the peptide with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) before and after its reduction were compared according to the method of Ellman [11]. Briefly, samples of less than 5 nmol of the peptide were treated in 1 M NaBH₄ solution, or water containing 0.1 M NaOH and 20 mM EDTA, for 1 h at 37 °C. Then 30 μ l of 6 M HCl and 100 μ l of 2 M Tris/HCl buffer were added to adjust the reaction mixture to pH 8.2. The solutions were then mixed

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); mBBR, 4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; CL, cardiolipin; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdGly, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine.

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with 20 μ l of 0.01 M DTNB solution and their A_{412} values were measured.

Preparation of liposomes containing trapped glucose and assay of their release of glucose

Liposomes were prepared by the method of Kinsky et al. [12]. Briefly, a single phospholipid or a mixture of phospholipids was dissolved in chloroform, and an aliquot containing 1 μ mol of phospholipids was dried completely in a rotary evaporator under reduced pressure. Then 0.1 ml of 0.3 M glucose solution was added and the lipids were dispersed with the aid of a vortex mixer to obtain multilamellar liposomes containing glucose. The liposomes were then washed twice with 0.4 ml of 5 mM Veronal buffer, pH 7.5, containing 0.15 M NaCl (Veronal-buffered saline) to remove untrapped glucose, and finally suspended in 0.5 ml of the same buffer.

In tests on the sensitivity of liposomes to peptides, various concentrations of the peptides dissolved in 10 μ l of 10 mM phosphate buffer, pH 6.0, containing 130 mM NaCl were added to 10 μ l of the liposome suspension. The mixtures were incubated at 37 °C for 30 min and then the amount of released glucose was determined [13]. Phospholipids in the liposomes were measured by determining the amount of phosphate by the method of Chen et al. [14]. Glucose released from the liposomes was measured with a Glucose C-Test kit (Wako Chemical Co., Japan), consisting of phenol, 4-aminoantipyrine, mutarotase, glucose oxidase and peroxidase. A red-coloured quinone derivative was formed with phenol and 4-aminoantipyrine in the presence of glucose, and from the absorbance at 492 nm of this compound, the amount of glucose in the reaction mixture was determined [3].

Assay of binding of the 7R–17K fragment to liposomes

This assay was performed in 100 μ l of Veronal-buffered saline containing 0.3 % BSA. The reaction mixture contained various amounts of liposomes and 0.42 nmol of the 7R–17K fragment labelled with 4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione (mBBR). After incubation for 1 h at 37 °C, the liposomes were collected by centrifugation for 10 min at 10000 g and washed twice with 100 μ l of Veronal-buffered saline containing 0.3 % BSA by repeated centrifugation. They were then suspended in 100 μ l of Veronal-buffered saline and solubilized by adding 10 μ l of 20 % (v/v) Triton X-100. The volume was increased by adding 0.9 ml of Veronal-buffered saline and the fluorescence of mBBR due to the liposome-bound 7R–17K fragment was measured with excitation and emission wavelengths of 385 nm and 479 nm respectively. The recovery of liposomes during this process, calculated from the recovery of phospholipids, was about 80 %.

Fluorescence-labelling of the 7R–17K fragment with mBBR

Fluorescence-labelling of the 7R–17K fragment was performed by modifying its cysteine residues with mBBR by the method of Fahey et al. [15]. Briefly, 0.1 μ mol of the 7R–17K fragment was dissolved in 40 μ l of Hepes/HCl (pH 8.0) containing 5 mM diethylenetriamine-*NNN'*-*N''*-penta-acetic acid and 2 mM mBBR, and kept in the dark for 10 min at room temperature to allow the reaction to proceed. Then 2 μ l of methanesulphonic acid was added to terminate the reaction and the resulting mBBR-labelled 7R–17K fragment was purified by h.p.l.c. in a

Synchropak RP-P (C_{18}) column. The fluorescence-labelled 7R–17K fragment was dissolved in distilled water and stored at 4 °C.

Assay of haemolytic activity

Packed sheep erythrocytes (0.2 ml) were washed three times with 0.8 ml of PBS (130 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) by repeating centrifugation at 2000 g for 5 min, and were resuspended in 10 ml of the same PBS solution. The resulting erythrocyte suspension (25 μ l) and sample solution (25 μ l) containing various concentrations of the peptides were mixed and incubated at 37 °C for 2 h. After centrifugation at 2000 g for 5 min, the A_{576} values of the supernatants were measured. Melittin (Boehringer–Mannheim) was used as a positive control.

RESULTS

Preparation of four fragments of sapecin B and their antibacterial activities

From structural comparison of sapecin B and charybdotoxin, amino acid residues 7–17 of sapecin B were expected to form a helical structure and residues 29–34 to form a sheet structure in solution. We synthesized four peptides corresponding to amino acid residues 1–6, 7–17, 18–28 and 29–34 of sapecin B having amidated amino acid residues at their C-terminal, as shown in Figure 1, and examined their antibacterial activities against *S. aureus*. Only the 7R–17K fragment (amino acid residues 7–17 of sapecin B) repressed the growth of *S. aureus*, its antibacterial activity being almost comparable with that of sapecin B: their ID_{50} values (concentrations for 50 % growth inhibition) being 0.7–1.5 μ M. The three other peptides had essentially no antibacterial activity at concentrations less than 10 μ M (Figure 2). These results suggested that the amino acid sequence of residues 7–17 of sapecin B is important for its antibacterial activity.

As reported previously, sapecin B is toxic to Gram-positive bacteria such as *S. aureus*, but its antibacterial effect on Gram-negative bacteria such as *E. coli* is not so marked [10]. However, as summarized in Table 1, the 7R–17K fragment repressed the growth of *E. coli*, as well as that of *S. aureus*. Surprisingly, this fragment also repressed the growth of a fungus, *Candida albicans*,

Figure 1 Fragmentation of sapecin B and synthetic peptides

Sapecin B was divided into four parts and amidated peptides shown in boxes corresponding to each fragment were synthesized. The 7R–17K fragment and the 29V–34Q fragment correspond to the helix region and sheet region of sapecin B respectively. The α -carboxyl group of glutamine in the synthetic 29V–34Q fragment was not amidated.

Figure 2 Antibacterial activities of synthetic peptides

The antibacterial activities of amidated fragments of sapecin B were measured using *S. aureus* ATCC 36232 as a target bacterium. Increasing amounts of test samples were added to the medium and relative bacterial growth was determined after incubation for 180 min at 37 °C. ●, Sapecin B; □, 1L-6D fragment; ○, 7R-17K fragment; △, 18G-28K fragment; ▽, 29V-34Q fragment.

Table 1 Comparison of ID₅₀ values of the 7R-17K fragment and sapecin B against various micro-organisms

Strain	7R-17K fragment (μM)	Sapecin B (μM)
<i>S. aureus</i> ATCC 6538P	1.5	0.7
<i>E. coli</i> K-12 594	3.0	> 50
<i>C. albicans</i> ATCC 36232	15.0	> 80

although the ID₅₀ value of this activity was one order of magnitude higher than that of its antibacterial activity.

To determine whether the growth inhibition of *C. albicans* by the 7R-17K fragment is due to a fungicidal effect or simply a fungistatic effect, we counted viable cells. For this, a fixed number of *C. albicans* cells were treated with increasing amounts of the 7R-17K fragment for 1 h and then plated. As shown in Figure 3, numbers of colonies decreased markedly with increases in the peptide concentration, indicating that this peptide has fungicidal activity. We also found that the viable numbers of *S. aureus* and *E. coli* cells decreased drastically from 2×10^5 to 2×10^2 on their treatment with 10 μM of this peptide. Thus, the 7R-17K fragment seems to be toxic to various micro-organisms, and kills at least the two bacteria and one fungus so far tested.

Interaction of the 7R-17K fragment with phospholipids

Previously, we have found that sapecin interacts with cardiolipin (CL) in bacterial membranes and that the susceptibilities of bacteria to sapecin partly depend on their content of this phospholipid [3]. Therefore, we examined whether the 7R-17K fragment interacted with bacterial phospholipids. A fluorescence-labelled 7R-17K fragment was necessary for this experiment, so we first examined whether the two thiol groups in the 7R-17K

Figure 3 Candidacidal activity of the 7R-17K fragment

Increasing amounts of the 7R-17K fragment were incubated with a fixed number of *C. albicans* ATCC 36232 cells (2×10^5 cells/ml) suspended in Sabouraud medium for 1 h at 37 °C. Then the cell suspension was plated after dilution, incubated for 2 days at 37 °C, and numbers of colonies formed were counted.

fragment are free or form a disulphide bridge. For this, we compared the reactivity of this fragment with DTNB with and without reduction with NaBH₄. The reactivity of this fragment with DTNB was not affected by its reduction, and was twice that of reduced glutathione on a molar basis, indicating that the two thiol groups in the 7R-17K fragment are free. Therefore, we labelled this fragment with mBBBr, which reacts with free thiol groups in a peptide, and purified the resulting labelled 7R-17K fragment to homogeneity using h.p.l.c. The labelled fragment showed no reactivity with DTNB, indicating that its two thiol groups were labelled with mBBBr. This was confirmed by sequencing the labelled peptide.

We examined the binding of labelled 7R-17K fragment to phospholipids. Liposomes were prepared by mixing phosphatidylcholine (PtdCho) and various other phospholipids in molar ratios of 0.6 to 0.4. A fixed amount of the labelled 7R-17K fragment was incubated with increasing amounts of liposomes, the liposomes were then washed well, and the amount of bound peptide was measured by fluorescence spectrophotometry. mBBBr itself had no affinity for any of the liposomes used, so the fluorescence detected was due to the bound 7R-17K fragment. As shown in Figure 4, significant binding was detected with all acidic phospholipids except phosphatidylserine (PtdSer). These results indicate that the binding specificity of this peptide is not so strict as that of sapecin, which binds exclusively to CL, but the binding efficiency to CL was higher than those to phosphatidylinositol (PtdIns) and phosphatidylglycerol (PtdGly). The fragment showed no significant binding to neutral phospholipids such as phosphatidylethanolamine (PtdEtn) and PtdCho.

As reported previously, *E. coli* is less sensitive than *S. aureus* to sapecins; this is partly explained by the difference in their membrane contents of CL [1,3,10]. However, as the 7R-17K fragment of sapecin B was active against both *S. aureus* and *E. coli* and bound to various acidic phospholipids, we examined whether it disrupted liposomes. For this, we prepared two types of liposomes with similar phospholipid compositions to those of the membranes of *S. aureus* and *E. coli* respectively, each containing trapped glucose, and examined the effects of the 7R-17K fragment and sapecin B on them by measuring the release of this glucose. As shown in Figure 5, an increase in the

Figure 4 Binding of the 7R-17K fragment to liposomes

Liposomes were prepared by mixing PtdCho and various single phospholipids in molar ratios of 0.6 to 0.4 and the bindings of increasing amounts of these liposomes to a fixed amount of fluorescence-labelled 7R-17K fragment were measured. After incubation of liposomes with the fluorescence-labelled 7R-17K fragment, the liposomes were washed well. Emission of fluorescence was measured at 479 nm with excitation at 385 nm, after solubilizing the liposomes with Triton X-100. The phosphorus content of 10 μ l of liposome suspension varied from 14 to 17 nmol depending upon the phospholipid. $\circ$, PtdGly; $\triangle$, CL; $\square$, PtdIns; $\blacksquare$, PtdCho; $\blacktriangle$, PtdSer; $\bullet$, PtdEtn.

Figure 5 Release of glucose from liposomes in the presence of the 7R-17K fragment or sapecin B

Two types of liposomes with entrapped glucose were prepared from PtdEtn, PtdGly and CL. Their phospholipid compositions resembled those of *S. aureus* (molar ratio of PtdGly:CL, 3:1, v/v) and *E. coli* (PtdEtn:PtdGly:CL, 7:2:1, by vol.). Increasing amounts of the 7R-17K fragment (a), or sapecin B (b) were incubated with these liposomes for 1 h at 37 °C. Then the amount of glucose released from the liposomes was measured. Phospholipid composition: $\circ$, like that of *S. aureus*; $\triangle$, like that of *E. coli*.

Table 2 Antibacterial activities of synthetic analogues of the 7R-17K fragment

Peptide	Sequence	ID ₅₀ (μ M)	
		<i>S. aureus</i> ATCC 6538P	<i>E. coli</i> K-12594
7R-17K	RSLCLLHCRLK-NH ₂	1.5	3.0
Analogue 1	RSLCLLHCRLK-COOH	8.6	14.0
Analogue 2	RSLLLHLRLK-NH ₂	0.3	1.9
Analogue 3	RLKCLLLCRLK-NH ₂	0.2	1.5
Analogue 4	RLKCLLLCRLK-COOH	0.4	2.2
Sapecin B		0.7	> 50.0

concentration of 7R-17K fragment of sapecin B resulted in an increase in the release of glucose from liposomes formed from PtdGly and CL in a molar ratio of 3:1, resembling the phospholipid composition of *S. aureus*. However, the effective dose causing 50% disruption of the liposomes (ED₅₀) value of the 7R-17K fragment was one-tenth of that of sapecin B. As expected, the 7R-17K fragment also induced the release of glucose from liposomes with the phospholipid composition of *E. coli* membranes (molar ratio of PtdEtn, PtdGly and CL, 7:2:1, by vol.), although its ED₅₀ value for these liposomes was much higher than that for *S. aureus*-membrane-type liposomes. No appreciable release of glucose was detected when these liposomes were treated with sapecin B, even at concentrations of above 100 μ g/ml. These results are consistent with the antibacterial activities of these compounds shown in Table 1. Thus, we conclude that the antibacterial effect of the 7R-17K fragment is due to its direct interaction with the bacterial membranes.

Antibacterial activities of analogues of the 7R-17K fragment

The above results showed that the 7R-17K fragment of sapecin B had antimicrobial activity, and that this activity seemed to be due to its interaction with acidic phospholipids in bacterial membranes. As this fragment presumably interacts with bacterial membranes via its hydrophobic amino acid residues and/or positively charged amino acid residues, we thought that its antibacterial activity might be increased by changing these amino acid residues. To test this possibility, we synthesized four analogues of this peptide and examined their antibacterial activities. Analogue 1 was essentially the same as the 7R-17K

Table 3 Surface activities of antimicrobial peptides

Peptides or SDS were dissolved in distilled water at concentrations of 0.154 mM. Surface tensions were measured in a platinum ring connected to a precision electrobalance at 25 °C by the method of Harkins and Jordan [16].

Sample	Surface tension (10 ⁻⁵ N/cm)
7R-17K fragment	65.91
Analogue 1	66.88
Analogue 2	61.95
Analogue 3	61.75
Analogue 4	62.29
SDS	68.90
Distilled water	72.29

Table 4 Antifungal activities of the synthetic 7R–17K fragment, its analogues and sapecin B against various yeasts

Organism		Minimum inhibitory concentration (μM)				
		7R–17K	Analogue 2	Analogue 3	Analogue 4	Sapecin B
<i>Candida albicans</i>	ATCC 36232	50	6	12	25	> 80
<i>Candida lipolitica</i>	IFO 0717	12	6	6	25	> 80
<i>Pichia pastoris</i>	IFO 0948	12	6	6	25	> 80
<i>Rhodotorula glutinis</i>	IFO 1099	1.5	1.5	1.5	3	> 80
<i>Saccharomyces cerevisiae</i>	IFO 1136	50	6	25	50	> 80

Figure 6 Haemolytic activities of antimicrobial peptides

Sheep erythrocytes were incubated with increasing amounts of each peptide for 2 h at 37 °C. After centrifugation, the A_{576} values of the resulting supernatants were measured. Complete lysis was achieved by incubating the cells in distilled water. The peptides used were: $\square$, analogue 1; ∇ , analogue 2; $\triangle$, analogue 3; $\circ$, analogue 4; $\diamond$, the 7R–17K fragment; $\blacksquare$, melittin (positive control).

fragment except that the C-terminal lysine was not amidated. In analogue 2, the two cysteine residues of the 7R–17K fragment were replaced by leucine residues to increase hydrophobicity. In analogue 3, the third leucine residue was replaced by lysine to increase electropositivity in the N-terminal region, and the second serine and seventh histidine residues were replaced by leucine residues to increase hydrophobicity. Analogue 4 was the same as analogue 3 except that its C-terminal lysine was not amidated.

As shown in Table 2, the antibacterial activities of analogues 2 and 3 against *S. aureus* were 5–7-fold greater than that of the 7R–17K fragment. However, their antibacterial activities against *E. coli* were at most twice that of the original peptide. Judging from the activities of the 7R–17K fragment and analogue 1, amidation of the C-terminal lysine residue of the peptide seemed to increase the activity. A similar tendency was observed with analogues 3 and 4.

As these peptides are supposed to interact with bacterial membranes, they are expected to have high surface activity. To confirm this, we measured the surface tensions of the peptide solutions at concentrations of 0.154 mM by the method of Harkins and Jordan [16]. These solutions had lower surface tensions than that of SDS solution, as shown in Table 3, and a good correlation was observed between the surface tension and antibacterial activity (cf. Table 2).

As the 7R–17K fragment had antifungal activity against *C. albicans*, we examined the effects of these analogues on various fungi. For this, we determined the minimum inhibitory concentration of each peptide. As summarized in Table 4, all these peptides significantly inhibited growth of the fungi tested, but the antifungal activities of analogues 2 and 3 were higher than those of other peptides. In particular, their antifungal activities against *C. albicans* were 4–8-fold greater than that of the 7R–17K fragment. Of these fungi, *Rhodotorula glutinis* was especially sensitive to these peptides, the minimum inhibitory concentration being below 3 μM . Peptides having much higher antibacterial and/or antifungal activity could probably be designed on the basis of these results.

As these peptides are surface-active and repress the growth of bacteria and fungi, we thought that they could also be effective on mammalian cells. To test this possibility, we examined their haemolytic activities on sheep erythrocytes. Contrary to expectation, no appreciable haemolytic activity was detected under conditions in which melittin caused significant haemolysis (Figure 6). Thus, these peptides are probably not so effective on mammalian cells despite having potent antimicrobial activity.

DISCUSSION

Several antibacterial proteins have been isolated from various insects and characterized [17–23]. The primary targets of most of these proteins are thought to be the bacterial membrane. In fact, we demonstrated that sarcotoxin I (a cecropin-type antibacterial protein of *Sarcophaga*) has ionophore activity, causing loss of the membrane potential of *E. coli* [24]. We also demonstrated that sapecin specifically interacts with CL in bacterial membranes [3]. Several attempts have been made to modify the activities of these proteins. A hybrid analogue of cecropin A and D, and the D-enantiomers of cecropin A, magainin-2-amide and melittin were synthesized chemically [25,26]. Cecropin–melittin hybrids were also synthesized [27]. Rosenthal et al. substituted arginine residues in dipterocins by canavanine, an arginine antagonist [28]. However, there are no reports of fragmentation of a known antibacterial protein to identify its active site.

In this study, we synthesized four amidated peptides which together constitute the whole of sapecin B, and found that one peptide corresponding to the helix region of sapecin B, as judged by analogy to charybdotoxin, had potent antibacterial activity. The antibacterial activity of this hendecapeptide against *S. aureus* was almost comparable with that of intact sapecin B. However, unlike sapecin B, this peptide also showed antimicrobial activity against *E. coli* and various yeasts including *C. albicans*. This finding suggests two possibilities: one is that the 7R–17K fragment is the active site of sapecin B and so sapecin B is

potentially toxic to both Gram-positive and Gram-negative bacteria, and also to fungi, but that its other regions interfere with the interaction with Gram-negative bacteria and/or fungi, and so it shows antibacterial activity only against Gram-positive bacteria. The other possibility is that sapecin B is active as a complete molecule, but that the 7R–17K fragment incidentally shows antimicrobial activity, so the antimicrobial activities of intact sapecin B and this peptide are independent. At present, it is difficult to know which possibility is correct, as the antimicrobial specificities of the two substances are different. On treatment of *S. aureus*-membrane-type liposomes with the 7R–17K peptide or sapecin B, entrapped glucose was released. The concentrations of the peptide causing liposome disruption and inhibiting bacterial growth were almost the same. However, the concentration of sapecin B causing liposome disruption was higher than that inhibiting bacterial growth. These facts support the latter possibility. If the former possibility is correct, it may be possible to identify the active sites of other antibacterial proteins such as sarcotoxin I in the same way.

It is noteworthy that the 7R–17K fragment showed antifungal activity as well as antibacterial activity. Several groups have reported isolations of small antibacterial peptides from various organisms: tachyplesin (17 amino acid residues) [29], indolicidin (13 amino acid residues) [30] and bactenecin (12 amino acid residues) [31]. However, none of these peptides showed antifungal activity. Small peptides having potent antifungal activity such as the 7K–17R fragment may be potentially important as lead compounds to design better antifungal agents.

We showed that the antimicrobial activity of the 7R–17K fragment could be increased by changing a few of its amino acid residues. For this, we synthesized analogues of this peptide with reference to hydrophobic and positively charged amino acid residues. We found a correlation between the antibacterial activities and surface activities of these peptides. As their surface activities were due to their amphipathic characters, the amphipathicities of these peptides seemed to be related to their antibacterial activities. We also found that amidated peptides had higher activity than non-amidated ones. Probably, amidated peptides are more resistant to microbial proteinases than non-amidated ones and this may explain their higher activities.

This work was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science and Culture of Japan, and by a Grant-in-Aid BMP 93-IV-2-8 (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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