Identification and characterization of the antimicrobial peptide corresponding to C-terminal β-sheet domain of tenecin 1, an antibacterial protein of larvae of *Tenebrio molitor*

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An active fragment was identified from tenecin 1, an antibacterial protein belonging to the insect defensin family, by synthesizing the peptides corresponding to the three regions of tenecin 1. Only the fragment corresponding to the C-terminal β-sheet domain showed activity against fungi as well as Gram-positive and Gram-negative bacteria, whereas tenecin 1, the native protein, showed activity only against Gram-positive bacteria. CD spectra indicated that each fragment in a membrane-mimetic environment might adopt a secondary structure corresponding to its region in the protein. The leakage of dye from liposomes induced by this fragment suggested that this fragment acts on the membrane of pathogens as a primary mode of action. A comparison between the structure and the activity of each fragment indicated that a net positive charge was a prerequisite factor for activity. To the best of our knowledge this is the first report in which the fragment corresponding to the β-sheet region in antibacterial proteins, which consists of α-helical and β-sheet regions, has been identified as a primary active fragment.

INTRODUCTION

The recent emergence of multidrug-resistant bacteria has stimulated the development of novel antibacterial molecules with unexploited mechanisms of action (reviewed in [1]). A large number of peptides produced in the host defence system have been isolated and their functions characterized [2–5]. The mode of biological action of these molecules suggests that interactions between peptides and lipid membranes of cells have a major role in their function [6–8]. In spite of the diversity of the amino acid sequence, most antibacterial peptides and proteins have a net positive charge and amphiphilic α-helical or β-sheet structures, which are regarded as structural requirements for biological activity [9–11]. Structurally, the antibacterial peptides can be divided into linear and cyclic forms with disulphide bridge(s). Generally, disulphide bridge(s) in proteins and peptides are regarded as an important factor in their structure and biological activity. However, for antibacterial peptides the requirement of disulphide bridge(s) for activity is not clear: some antibacterial peptides did not lose activity by a reduction of disulphide bridges; other peptides lost activity [12,13]. Many studies have been performed to identify a linear active fragment from the cyclic antibacterial peptides with disulphide bridges through random digestion by enzymes [12,13], chemical modification [14] or the random synthesis of the truncated form of the native peptides [14,15]. However, these approaches were lacking in rational design for the screening of an active fragment and thus demanded much labour and time for the identification process.

The insect defensin family of proteins, which have molecular masses of 3–4 kDa and three disulphide bridges, showed strong bactericidal activity against various Gram-positive bacteria and less activity against Gram-negative bacteria [16,17]. NMR studies [18–20] have shown that the insect defensin family shares a common structural feature of an N-terminal loop and a short amphipathic α-helix followed by a C-terminal anti-parallel β-sheet structure connected by disulphide bridges. Among the insect defensin family, tenecin 1 isolated from the haemolymph of larvae of the coleopteran *Tenebrio molitor* has a potent bactericidal activity against Gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus* [21].

In the present study, a tentative structure of tenecin 1 was modelled from the tertiary structure of insect defensin A [19], which has a highly similar primary amino acid sequence and the same disulphide bridge connectivity. Considering the amphipathic secondary structure as a prerequisite factor for antibacterial activity, we divided tenecin 1 into three regions and synthesized the peptides corresponding to the 15-mer N-terminal loop, the 11-mer α-helix and the 15-mer β-sheet region. CD spectra indicated that each fragment of tenecin 1 in the presence of trifluoroethanol (TFE) might resemble the secondary structure corresponding to its region of tenecin 1. However, the fragment corresponding to the α-helix and N-terminal loop regions did not show antibacterial activity, whereas the fragment corresponding to the C-terminal β-sheet region was identified as an active fragment. Interestingly, this active fragment showed activity against fungi as well as Gram-positive and Gram-negative bacteria, whereas tenecin 1, the native protein, showed activity only against Gram-positive bacteria. Further truncation and modification of this active fragment provided information about the minimal peptide length and amino acid residues necessary for antibacterial and antifungal activity.

MATERIALS AND METHODS

Peptide synthesis

Each peptide was prepared by stepwise solid-phase synthesis on an Applied Biosystems model 431A automatic peptide synthesizer. The peptide chain was assembled on 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid resin. All fluorenylmethoxy-

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; DPX, *p*-xylylenebis(pyridinium) bromide; TFE, trifluoroethanol.

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carbonyl amino acid derivatives were purchased from Novabiochem (San Diego, CA, U.S.A.). The fluorenylmethoxycarbonyl group was employed for the protection of the α-amino group; the side chain protections were triphenylmethyl for Cys, Gln, Asn and His, t-butyl for Thr, Glu, Ser and Asp, t-butyloxycarbonyl for Lys, and 2,2,5,7,8-pentamethylchroman-6-sulphonyl for Arg. Cleavage of the peptide from the resin was achieved by treatment with a mixture of trifluoroacetic acid/thioanisole/ethane dithiol/water (80:5:2.5:5, by vol.) at room temperature for 12 h. After filtration and washing of the resin with trifluoroacetic acid, a gentle stream of nitrogen was used to remove excess trifluoroacetic acid. The crude peptide was triturated with diethyl ether chilled to -20 °C and then centrifuged at 3000 ***g*** for 10 min at -10 °C. The synthetic peptide was purified by preparative HPLC with a Vydac C_{18} column. Amino acid analysis and electrospray ionization MS on Platform II from Micromass (Manchester, U.K.) were used for the further characterization of the purified peptide.

Identification of free thiol group and synthesis of intra-disulphide bridge(s)

The Ellman assay and HPLC–MS were used to characterize the redox state of the cysteine residue in the peptide. If necessary, purified peptides were reduced with 50 mM of dithiothreitol (Aldrich, Milwaukee, WI, U.S.A.) in 10 mM phosphate buffer, pH 7.4, at 50 °C for 4 h and then purified by HPLC. Linear tenecin 1 was synthesized and purified by the method described above. The oxidation of linear tenecin 1 was performed by using the glutathione system (reviewed in [22]), and disulphide pairs of oxidized tenecin 1 were determined by the same method as described for the characterization of sapecin B [3]. The intra-disulphide bridge of TE-(17-27) was prepared as follows. Linear TE-(17-27) was diluted below 20 μM in 0.01 M phosphate buffer, pH 6.8, in the presence of 5 % (v/v) DMSO (Aldrich) [23]. After being stirred at room temperature, the oxidation of TE-(17-27) was monitored by the Ellman assay. After completion of the oxidation, the excess solvent was removed by rotary evaporator and the peptide was purified by HPLC.

Antimicrobial assay

Antimicrobial assay *in vitro* was performed with a modified microdilution technique [24,25] in a 96-well microplate (Nunc, Roskilde, Denmark). Antibiotic medium 3 (M3; pH 7.0 at 25 °C; Difco) was used as the antibacterial assay medium. Freshly grown cells on antibiotic medium 3 agar plates were suspended in physiological saline at 10^4 cells/ml of 2-fold concentrated medium and used as the inoculum. Just before the assay, solid samples were dissolved, added to the wells (100 μl per well) and serially diluted 1:1 (by vol.). After inoculation (100 μl per well, 5×10^3 cells per ml), the plates were incubated at 37 °C for 24 h and the absorbance at 620 nm was measured with an ELISA reader (Spectra; SLT, Salzburg, Austria) to assess cell growth. The antifungal assay was done in Sabraud/2 % (w/v) dextrose broth (SB; pH 5.6 at 25 °C; Merck) and the plates were incubated at 30 °C for 24 h.

Molecular modelling of tenecin 1

Tenecin 1 has 66.7 % sequence identity with insect defensin A, whose structure was solved by two-dimensional NMR (pdbica.ent; Brookhaven Protein Data Bank, entry code 1 ICA). On the basis of the structure of insect defensin A, the structure of tenecin 1 was preliminarily designed by using the homology module of Insight II/Discover (Biosym, San Diego, CA, U.S.A.). The steepest descent, conjugated gradient and Va09a minimization algorithms sequentially refined the structure of tenecin 1. All interactions were calculated by using the consistent valence force field (CVFF) potential functions; the dielectric constant of electrostatic interaction was set at 1. The cutoff of 0.1 nm was employed to decrease the calculation time of non-bonded interactions. The refined structure is shown in a ribbon diagram (see Figure 2).

Haemolytic assay

The detailed method has been described elsewhere [26]. Packed mouse erythrocytes were washed three times with buffer [150 mM KCl/5 mM Tris/HCl (pH 7.4)] and then suspended in 10 volumes of the same buffer (stock cell suspension). The stock cell suspension was diluted 1:25 with the same buffer, preincubated at 37 °C for 15 min and then mixed with increasing amounts of the test samples. After incubation for 1 h at 37 °C, the samples were centrifuged at 4000 ***g*** for 5 min and the absorbance of the supernatant was determined at 540 nm. Haemolysis effected by 0.1 % (v/v) Triton X-100 was taken as 100 %.

CD measurements

CD spectra were recorded on a Jasco 715 spectropolarimeter (Tokyo, Japan) with a quartz cell of 1 mm path length. Sample solutions were prepared as follows: peptide powder was dissolved in TFE or distilled water, filtered and mixed with the same volume of 20 mM sodium phosphate buffer, pH 7.4. The concentration of each peptide was determined on the basis of the amino acid analysis and liquid chromatography–electrospray ionization MS data. CD spectra were measured from 250 to 190 nm at 22 °C. The CD data were expressed as mean residue ellipticity. The percentages of α-helix, β-sheet and random coil were calculated from the CD spectra by a program based on the method of Chen et al. [27].

Leakage assay of liposomes

Liposomes were prepared by evaporation of the lipid mixture in chloroform with nitrogen gas. The resulting thin film was hydrated in a buffer (pH 7.4) that contained 12.5 mM 8-amino-naphthalene-1,3,6-trisulphonic acid disodium salt (ANTS), 45 mM *p*-xylylenebis(pyridinium) bromide (DPX), 68 mM NaCl and 10 mM Hepes, then agitated for 30 min and vortex-mixed vigorously for 10 min. The resulting multilamellar vesicles were sonicated and shaken for 1 h at room temperature. The suspension was freeze–thawed for five cycles. Liposomes were separated from the unencapsulated material on a Sephadex G-50 (Pharmacia) column that had been equilibrated with 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl and 1 mM EGTA. The assay was based on the quenching of ANTS fluorescence by DPX. Leakage was measured with liposomes containing both ANTS and DPX [28,29] by the change in emission of ANTS. The fluorescence from liposomes containing both 12.5 mM ANTS and 45 mM DPX was set as the baseline and that from liposomes lysed with Triton X-100 was set as 100 % leakage. Fluorescence (excitation at 360 nm; emission at 535 nm) was measured [30] with a Jasco FP-777 spectrofluorimeter (Tokyo, Japan).

RESULTS

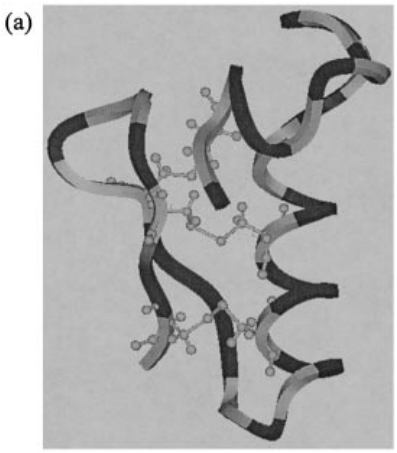
Antimicrobial activity of synthetic fragments of tenecin 1

As shown in Figure 1, tenecin 1 and insect defensin A share a highly similar amino acid sequence and the same disulphide

Tenecin 1	VTCDI	LSVEA	KGVKL	NDAAC	AAHCL	FRGRS	GGYCN	GKRVC	VCR
Insect defensin A	ATCDL	LS	GTGI	NHSAC	AAHCL	LRGNR	GGYCN	GKGVC	VCR
Peptide B	VTCDL	LGFEI	AGTKL	NSAAC	GAHCL	ALGRR	GGYCN	SKSVC	VCR
Peptide C	FTCDV	LGFEI	AGTKL	NSAAC	GAHCL	ALGRT	GGYCN	SKSVC	VCR
Royalicin	VTCDL	LSF	KGQV	NDSAC	AANCL	SLGKA	GGHCE	KGVC	ICR
Sapecin	ATCDL	LS	GTGI	NHSAC	AAHCL	LRGNR	GGYCN	GKAVC	VCR
Sapecin C	ATCDL	LS	GIGV	QHSAC	ALHCV	FRGNR	GGYCT	GKGIC	VCR
Sapecin B	LTC		EI	DRSLC	LLHCR	LKGYL	RAYCS	QQKVC	RCV

Figure 1 Primary structures of tenecin 1 [21] and related insect defensin family proteins

Broken lines indicate where disulphide bridges and gaps were introduced to align the maximal sequence similarity. Sapecin was purified from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina* [3]. Sapecin B and sapecin C were purified from the culture medium of NIH-Sape-4 [4]. Insect defensin A [45] and royalicin [46] were isolated from the dipteran insects *Pormia terranova* and *Apis mellifera* respectively. Peptide A and Peptide C were isolated from the tenebrionid beetle *Zophobas atratus* [47].



(b)

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                      10        20        30        40
Tenecin 1          VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR
                   :::.:::    :. .:..:::::::::::..:::::::: :::::
Insect defensin A  ATCDLLS----GTGINHSACAAHCLLRGNRGGYCNGKGVCVCRN
                         10        20        30        40
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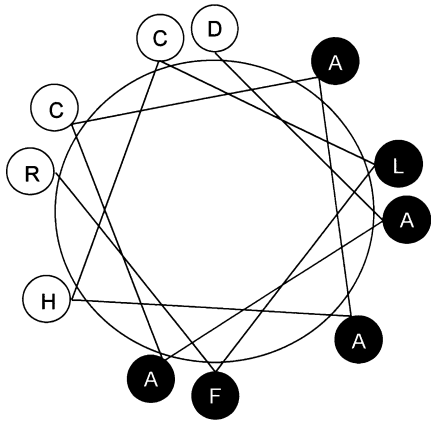
Figure 2 Molecular model of tenecin 1 built from the three-dimensional structure of insect defensin A

(**a**) Ribbon diagram of tenecin 1 generated by computer modelling (black, hydrophobic residues; grey, hydrophilic residues). Disulphide bridges are shown in ball and stick representation. (**b**) Sequence-based alignment of tenecin 1 and insect defensin A.



TE-(17-27)

Figure 3 Edmundson helical wheel diagram for TE-(17-27)

bridge connectivity. The three-dimensional molecular model of tenecin 1 was built by modelling from the NMR structure [19] of insect defensin A. As shown in Figure 2, this model exhibits the characteristic structural features of the insect defensin family, which consists of an N-terminal loop region and a short amphipathic α-helix region in the middle, followed by a C-terminal anti-parallel β-sheet region and disulphide bridges that connect the α-helix and β-sheet regions. Generally, most of antibacterial proteins and peptides have an amphiphilic α-helical structure or β structure for the interaction with the lipid membrane of the pathogenic cells as a requirement for antibacterial activity [9–11]. Insect defensin A and sapecin B were reported to act on the lipid membrane of pathogenic cells in biological processes [31,32]. Tenecin 1, with a structure similar to that of insect defensin A, should possess one or more structural motifs, which could interact with the lipid membrane. As shown in Figure 2, a short amphipathic α-helical region and amphipathic β-sheet region in tenecin 1 might satisfy the structural requirement for the interaction with the lipid membrane. We also analysed an α-helical region of tenecin 1 by Edmundson wheel projection, as shown in Figure 3. This analysis suggested that the peptide corresponding to the α-helix region might form a perfect amphipathic α-helical structure. As shown in Table 1, a hydrophobic moment [33] of 0.48 was calculated from the peptide corresponding to the α-helix region, which was comparable to the characteristics of the reported membrane-active peptides [34,35]. Considering the expected secondary structure of each fragment, we synthesized the peptides corresponding to the regions of tenecin 1 (Table 1) and assayed them for antibacterial and antifungal activity. Even though TE-(17-27), corresponding to the α-helical region, satisfied the general criteria for the characteristic of the membrane-disturbing peptide, this peptide did not

Table 1 Primary structures of synthetic fragments of tenecin

Hydrophobic moment was calculated by the method of Eisenberg [33]. The bold cysteine residues in TEC-(17-27) are linked by an intra-disulphide bridge. The underlined cysteine residues in TEC-(33-43) were blocked by an acetamidomethyl group. Abbreviation: TE, tenecin 1.

Peptide	Amino acid sequence	Hydrophobic moment, μ_H
TE-(1-15)	VTCDILSVEAKGVKL-NH_2	0.32
TE-(17-27)	DAACAAHCLFR-NH_2	0.48
TEC-(17-27)	DAA**C**AAH**C**LFR-NH_2	–
TE-(16-32)	NDAACAAHCLFRGRSGG-NH_2	0.21
TE-(29-43)	RSGGYCNGKRVCVCR-NH_2	0.12
TEC-(29-43)	RSGGYCNGKRVCVCR-CO_2H	0.12
TE-(33-42)	YCNGKRVCVC-NH_2	0.19
TEC-(33-43)	YC̲NGKRVC̲VC̲R-NH_2	–
TE-(34-43)	C̲NGKRVC̲VC̲R-NH_2	0.09
TE-(35-43)	NGKRVCVCR-NH_2	0.10
TE-(34-42)	CNGKRVCVC-NH_2	0.30

show antibacterial activity up to a concentration of 100 μg/ml (Table 2). We prepared the intra-disulphide bridge (i,$i+4$) of TE-(17-27) to prevent complications from aggregation by inter-disulphide bridge formation in the biological assay and to stabilize an α-helical structure [36]. However, oxidized TE-(17-27), like the reduced form, was inactive.

As shown in Table 2, the extension of TE-(17-27) by six residues (residues 16–32), corresponding to the α-helical and turn regions, did not have antibacterial activity at comparable concentrations. TE-(1-15), corresponding to the N-terminal loop region, also did not show antibacterial activity. However, TE-(29-43), corresponding to the β-sheet region, showed antimicrobial activity. Interestingly, TE-(29-43) inhibited the growth of most test micro-organisms, including fungi as well as Gram-positive and Gram-negative bacteria, at concentrations ranging from 10 to 30 μg/ml, whereas tenecin 1 inhibited the growth of Gram-positive bacteria only. After TE-(29-43)-treated *Candida albicans* cells were washed and reincubated in peptide-free media, no regrowth was observed, which indicated that the inhibitory activity of TE-(29-43) was irreversible. TE-(29-43) showed more potent activity against most test micro-organisms than did magainin II, a well-characterized antimicrobial peptide [37].

Search for the minimal active fragment

To find a minimal active fragment, TE-(29-43) was further truncated and assayed against bacteria and fungi. As shown in Table 2, the C-terminal amidated form of TE-(29-43) showed almost the same activity as the C-terminal acid form, so the peptides corresponding to the truncated form of TE-(29-43) were synthesized in the C-terminal amide form for convenience of synthesis. TE-(33-43) and TE-(34-43) showed a similar potency to TE-(29-43) against all test micro-organisms. However, the deletion of the cysteine residue from the N-terminus or the arginine residue from the C-terminus of TE-(34-43) resulted in a loss of antimicrobial activity. The capping of three free thiol groups of TE-(33-43) by acetamidomethyl groups also caused complete loss of activity. TE-(33-42), consisting of ten amino acid residues, was synthesized and its activity was measured. TE-(33-42) and the truncated form of the TE-(34-43) showed no activity, which indicated that the C-terminal decapeptide was a minimal active fragment and that the C-terminal Arg and N-terminal Cys residues of TE-(34-43) were required simultaneously for activity.

Table 2 Antimicrobial activities of synthetic fragments of tenecin 1

Abbreviation: n.a., not assayed.

Organism	Minimal inhibition concentration (μg/ml)												
	TE-(1-15)	TE-(17-27)	TE-(16-32)	TE-(29-43)	TE-(33-43)	TE-(34-43)	TE-(35-43)	TE-(34-42)	TE-(33-42)	TEC-(29-43)-COOH	TEC-(33-43)	TEC-(17-27)	Tenecin 1	Magainin II
Staphylococcus aureus ATCC 6538	> 100	> 100	> 100	25	30	30	> 100	> 100	> 100	25	> 100	> 100	6.25	40
Methicillin-resistant *S. aureus*	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	50	> 100
Staphylococcus epidemidis ATCC 12228	> 100	> 100	> 100	12.5	15	15	> 100	> 100	> 100	12.5	> 100	> 100	n.a.	10
Micrococcus luteus ATCC 9341	> 100	> 100	> 100	25	30	12.5	> 100	> 100	> 100	25	> 100	> 100	3.12	50
Bacillus subtilis ATCC 6633	> 100	> 100	> 100	12.5	15	15	> 100	> 100	> 100	12.5	> 100	> 100	n.a.	20
Mycobacterium smegmatis ATCC 607	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	n.a.	> 100	> 100	n.a	> 100
Corynebacterium diphtheriae ATCC 8024	> 100	> 100	> 100	12.5	7.5	7.5	> 100	> 100	> 100	n.a.	> 100	> 100	n.a.	5
Escherichia coli ATCC 2592	> 100	> 100	> 100	25	15	7.5	> 100	> 100	> 100	50	> 100	> 100	> 100	20
Pseudomonas aeruginosa ATCC 9027	> 100	> 100	> 100	25	50	50	> 100	> 100	> 100	50	> 100	> 100	> 100	20
Shigella flexneri ATCC 203	> 100	> 100	> 100	12.5	15	30	> 100	> 100	> 100	n.a.	> 100	> 100	n.a.	20
Candida albicans ATCC 36232	> 100	> 100	> 100	12.5	15	15	> 100	> 100	> 100	25	> 100	> 100	> 100	5

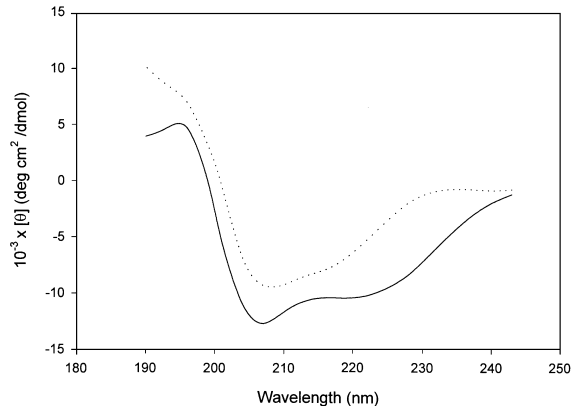


Figure 4 CD spectra of TE-(17-27) and TE-(29-43) in the presence of TFE

Spectra of TE-(17-27) (100 μg/ml) and TE-(29-43) (100 μg/ml) in buffer with TFE 50% (v/v) were recorded on a Jasco J-715 spectropolarimeter in a 1 mm cell at 22 °C. Peptides: TE-(17-27) (solid line) and TE-(29-43) (dotted line).

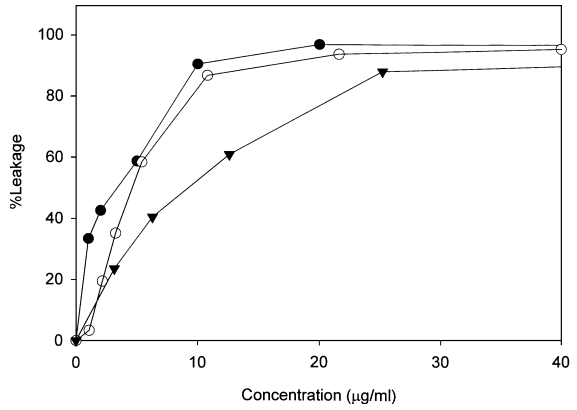


Figure 5 Peptide-induced leakage of dye from liposomes

The phospholipid composition of liposomes resembled that of Gram-positive bacteria [phosphatidylglycerol/cardiolipin (3:1, mol/mol)]. Peptides were incubated with liposomes for 1 h at 37 °C. Peptides: ●, melittin; ○, TE-(29-43); ▼, TE-(33-43).

CD measurements

To investigate the secondary structure of each fragment, CD spectra of each peptide were measured in the presence and the absence of TFE, a membrane-mimetic solvent. CD spectra indicated that each fragment in the absence of TFE had a random structure and TE-(1-15) still adopted a random structure in the presence of TFE (results not shown). As shown in Figure 4, the CD spectrum of TE-(17-27) measured in the presence of TFE exhibited double minimum bands at 208 and 222 nm, characteristic of an α-helix. The α-helical content of TE-(17-27) was calculated to be 37% by the method of Chen et al. [27]. This result was compatible with the prediction of the α-helical wheel projection and the molecular model of tenecin 1. As shown in Figure 4, the CD spectrum of TE-(29-43), measured in the presence of TFE, showed a shallow negative band around 210 nm without a band at 222 nm, which suggests that TE-(29-43) prefers forming the β-sheet structure; 46% β-sheet and 37% random structures were calculated from the CD spectrum of TE-(29-43). These results suggest that each fragment in a membrane-mimetic environment might resemble the secondary structure corresponding to its region in tenecin 1.

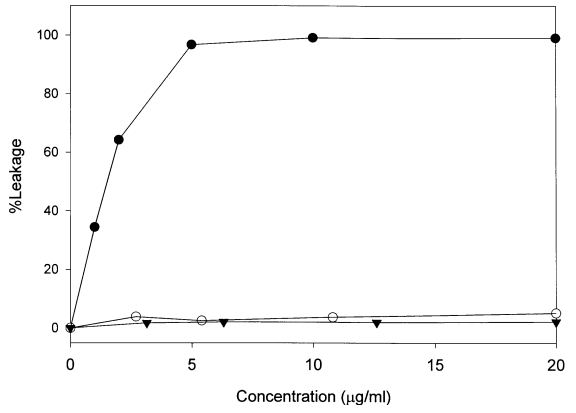


Figure 6 Haemolytic activities measured by leakage assay

The phospholipid composition of liposomes resembled that of human erythrocyte membrane [phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine/sphingomyelin/cholesterol (25:22:10:18:25, molar ratios)]. Peptides were incubated with liposomes for 1 h at 37 °C. Peptides: ●, melittin; ○, TE-(29-43); ▼, TE-(33-43).

Leakage assay with synthetic fragments

Generally, members of the insect defensin family have been reported to act on the lipid membrane of pathogens in biological processes [31,32]. The active fragment showed activity against fungi as well as bacteria, whereas tenecin 1, the native protein, showed activity only against Gram-positive bacteria. To elucidate the mechanism of the active fragments, TE-(33-43) and TE-(29-43) were examined for their ability to induce the leakage of dye from phospholipid liposomes that mimicked the membrane of Gram-positive bacteria. Various concentrations of each peptide were interacted with liposomes that consisted of phosphatidylglycerol/cardiolipin (3:1); after 1 h the leakage of liposome-enclosed ANTS was assayed. Melittin, a membrane-active peptide [38], was used as a control. As shown in Figure 5, the leakage potency of melittin was similar to that of TE-(33-43) and TE-(29-43) at given concentrations. A comparison of the leakage potency and the activity of the fragment against Gram-positive bacteria suggests that these active peptides kill pathogenic micro-organisms through membrane leakage as a primary mode of action. Melittin is known to form an ion channel to induce cell lysis. However, in view of the short length and secondary structure of TE-(29-43) in the presence of TFE, it would be difficult to envisage that this peptide forms an ion channel. We also tested the interaction of TE-(33-43) and TE-(29-43) with the liposome phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine/sphingomyelin/cholesterol (25:22:10:18:25, molar ratios), which resembled the membranes of erythrocytes. As shown in Figure 6, TE-(33-43) and TE-(29-43) induced no leakage from liposome at concentrations up to 100 μg/ml, whereas 5 μg/ml melittin, a cytotoxic peptide for erythrocytes [38], caused 100% lysis. This result indicates that the active fragments show a great selectivity between the lipid membrane of *C. albicans* and erythrocytes.

Haemolytic activity of the active fragment

The leakage study described above suggests that TE-(33-43) and TE-(29-43) have a great specificity for the membrane. Figure 7

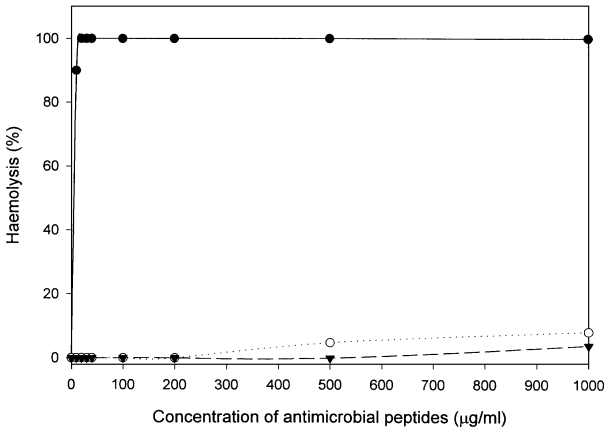


Figure 7 Haemolytic activities of antimicrobial fragments of tenecin 1

Erythrocytes were incubated in phosphate buffer with various concentrations of fragments of tenecin 1 for 1 h at 37 °C. Peptides: ●, melittin; ○, TE-(29-43); ▼, TE-(33-43).

shows haemolysis of mouse erythrocytes as a function of the concentration of TE-(33-43), TE-(29-43) and melittin. Melittin, a cytotoxic peptide for erythrocytes [38], caused 100 % lysis at 10 μg/ml, whereas TE-(33-43) and TE-(29-43) did not, even at 1 mg/ml, which was at least 40-fold the minimal inhibitory concentration against *C. albicans*. This result is consistent with the leakage assay result. Although the membrane composition of erythrocytes is very close to that of *C. albicans*, the active fragments did not show activity against erythrocytes but did against *C. albicans*.

DISCUSSION

In the present study we suggest a novel approach in which the active fragment can be identified efficiently from tenecin 1 on the basis of screening an amphipathic structure that is known as a general criterion for activity. Many studies of membrane-active peptides (reviewed in [1]) indicate that the structure of the peptides developed in the lipid membrane is critical to their activity. To obtain structural information about the peptide in the lipid membrane, many membrane-mimetic conditions have been applied. CD spectra measured in TFE suggest that each fragment in the lipid membrane might resemble the secondary structure corresponding to its region of the native protein. However, the complexity of the lipid membrane and the validity of the structural information in membrane-mimetic environments make it difficult to ascertain the structure in the lipid membrane.

Fragment TE-(17-27), corresponding to the α-helical region of tenecin 1, had an α-helical structure in the membrane-mimetic condition and showed a perfect amphipathic structure in an α-helical wheel diagram. However, this fragment had no antibacterial activity, whereas fragment TE-(29-43), corresponding to the β-sheet region, showed antifungal activity as well as antibacterial activity. This result is different from that of Yamada and Natori [15], in which the fragment corresponding to the α-helical region of sapecin B, which also belongs to the insect defensin family, showed broad antibacterial activity. To our knowledge, the present results are the first report in which the peptide corresponding to the β-sheet region in an antibacterial protein that consists of α-helical and β-sheet regions has been identified as a primary active fragment.

We should consider why the fragment corresponding to the β-sheet region in tenecin 1 has antimicrobial activity, whereas the fragment corresponding to the α-helical region does not. As we describe in the Introduction section, an amphipathic structure and a net positive charge are fundamental to antimicrobial activity. Fragments corresponding to both α-helical and β-sheet regions of tenecin 1 satisfy a structural requirement for activity, and thus a net positive charge is a critical factor for activity; the net positive charge of the β-sheet fragment is $+5$, whereas the net positive charge of the α-helical fragment is $+1$. We observed the requirement of high net positive charge for activity in the truncation of the fragment corresponding to the β-sheet region of tenecin 1. The deletion of the arginine residue from the C-terminus of TE-(34-43) and TE-(33-43) resulted in a complete loss of activity, which also supports the requirement of a net positive charge for antimicrobial activity. The amidated peptide of TE-(29-43) showed almost the same activity as the acid form; however, the amidated peptide of TE-(33-43) showed much higher activity than the acid form (results not shown). The amidated peptide has a net positive charge increased by one and shows increased resistance against microbial proteinases compared with the acid form. If the increase in activity of amidated peptide is due to the increase in resistance against proteinases, then the activities of TE-(29-43) (net charge $+5$) and TE-(33-43) (net charge $+4$) should also rise. However, the activity of only TE-(33-43), with a low net positive charge, is increased. This result strongly suggests that the increase in net positive charge rather than the increase in stability is a major reason for the increase in activity of the amide form and that the net positive charge is a critical factor for activity. Zhong et al. [39] have also reported the importance of net positive charge in the design and synthesis of antimicrobial peptides.

We contemplate the reason why the fragment derived from tenecin 1 shows activity against fungi as well as Gram-positive and Gram-negative bacteria, whereas tenecin 1 shows activity only against Gram-positive bacteria. One possible explanation is that the C-terminal β-sheet region of tenecin 1 is a primary active domain and the other region (α-helical and N-terminal loop regions) might have an important role in potency and specificity. The other possible explanation is that this peptide just satisfies the general criteria for antimicrobial activity, such as a net positive charge and an amphipathic structure. At present, it is difficult to decide which explanation is better. However, the former explanation seems to be less reasonable because the synthesized peptide corresponding to the C-terminal region of insect defensin A showed activity against Gram-negative bacteria and fungi but not against Gram-positive bacteria (results not shown), whereas insect defensin A itself was reported to show activity only against Gram-positive bacteria [19].

The result of searching for the minimal active fragment of tenecin 1 indicates that the C-terminal decapeptide is the minimal fragment for antimicrobial activity. McLean et al. [40] also reported that the minimal peptide length for the interaction of amphipathic α-helical peptides with phosphatidylcholine liposomes was the decapeptide. Generally, the biological actions of insect defensin proteins are reported to be on the lipid membrane [31,32]. From our mechanism study, the active fragment killed micro-organisms by changing the permeability of the target membrane. The capping of three cysteine residues of TE-(33-43) by acetamidomethyl groups resulted in a loss of activity, as with tachyplesin [41] and protegrin 1 [42]. We carefully examined the redox state of thiol groups in each fragment because this redox state seemed to be important for activity. All cysteine residues of the active fragments in this work were confirmed to be in the reduced state by the Ellman assay and MS (results not shown). To investigate whether the reduced state of

cysteine residues was changed in each experiment, we monitored the state of cysteine residues of TE-(29-43) in 10 mM phosphate buffer, pH 7.4, at room temperature by using the Ellman assay. This assay showed that the reduced state of cysteine residues in TE-(29-43) did not change over a period of 24 h (results not shown). Considering the above result and the low minimal inhibitory concentration (less than 30 μg/ml), at which the peptide is too dilute to form the dimer by inter-disulphide bridge(s), we expect that the reduced state of the active fragment is the active form.

It was reported that the peptide corresponding to the α-helical region in antibacterial proteins has been identified as the primary active fragment [15,43,44]. However, it is a novel finding that the peptide corresponding to the β-sheet region in an antibacterial protein consisting of α-helical and β-sheet regions is identified as a primary active fragment. The present study also indicated that a net positive charge as well as an amphipathic structure was required for antimicrobial activity. Even though several small active peptides of various lengths from 17-mer to 12-mer were isolated from Nature (reviewed in [1]), few of them showed antibacterial and antifungal activity. The active fragments identified in this work are very small (between 15-mer and 10-mer) but show activity against fungi and bacteria and no haemolytic activity. In view of the recent emergence of multidrug-resistant bacteria and the increase in fungal infections, these active fragments with a unique structure open a promising lead for the further development of novel antibacterial and antifungal agents.

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