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Conformation-dependent antibiotic activity of tritrpticin, a cathelicidin-derived antimicrobial peptide

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Abstract

Tritrpticin, a Trp-rich cationic antimicrobial peptide with a unique amino acid sequence (VRRFPWWWPFLRR), is found in porcine cathelicidin cDNA. Tritrpticin has a broad spectrum of antibacterial and antifungal activities and hemolytic activity comparable to that of indolicidin. To investigate the mechanism of the bacterial killing action of tritrpticin and to identify structural features important for bacterial cell selectivity, we designed several tritrpticin analogs with amino acid substitutions of the Pro and Trp residues. Circular dichroism studies revealed that the substitution of Pro → Ala (TPA) or Trp → Phe (TWF) leads to significant conformational changes in SDS micelles, converting the β-turn to α-helix or to poly-L-proline II helix, respectively. Compared to tritrpticin, TPA retained most of its antimicrobial activity, but showed enhanced hemolytic and membrane-disrupting activities. In contrast, TWF showed a 2–4-fold increase in antimicrobial activity against Gram-negative bacteria, but a marked decrease in both hemolytic and membrane-disrupting activities. Taken together, our findings suggest that compared with the β-turn and α-helical structures, the poly-L-proline II helix is crucial for effective bacterial cell selectivity in tritrpticin and its analogs.

Keywords: Antimicrobial peptide; Tritrpticin; α-Helix; β-Turn; Poly-L-proline II helix

Antimicrobial peptides are widely distributed among multicellular organisms and have been recognized as an important component of the non-specific host defense system against invading pathogens [1–3]. A major class of mammalian antimicrobial peptides is the cathelicidin family, which are synthesized in the granules of myeloid cells. The cathelicidin-derived antimicrobial peptides have highly conserved preproregions, whereas the C-terminal domains, which correspond to the antimicrobial activity, are highly varied. These peptides show a significant diversity in structure and species distribution [4–6]. However, based on their common structural features, they have been classified into three distinct groups. The first group contains amphipathic α-helical peptides such as CAP18 [7], CRAMP [8,9], and PMAP-23 [10,11]; the second group has Pro/Arg-rich or Trp-rich peptides including PR-39 [12–14], Bac5, and Bac7 [15–17], and indolicidin [18–20]; and the third peptide group includes Cys-containing β-sheet peptides such as protegrins [21–23]. Although the exact antibacterial mechanism of the peptides has not been fully determined, most α-helical and β-sheet cathelicidin-derived antimicrobial peptides act as membrane-active molecules that permeabilize the negatively charged plasma membrane [24–28]. In contrast, the mechanism of the Pro/Arg-rich peptides such as PR-39 is not linked to membrane lysis. PR-39 is thought to kill bacteria by interfering with DNA or protein synthesis [13,29]. Likewise, Bac5 and Bac7 cause a rapid decrease in RNA and protein synthesis [30]. The cathelicidins, which use diverse bacterial killing mechanisms, have been extensively studied for their potential as novel therapeutic agents.

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Tritrpticin, a member of the cathelicidin family, is a 13-amino acid antimicrobial peptide with the sequence VRRFPWWWPFLRR. Tritrpticin is known to have a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria as well as some fungi [31]. The primary structure of tritrpticin is remarkable because of its high content of Arg (30%), Trp (23%), and Pro (15%). Therefore, tritrpticin belongs to the group of Trp- or Pro/Arg-rich antimicrobial peptides. Trp and Pro residues are known to play important roles in the assembly and structure of membrane proteins [32–34]. The secondary and tertiary structures of tritrpticin in the absence or presence of SDS micelles have been determined by circular dichroism (CD) and nuclear magnetic resonance (NMR) analyses. Schibli et al. [35] have reported that tritrpticin has a random coil conformation in Tris buffer, whereas in SDS micelles it forms two adjacent turns around the two Pro residues at peptide positions 5 and 9. The unique structure caused by the two Pro residues creates a stable amphipathic turn structure in which Trp residues are buried in the micelle with Arg residues present on the opposite side of the structure. In contrast, the study by Nagpal et al. [36] suggested that tritrpticin adopts a β-turn conformation in aqueous buffer and undergoes functional activation through a conformational transition from β-turn to poly-L-proline II helix as an initial event in bacterial killing. Although the mode of antimicrobial action of tritrpticin is poorly understood, the high content of Trp in tritrpticin suggests a similar mode of action to the Trp-rich bovine antimicrobial peptide indolicidin.

To gain a better understanding of the antimicrobial and hemolytic mechanism of the action of tritrpticin and to provide a basis for understanding its selectivity for bacterial cells, we designed and synthesized several tritrpticin analogs with amino acid substitutions of the Trp and Pro residues of the peptide. Using these peptide analogs, we assessed the functionally relevant structural features of tritrpticin by examining the effect of these structural changes, as determined by CD analysis, on the antibacterial, antifungal, hemolytic, and liposome-disrupting activities of the peptide analogs.

Materials and methods

Microorganisms. *Escherichia coli* KCTC 1682, *Salmonella typhimurium* KCTC 1926, *Pseudomonas aeruginosa* KCTC 1637, *Bacillus subtilis* KCTC 3068, *Staphylococcus epidermidis* KCTC 1917, *Staphylococcus aureus* KCTC 1621, and *Candida albicans* KCTC 7965 were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Taejon, Korea).

Peptide synthesis. Tritrpticin and its analog derivatives were synthesized by the solid phase method using Fmoc(fluoren-9-ylmethoxycarbonyl)-chemistry [37]. Fmoc-protected peptides were deprotected and cleaved using a mixture of TFA (trifluoroacetic acid), phenol, H_2O, thioanisole, and 1,2-ethanedithiol (82.5:5:5:5:2.5, v/v) for 3 h at room temperature. HPLC analysis was performed on a Shimadzu LC-6AD system and Shimadz LC-10Avp system with ODS column ($4.6 \times 250\,mm^2$). Peptide concentrations in stock solutions were determined by amino acid analysis. The molecular weight of all synthetic peptides was confirmed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Table 1).

Antimicrobial activity. Antimicrobial activity of the peptides against seven selected organisms, including Gram-positive and Gram-negative bacteria and *C. albicans*, was determined by the broth microdilution assay. Briefly, single colonies of bacteria and fungi were inoculated into the culture medium (LB broth for bacteria and YM broth for *C. albicans*) and cultured overnight at 37 °C (or 30 °C for *C. albicans*). An aliquot of this culture was transferred to 10 ml fresh culture medium and incubated for an additional 3–5 h at 37 °C (for bacteria) or 30 °C (for *C. albicans*) to obtain mid-logarithmic phase organisms. A 2-fold dilution series of peptides in 1% peptone was prepared. A set of serial dilutions (100 μl) were added to 100 μl of 2×10^6 CFU/ml in 96-well microtiter plates (Falcon) and then incubated at 37 °C (or 30 °C for *C. albicans*) for 16 h. The lowest concentration of peptide that completely inhibited growth of the organisms was defined as the minimal inhibitory concentration (MIC). The MICs were the average of triplicate measurements in three independent assays.

Hemolytic activity. The hemolytic activities of the peptides were determined using human red blood cells (hRBC). The hRBC were centrifuged and washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer, pH 7.0/150 mM NaCl). One hundred microliters of the hRBC suspended 4% (v/v) in PBS plated into sterilized 96-well plates and then 100 μl peptide solution was added to each well. The plates were incubated for 1 h at 37 °C and centrifuged at 1000*g* for 5 min. Aliquots (100 μl) of the supernatant were transferred to 96-well plates, where hemoglobin release was monitored using ELISA plate reader (Molecular Devices, Sunnyvale, California) by measuring the absorbance at 414 nm. Percent hemolysis was calculated by the following formula: % hemolysis = $[(Abs_{414\,nm}$ in the peptidesolution $- Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% Triton X-100 $- Abs_{414\,nm}$ in PBS)] × 100. Zero percentage and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

Table 1
Amino acid sequences of tritrpticin, its analogs, and indolicidin and molecular weights determined by MALDI-TOF MS

Peptide	Amino acid sequence	Calculated	Observed
Tritrpticin	VRRFPWWWPFLRR	1902.4	1903.3
TPG	VRRFGWWWGFLRR	1822.3	1823.2
TPA	VRRFAWWWAFLRR	1850.3	1850.8
TWF	VRRFPFFFPFLRR	1785.3	1786.1
TWA	VRRFPAAAPFLRR	1557.0	1557.2
Indolicidin	ILPWKWPWWPWRR-NH_2	1907.4	1907.1

Circular dichroism (CD) spectroscopy. The CD spectra of the peptides were recorded using a Jasco J-715 CD spectrophotometer (Tokyo, Japan) with a 1 mm path length cell. Wavelengths from 185 to 250 nm were measured, with 0.1 nm step resolution, 50 nm/min speed, 0.5 s response time, and 1 nm bandwidth. CD spectra of the peptides were collected and averaged over four scans in 10 mM sodium phosphate buffer (pH 7.0) and 30 mM SDS (sodium dodecyl sulfate) micelles, respectively, at 25 °C. The mean residue ellipticity [θ] (given in units of deg cm^2 dmol^{-1}) was calculated using $[\theta] = [\theta]\text{obs}\,(\text{MRW}/10lc)$, where [$\theta$]obs is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/ml, and l is the optical path length of the cell in cm. The spectra are expressed as molar ellipticity [θ] vs. wavelength.

Preparation of liposomes. Small unilamellar vesicles (SUVs) composed of POPC/POPG (3:1, w/w) were prepared for dye-leakage and tryptophan fluorescence experiments as follows. Phospholipid (7.5 mg) was dissolved in chloroform and dried with a stream of nitrogen to form a thin lipid film on the wall of a glass tube. The resulting lipid film, after being dried under vacuum overnight, was hydrated with 2 ml Tris–HCl buffer [10 mM Tris–HCl (pH 7.4), 154 mM NaCl, and 0.1 mM EDTA]. The suspension was sonicated under nitrogen in an ice bath for 30 min at 25 °C using a titanium tip sonicator. The lipid concentration was 0.5 mM. Calcein-entrapped liposomes for dye-leakage experiments were prepared by the following procedure. First, the dried lipid was hydrated with 2 ml Tris–HCl buffer containing 70 mM calcein, after which the suspension was vortex-mixed for 10 min. The resultant lipid dispersions were then sonicated in ice water for 20–30 min with a titanium-tipped sonicator until clear. Calcein-entrapped vesicles were separated from free calcein by gel filtration using Sephadex G-50 column with the same buffer.

Dye-leakage. Tris–HCl buffer (pH 7.4, 2 ml) in a cuvette was added to 20 μl of the vesicles containing 70 mM calcein to give a vesicle solution with a final concentration of 70 μM lipid. The fluorescence intensities of calcein released from liposomes were monitored at 520 nm (excited at 490 nm) on a Jasco FP-750 spectrofluorimeter (Tokyo, Japan) and measured 2 min after the addition of the peptides. To measure the fluorescence intensity for 100% dye-leakage, 20 μl Triton X-100 (20% in Tris buffer) was added to dissolve the vesicles. The percentage of dye-leakage caused by the peptides was calculated using the equation: $\%\ \text{leakage} = 100 \times (F - F^0)/(F^t - F^0)$, where F^0 and F^t are the initial fluorescence intensities observed without the peptides and after Triton X-100 treatment, respectively, and F is the fluorescence intensity achieved by the peptides.

Tryptophan fluorescence. Tryptophan fluorescence measurements were made on a Jasco FP-750 spectrofluorimeter (Tokyo, Japan). Each peptide (2 μg/ml) was added to 1 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM liposomes and the peptide/liposome mixture was allowed to interact at 25 °C for 10 min. The fluorescence was excited at 280 nm and the emission was scanned from 300 to 400 nm. The fluorescence spectrum of each peptide with liposomes was subtracted from the spectrum of the liposomes alone.

Results

Comparison of antimicrobial and hemolytic activities of the peptides

Tritrpticin and its peptide analogs (Table 1) were examined for their lytic activities against bacteria, *C. albicans*, and human erythrocytes. The activities of the peptides for bacteria and *C. albicans* expressed as the minimal inhibitory concentrations (MIC) are summarized in Table 2 and the dose–response relationship of the hemolytic activity is depicted in Fig. 1. Compared to indolicidin, tritrpticin exhibited a similar or slightly weaker antimicrobial activity (MIC: 8–64 μg/ml), but relatively potent hemolytic activity (37% hemolysis at 100 μg/ml). TPG in which Pro was substituted with Gly showed antibacterial and hemolytic activities comparable to those of tritrpticin, whereas TPA, in which Pro was replaced with Ala, retained most of its antimicrobial activity, but had significantly enhanced hemolytic activity (63% hemolysis at 100 μg/ml). In contrast, TWF (Trp → Phe substitution) had antimicrobial activities against Gram-positive bacteria equivalent to tritrpticin, but this analog showed a 2–4-fold greater antimicrobial activity against Gram-negative bacteria. However, TWF did not show any hemolytic activity, even at 200 μg/ml, and TWA (Trp → Ala substitution) was totally inactive to bacterial cells and human erythrocytes. These results indicate that Trp residues in tritrpticin are not essential for its antimicrobial activity, but appear instead to be responsible for hemolytic activity. In addition, the hydrophobicity of the amino acid residue in the central position of the peptide appears to be important for the antimicrobial activity of tritrpticin.

Table 2
Antimicrobial activities of tritrpticin, its analogs, and indolicidin (MIC: μg/ml)

Microorganism	MIC (μg/ml)					
	Tritrpticin	TPG	TPA	TWF	TWA	Indolicidin
Gram-negative						
E. coli	32	32	32	8	>64	16
S. typhimurim	32	32	32	8	>64	16
P. aeruginosa	32	64	64	16	>64	32
Gram-positive						
B. subtilis	8	16	16	8	>64	4
S. aureus	16	16	32	16	>64	8
S. epidermidis	8	8	16	8	>64	4
Fungus						
C. albicans	32	32	32	16	>64	16

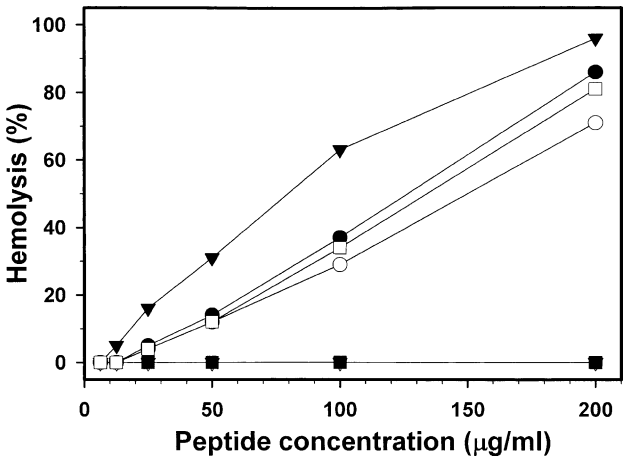

Fig. 1. Dose–response of hemolytic activity of the peptides toward human erythrocytes. Assays were performed as described in the Materials and methods. Designations are as follows: tritrpticin (●); TPG (○); TPA (▼); TWF (▽); TWA (■); indolicidin (□). TWF and TWA do not show any hemolysis at 200 μg/ml.

Structural analysis of the peptides

The secondary structures of tritrpticin and its analogs were determined by CD spectroscopy in aqueous buffer or SDS micelles. The CD spectra of tritrpticin and its analogs are presented in Figs. 2 and 3. The CD spectra of tritrpticin showed a positive band at 212 nm and a negative band at 225 nm in aqueous buffer or SDS micelles. The presence of a positive band suggests a characteristic β-turn conformation and the negative band is likely due to the presence of Trp residues [36]. Interestingly, TPA (Pro → Ala substitution) showed a CD spectrum with negative minimum bands at 207 and 218 nm and a positive maximum band at 195 nm in SDS micelles. These bands are consistent with the formation of an α-helix in a membrane-mimetic environment, as has been previously observed for other antimicrobial peptides [38,39]. In contrast, the CD spectrum of TWF (Trp → Phe substitution) showed no signal at 212 nm, indicating the disappearance of the β-turn, but instead showed in both aqueous buffer and SDS micelles a negative signal at 204 nm, corresponding to poly-L-proline II extended helix. However, the CD spectra of TWA (Trp → Ala substitution) exhibited a minimum at around 200 nm, which is indicative of a highly disordered conformation. Thus, it appears that the Pro → Ala or Trp → Phe substitution leads to the conversion of the peptide conformation from β-turn to α-helix or poly-L-proline II helix, respectively. These structural studies suggest that the presence of Pro and Trp in tritrpticin is important for the peptide to be able to adopt a stable amphipathic turn conformation.

Peptide-induced dye-leakage

The membrane-disrupting ability of tritrpticin and its analogs was investigated by examining the dye-leakage from POPC/POPG (3:1) liposomes. Upon addition of tritrpticin and its analogs to the liposomes, the entrapped calcein was released into the buffer due to lysis induced by the peptides. Relative lytic efficiencies were determined by complete disruption of the vesicles with Triton X-100, which correspond to the total fluorescence. Dose–response curves for tritrpticin TPG, TPA, TWF, and TWA with POPC/POPG (3:1) liposomes are shown in Fig. 5. Tritrpticin and TPG showed relatively strong leakage activities (74% and 54% leakage, respectively, at 8 μg/ml). TPA, which adopts an α-helical structure, caused an almost total disruption of the vesicles at a peptide concentration of 8 μg/ml. However, the peptide analogs with either Phe or Ala substitutions showed considerably reduced membrane-lytic activity. Thus, TWF showed very weak leakage activity (7% leakage at 8 μg/ml) and TWA did not cause any leakage. These results suggest that the Trp residues in tritrpticin play an important role in promoting membrane-lytic activity.

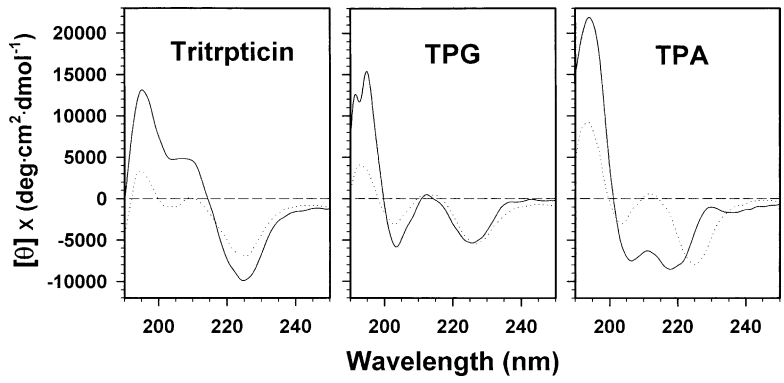

Fig. 2. CD spectra of tritrpticin, TPG, and TPA. Spectra were recorded at 25 °C in aqueous buffer (···) and in 30 mM SDS micelles (—). Each peptide was used at a concentration of 50 μg/ml.

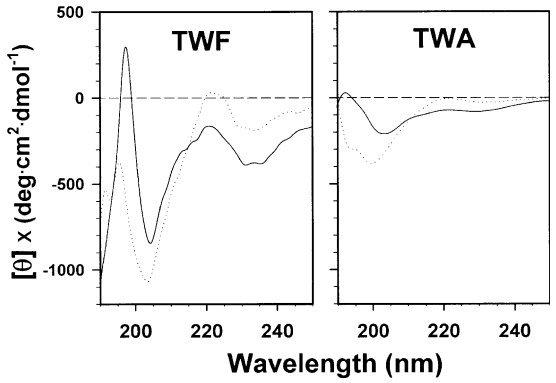

Fig. 3. CD spectra of TWF and TWA. Spectra were recorded at 25 °C in aqueous buffer (···) and in 30 mM SDS micelles (—). Each peptide was used at a concentration of 50 μg/ml.

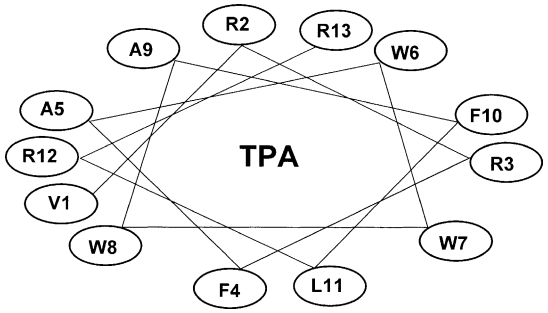

Fig. 4. Schiffer–Edmundson helical wheel representation of TPA.

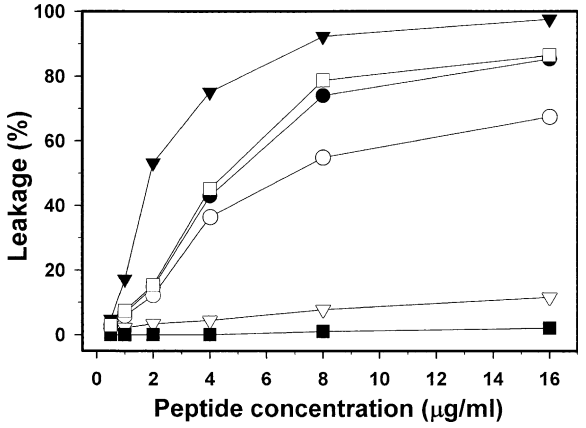

Fig. 5. Leakage of the fluorescent probe calcein from POPC/POPG (3:1, w/w) liposomes is defined as the percent leakage after 5 min at a lipid concentration of 70 μM. Symbols: tritrpticin (●); TPG (○); TPA (▼); TWF (▽); TWA (■); indolicidin (□).

Binding of peptides to lipid vesicles

Tritrpticin, TPG, and TPA each have three Trp residues. The fluorescence emission characteristics of Trp are sensitive to its immediate environment and have been used to monitor the binding of peptides to lipid vesicles. We monitored the fluorescence change of Trp residues of the peptides in buffer and in the presence of POPC/POPG (3:1) liposomes. When POPC/POPG (3:1) liposomes were added to the aqueous solutions containing tritrpticin and TPG, the fluorescence spectra of the peptides showed a significant blue shift of the Trp fluorescence maxima and increased intensities (Fig. 6). Unexpectedly, TPA showed the lowest blue shift (5 nm) without, any increase in quantum yield.

Discussion

Previous studies based on CD and NMR analyses of the secondary structure and antimicrobial mechanism of tritrpticin have been somewhat controversial. Schibli et al. [35] reported that while tritrpticin shows a disordered structure in Tris–HCl buffer, the peptide adopts an amphipathic helical turn structure in SDS micelles. From these observations, they suggested that the antibacterial action of tritrpticin might involve non-specific interactions with the cell membrane. However, Nagpal et al. [36] reported that tritrpticin adopts a β-turn conformation in aqueous buffer specifically binding to a negatively charged receptor exposed on the target bacterial membrane. In our study, we wished to gain a better understanding of the antimicrobial mechanism of the action of tritrpticin and define the structural requirements for its antimicrobial activity.

We found that tritrpticin strongly bound to liposomes formed either by neutral or negatively charged phospholipids and induced dye-leakage from the liposomes (data not shown). These results indicate that tritrpticin acts through non-specific binding to the cytoplasmic membrane rather than through a specific protein receptor. However, we observed that the membrane-lytic activities of tritrpticin analogs did not correlate with their antimicrobial activities. In particular, TPA (Pro → Ala substituted analog) retained or reduced antimicrobial activity only slightly, even though it displayed enhanced membrane-lytic activity. In contrast, TWF (Trp → Phe substituted analog) had a 2–4-fold increase in antimicrobial activity against Gram-negative bacteria while showing a considerable decrease in membrane-lytic activity.

Tritrpticin adopted a typical β-turn structure with a positive band at 212 nm in both aqueous solution and SDS micelles, whereas TPA assumed an α-helical CD spectrum with negative minimum bands at 207 and 218 nm and a positive maximum band at 195 nm in SDS micelles. The helical wheel diagram of TPA indicated that basic Arg residues and hydrophobic Trp residues are not separated (Fig. 4). TPA seems to have a relatively less amphipathic α-helical structure than tritrpticin, adopting instead a stable amphipathic turn structure. The slight decrease in antimicrobial activity of TPA may at least partially arise from its reduced amphipathicity due to a

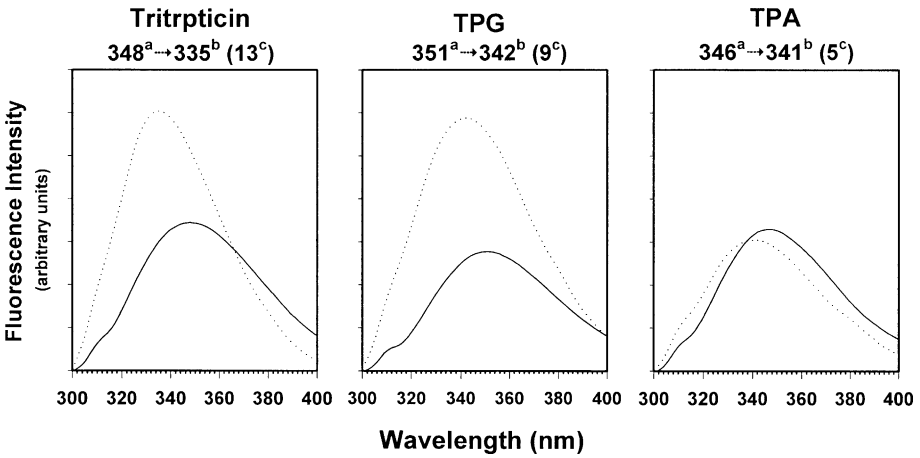

Fig. 6. Tryptophan fluorescence emission spectra of the peptides at 2 μg/ml in the presence of 10 mM Tris buffer (—) or in the presence of POPC/POPG (3:1, w/w) liposomes (···). a, Emission maxima in Tris–HCl buffer; b, emission maxima in liposome; c, blue shift in emission maximum.

significant conformational change in which the β-turn in tritrpticin is converted into an α-helical conformation in the peptide analog. Interestingly, TPA has the most hemolytic and membrane-disrupting activity, indicating that the α-helical conformation is more effective in membrane lysis than either the β-turn or the poly-L-proline II extended helix conformation. Recently, Friedrich et al. [39] found that CP10A, an indolicidin analog in which the three Pro residues were substituted with Ala residues, also adopted an α-helical structure. However, unlike TPA, CP10A showed a 2–8-fold increase in antimicrobial activity against most Gram-positive bacteria when compared to indolicidin [40]. The structural analysis using CD and NMR spectroscopy indicated that CP10A in the presence of lipid has a considerably more amphipathic structure than indolicindin. The increased amphipathicity in CP10A induced by the profound structural conversion from an extended boat-shaped structure to α-helical structure is likely to be responsible for its greater antimicrobial activity against Gram-positive bacteria. Therefore, it appears that the rather poor antimicrobial activity of TPA can be substantially improved by increasing its amphipathicity.

Trp residues in the antimicrobial peptides, such as indolicidin and melittin, have been reported to be crucial for their hemolytic activity [41,42]. Similarly, the analogs (TWF and TWA) with Trp → Phe or Ala substitutions were ineffective in lysing human erythrocytes. Notably, TWF, which adopted a poly-L-proline II helix structure, showed a 2–4-fold greater antimicrobial activity against Gram-negative bacteria. These results suggest that the antimicrobial mechanism of TWF might not be linked to membrane disruption. TWF is thought to kill bacteria by its inhibition of intracellular synthesis of protein, DNA, or RNA.

In summary, our study revealed two important findings: (i) The substitution of Pro → Ala (TPA) in tritrpticin caused a considerable conformational change, converting a β-turn into an α-helix. Although TPA showed high hemolytic and membrane-disrupting activities, it showed a similar or slightly reduced antimicrobial activity. (ii) TWF, which has a Trp → Phe substitution, had a 2–4-fold increase in antimicrobial activity against Gram-negative bacteria. The poly-L-proline II helical structure of TWF is likely responsible for its greater antimicrobial activity against Gram-negative bacteria. Overall, the structure–activity relationships of tritrpticin established in this study indicate that the poly-L-proline II helical structure of the antimicrobial peptides is crucial for effective bacterial cell selectivity compared with the β-turn and α-helical structures.

Acknowledgments

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References

[1] M. Zasloff, Antibiotic peptides as mediators of innate immunity, Curr. Opin. Immunol. 4 (1992) 3–7.
[2] R.I. Lehrer, A.K. Lichtenstein, T. Ganz, Defensins: antimicrobial and cytotoxic peptides of mammalian cells, Annu. Rev. Immunol. 11 (1993) 105–128.
[3] H.G. Boman, Peptide antibiotics and their role in innate immunity, Annu. Rev. Immunol. 13 (1995) 61–92.
[4] M. Zanetti, R. Gennaro, M. Scocchi, B. Skerlavaj, Structure and biology of cathelicidins, Adv. Exp. Med. Biol. 479 (2000) 203–218.
[5] T. Ganz, R.I. Lehrer, Antimicrobial peptides of vertebrates, Curr. Opin. Immunol. 10 (1998) 41–44.
[6] M. Zanetti, R. Gennaro, D. Romeo, Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain, FEBS Lett. 374 (1995) 1–5.
[7] J.W. Larrick, J.G. Morgan, I. Palings, M. Hirata, M.H. Yen, Complementary DNA sequence of rabbit CAP18—a unique

lipopolysaccharide binding protein, Biochem. Biophys. Res. Commun. 179 (1991) 170–175.
[8] R.L. Gallo, K.J. Kim, M. Bernfield, C.A. Kozak, M. Zanetti, L. Merluzzi, R. Gennaro, Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse, J. Biol. Chem. 272 (1997) 13088–13093.
[9] S.Y. Shin, S.W. Kang, D.G. Lee, S.H. Eom, W.K. Song, J.I. Kim, CRAMP analogues having potent antibiotic activity against bacterial, fungal, and tumor cells without hemolytic activity, Biochem. Biophys. Res. Commun. 275 (2000) 904–909.
[10] M. Zanetti, P. Storici, A. Tossi, M. Scocchi, R. Gennaro, Molecular cloning and chemical synthesis of a novel antibacterial peptide derived from pig myeloid cells, J. Biol. Chem. 269 (1994) 7855–7858.
[11] J.H. Kang, S.Y. Shin, S.Y. Jang, K.L. Kim, K.S. Hahm, Effects of tryptophan residues of porcine myeloid antibacterial peptide PMAP-23 on antibiotic activity, Biochem. Biophys. Res. Commun. 264 (1999) 281–286.
[12] P. Storici, M. Zanetti, A novel cDNA sequence encoding a pig leukocyte antimicrobial peptide with a cathelin-like pro-sequence, Biochem. Biophys. Res. Commun. 196 (1993) 1363–1368.
[13] H. Boman, B. Agerberth, A. Boman, Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine, Infect. Immun. 61 (1993) 2978–2984.
[14] V. Cabiaux, B. Agerberth, J. Johansson, F. Homble, E. Goormaghtigh, J. Ruysschaert, Secondary structure and membrane interaction of PR-39, a Pro + Arg-rich antibacterial peptide, Eur. J. Biochem. 224 (1994) 1019–1027.
[15] R.W. Frank, R. Gennaro, K. Schneider, M. Przybylski, D. Romeo, Amino acid sequences of two proline-rich bactenecins. Antimicrobial peptides of bovine neutrophils, J. Biol. Chem. 265 (1990) 18871–18874.
[16] P.A. Raj, M. Edgerton, Functional domain and poly-L-proline II conformation for candidacidal activity of bactenecin 5, FEBS Lett. 368 (1995) 526–530.
[17] P.A. Raj, E. Marcus, M. Edgerton, Delineation of an active fragment and poly(L-proline) II conformation for candidacidal activity of bactenecin 5, Biochemistry 35 (1996) 4314–4325.
[18] M.E. Selsted, M.J. Novotny, W.L. Morris, Y.Q. Tang, W. Smith, J.S. Cullor, Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils, J. Biol. Chem. 267 (1992) 4292–4295.
[19] C. Subbalakshmi, N. Sitaram, Mechanism of antimicrobial action of indolicidin, FEMS Microbiol. Lett. 160 (1998) 91–96.
[20] C. Subbalakshmi, E. Bikshapathy, N. Sitaram, R. Nagaraj, Antibacterial and hemolytic activities of single tryptophan analogs of indolicidin, Biochem. Biophys. Res. Commun. 274 (2000) 714–716.
[21] C. Zhao, L. Liu, R.I. Lehrer, Identification of a new member of the protegrin family by cDNA cloning, FEBS Lett. 346 (1994) 285–288.
[22] K.T. Miyasaki, R.I. Lehrer, Beta-sheet antibiotic peptides as potential dental therapeutics, Int. J. Antimicrob. Agents 9 (1998) 269–280.
[23] Y. Sokolov, T. Mirzabekov, D.W. Martin, R.I. Lehrer, B.L. Kagan, Membrane channel formation by antimicrobial protegrins, Biochim. Biophys. Acta 1420 (1999) 23–29.
[24] K. Matsuzaki, K. Sugishita, N. Fujii, K. Miyajima, Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2, Biochemistry 34 (1995) 3423–3429.
[25] T.J. Falla, D.N. Karunaratne, R.E. Hancock, Mode of action of the antimicrobial peptide indolicidin, J. Biol. Chem. 271 (1996) 19298–19303.
[26] D. Andreu, L. Rivas, Animal antimicrobial peptides: an overview, Biopolymer 47 (1998) 415–433.
[27] Z. Oren, Y. Shai, Mode of action of linear amphipathic α-helical antimicrobial peptides, Biopolymer 47 (1998) 451–463.
[28] R.M. Epand, H.J. Vogel, Diversity of antimicrobial peptides and their mechanisms of action, Biochim. Biophys. Acta 1462 (1999) 11–28.
[29] H.G. Boman, Innate immunity and the normal microflora, Immunol. Rev. 173 (2000) 5–16.
[30] M. Zanetti, R. Gennaro, D. Romeo, Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain, FEBS Lett. 374 (1995) 1–5.
[31] C. Lawyer, S. Pai, M. Watabe, P. Borgia, T. Mashimo, L. Eagleton, K. Watabe, Antimicrobial activity of a 13 amino acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides, FEBS Lett. 390 (1996) 95–98.
[32] M. Schiffer, C.H. Chang, F.J. Stevens, The functions of tryptophan residues in membrane proteins, Protein Eng. 5 (1992) 213–214.
[33] H. Lu, T. Marti, P.J. Booth, Proline residues in transmembrane alpha helices affect the folding of bacteriorhodopsin, J. Mol. Biol. 308 (2001) 437–446.
[34] K.A. Williams, C.M. Deber, Proline residues in transmembrane helices: Structural or dynamic role?, Biochemistry 30 (1991) 8919–8923.
[35] D.J. Schibli, P.M. Hwang, H.J. Vogel, Structure of the antimicrobial peptide tritrpticin bound to micelles: a distinct membrane-bound peptide fold, Biochemistry 38 (1999) 16749–16755.
[36] S. Nagpal, V. Gupta, K.J. Kaur, D.M. Salunke, Structure–function analysis of tritrypticin, an antibacterial peptide of innate immune origin, J. Biol. Chem. 274 (1999) 23296–23304.
[37] R.B. Merrifield, Solid phase synthesis, Science 232 (1986) 341–347.
[38] G.M. Holzwarth, P. Doty, The ultraviolet circular dichroism of polypeptides, J. Am. Chem. Soc. 87 (1965) 218–228.
[39] C.L. Friedrich, A. Rozek, A. Patrzykat, R.E.W. Hancock, Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria, J. Biol. Chem. 276 (2001) 24015–24022.
[40] C.L. Friedrich, D. Moyles, T.J. Beveridge, R.E.W. Hancock, Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria, Antimicrob. Agents Chemother. 44 (2000) 2086–2092.
[41] C. Subbalakshmi, V. Krishnakumari, R. Nagaraj, N. Sitaram, Requirements for antibacterial and hemolytic activities in the bovine neutrophil derived 13-residue peptide indolicidin, FEBS Lett. 395 (1996) 48–52.
[42] S.E. Blondelle, R.A. Houghten, Hemolytic and antimicrobial activities of the 24 individual omission analogues of melittin, Biochemistry 30 (1991) 4671–4678.