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Role of Amino Acid Residues within the Disulfide Loop of Thanatin, a Potent Antibiotic Peptide

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Thanatin, a 21-residue peptide, is an inducible insect peptide with a broad range of activity against bacteria and fungi. It has a C-terminal disulfide loop, like the frog skin secretion antimicrobial peptides of the brevinin family. In this study, we tried to find the effect of a number of amino acids between the disulfide bond. Thanatin showed stronger antibacterial activity to Gram negative bacteria than other mutants, except Th1; whereas, the mutant peptides with deletion had higher activity to Gram positive bacteria than thanatin. An increase in the number of amino acid(s) using the alanine residue decreased the antibacterial activity in all of the bacteria. Th1 with deletion of threonine at position 15 (Thr^{15}) showed similar antibacterial activity against Gram-negative bacteria, but had higher activity against the Gram positive bacteria. In order to study the structure-function relationship, we measured liposome disruption by the peptides and CD spectra of the peptides. Th1 also showed the highest liposome leaking activity and α-helical propensity in the sodium dodecyl sulfate solution, compared with other peptides. Liposome disruption activity was closely correlated with the anti-Gram positive bacterial activity. All of the peptides showed no hemolytic activity. Th1 was considered to be useful as an antimicrobial peptide with broad spectrum without toxicity.

Keywords: Antibiotic peptide, Disulfide loop, Structure-activity relationship, Thanatin

Introduction

Insects have at least two defense mechanisms: a cellular response (phagocytosis and encapsulation) and an inducible humoral response (synthesis of potent antimicrobial peptides) in response to bacterial challenge or body injury (Hoffmann, 1995; Hoffmann *et al.*, 1996). Many antimicrobial peptides have been identified as inducible components of the insect defense mechanism (Bulet *et al.*, 1999). These peptides are grouped into four families: cecropins (Steiner *et al.*, 1981; Zasloff *et al.*, 1988), defensins (Hoffmann and Hetru, 1992), small prolin-rich peptides (Casteels *et al.*, 1989), and large glycin-rich peptides (Lee *et al.*, 1996).

Recently, an antimicrobial peptide, thanatin (GSKKPVPIIY CNRRTGKCQRM), which does not belong to any of the above groups, was isolated from the hemipteran insect *Podisus maculiventris* (Fehlbaum *et al.*, 1996). It is identified as the first insect antimicrobial peptide to show broad antibiotic activity against Gram negative bacteria, Gram positive bacteria, and fungi without cytotoxicity. It has a sequence homology with the brevinin family of antimicrobial peptides that is isolated from frog skin (Morikawa *et al.*, 1992; Simmaco *et al.*, 1993; Clark *et al.*, 1994; Park *et al.*, 1994). Thanatin and brevinins contain a disulfide loop with a strong cation at their C-terminal region. However, thanatin contains six amino acid residues within the loop, and 3 more amino acid residues after the C-terminal cysteine residue. Brevinins, however, has five residues within the loop (Rana box), and no more residue after the C-terminal cysteine residue. The secondary structures of thanatin and brevinins are also different. Thanatin adopts a well-defined anti-parallel β-sheet structure from 8 to the C-terminus (Mandard *et al.*, 1998). However, brevinins have α-helical propensity in the amphipathic condition (Kwon *et al.*, 1998). It has been reported that the C-terminal truncation of the three residues (QRM) are critical to Gram negative bacteria and to some Gram positive bacteria, but the N-terminal truncation of the three residues (GSK) does not affect the antimicrobial activity (Fehlbaum *et al.*, 1996). The three dimensional structure of thanatin demonstrates that the residues are important for stabilizing the anti-parallel β-sheet structure (Mandard *et al.*, 1998). Further N-terminal truncations decrease the antimicrobial activity, depending on the bacterial types

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(Fehlbaum *et al.*, 1996). Previously, we found that the chimeric peptide (T-B1) with the brevinin-1 disulfide loop on the thanatin background elicits higher anti-Gram positive bacterial activity than thanatin, but shows lower activity against the Gram-negative bacteria (Shin *et al.*, 1999b). The peptide adapts more to an a-helical structure in the membrane mimicking condition than thanatin. However, the effect of the residues within the disulfide loop of thanatin has not yet been studied systematically.

In this paper, we tried to systematically identify the effect of amino acids within the disulfide loop of thanatin. We synthesized several peptides with deletion or insertion within the disulfide loop, and characterized the relationships between their structures and antibacterial activities. We also tested the liposome disruption activity and measured the secondary structures of thanatin and its analogues.

Material and Methods

Peptide synthesis and purification Peptide syntheses were performed by the solid phase method (Merrifield, 1986) using Fmoc as the Nα-amino protecting group (Lee *et al.*, 1994; Shin *et al.*, 2000b). All of the peptides were purified by HPLC on a reverse-phase C18-column. Peptide in the reduced form was taken up in the oxidation buffer [1 mg/ml peptide in 0.1 M ammonium acetate, pH 8.5, with or without 5% dimethylsulfoxide (DMSO)] and allowed to refold for 1 to 2 day(s) at room temperature under shaking. It was purified by reverse-phase HPLC. The sequence data of the synthetic peptides used in this study are listed in Table 1.

Determination of minimal inhibitory concentration (MIC) Gram negative bacteria (*Escherichia coli HB 101, Salmonella typhimurium*, and *Pseudomonas aeruginosa*) and Gram negative bacteria (*Bacillus subtilis and Staphylococcus aureus*) were obtained from the Korean Culture Type Collection (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), and cultured in a nutrient broth at 37°C overnight. The cultures were diluted to 1 : 20 in the same broth, cultured at 37°C until absorbance 0.4-0.5 at 650 nm, and diluted in a basal medium of 1% bactopeptone to 1:200. One hundred microliter of the serially 2 fold diluted peptides (0.31 to 20 mM) in 1% bactopeptone were mixed with 50 ml of the above bacterial suspension, then incubated overnight at 37°C in a shaking incubator. The MIC was defined as the lowest concentration of the peptide at which there was no growth of bacteria.

Hemolysis assays The hemolytic activity of the peptides were determined using human red blood cells (hRBCs) by the method reported previously (Kang *et al.*, 1996). Briefly, the hRBCs that were isolated from the heparinized blood were washed twice with PBS-H (30 mM sodium phosphate, 150 mM sodium chloride, pH 7.0), and diluted to 1% (V/V) solution in PBS-H. The RBC diluent was mixed with equal volumes of 400 mg/ml and the peptide solution in PBS-H. Following 1 h of incubation, the samples were centrifuged at 1,000 × *g* for 5 min. The supernatant was separated and its absorbance measured at 414 nm. PBS-H and 0.5% triton X-100 were used as blank and 100% hemolysis controls, respectively.

Liposome disruptions of the peptides The mixture of carboxyfluorocalcein-entrapped phosphatidylcholine (PC; Sigma, St. Louis, USA) and phosphatidylserine (PS; Sigma, St. Louis, USA) (4 : 1, w/w) were prepared by the reverse phase ether evaporation method (Shin *et al.*, 2000a) using 60 mM carboxyfluorocalcein in PBS (10 mM sodium phosphate and 150 mM sodium chloride, pH 7.4) and filtration using 400 nm polycarbonate filter. The unentrapped carboxyfluorocalcein was removed by gel filtration using Sephadex-G50 (Sigma, St. Louis, USA). The liposome suspension was mixed with the peptides (final peptide concentration: 0 to 50 μg/ml after mixing) in the black 96 well plates (Nunc, Roskilds, Denmark). The leakage of carboxyfluorocalcein was monitored by measuring fluorescence intensity at 525 nm (515 nm cutoff), exited at 490 nm on a Spectra Max Gemini dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, USA). The percent dye release by peptide was estimated by the equation: percent release = $(F^P - F^0)/(F^T - F^0) \times 100$, where F^P is the fluorescence intensity by the peptide, F^T is the fluorescence intensity by 0.1% triton-X100, and F^0 is the fluorescence intensity by PBS.

CD spectra The CD spectra of 0.1 mg/ml of peptides in 10 mM sodium phosphate, 30 mM sodium dodecyl sulfate (SDS), and 50% trifluoroethanol (TFE) in 10 mM sodium phosphate, pH 7.0, were recorded on a Jasco J720 spectropolarimeter (Jasco, Tokyo, Japan). The spectra were measured at 25°C using 1 mm path length cells. The scan was repeated three times. The mean residue ellipticity (MRE, [θ]) given in $deg \cdot cm^2 \cdot dmol^{-1}$ was calculated as the following: $[\theta] = [\theta]obs \times (MRW/10lc)$ where [θ]obs is observed ellipticity (millidegree); MRW is mean residue molecular weight of the peptide; *l* is pathlength of the cell (cm); and c is the peptide concentration (mg/ml).

Results and Discussion

Syntheses of thanatin derived peptides with modification between the disulfide bond Thanatin has been reported to have potent anti-bacterial activity against both Gram positive bacteria and Gram negative bacteria (Fehlbaum *et al.*, 1996). To investigate the function of the disulfide loop, we manually synthesized the peptides with deletion or insertion of amino acid residue(s) between the cysteine residues (Table 1), according to our previous method. The purity of each purified synthetic peptide was above 95%, as measured by HPLC. Over 90% disulfide bonds of thanatin, Th1, and Th3 were formed within a day in the aqueous solution (100 mM ammonium acetate, pH 8.5) in the absence of DMSO, but those of Th2, Th4, and Th5 were only partially formed (40% to 60%) under the same conditions. However, in the presence of 5% DMSO the disulfide bond formations of the peptides could be increased up to 90% (data not shown). The peptides with the disulfide bond were purified by reverse phase HPLC, and its disulfide bond content was conformed by the treatment of 20 mM dithiothreitol (DTT) to the purified peptides. The mixture of an oxidizing peptide (with disulfide bond) and its reduced form (DTT treated) showed two peaks separated slightly on HPLC (data not shown).

Table 1. Amino acid sequences of thanatin and its analogues.

Peptides	Sequences	Remarks
thanatin	GSKKPVPIIYC*NRRTGKC*QRM	Native
Th1	GSKKPVPIIYC*NRR-GK*CQRM	Delete 15T
Th2	GSKKPVPIIYC*NRRT-K*CQRM	Delete 16G
Th3	GSKKPVPIIYC*NRR--K*CQRM	Delete TG
Th4	GSKKPVPIIYC*NRRATGK*CQRM	Insert *A*
Th5	GSKKPVPIIYC*NRRAATGK*CQRM	Insert *AA*
Th6	GSKKPVPIIYA*NRRTGK*AQRM	Change Cs to *A*s

Table 2. Antibacterial and hemolytic activities of thanatin and its analogues

Peptides	Anti-bacterial activity: MIC (μM)					Percent Hemolysis
	Gram negative bacteria			Gram positive bacteria		
	E. coli	*S. typhimurium*	*P. aeruginosa*	*B. subtilis*	*S. aureus*	
thanatin	0.64[a]	1.3	2.6	10.4	20.8	0
Th1	0.64	1.3	2.6	5.2	10.4	0
Th2	2.6	1.3	5.2	5.2	20.8	0
Th3	2.6	1.3	10.4	5.2	10.4	0
Th4	5.2	2.6	5.2	20.8	NA	0
Th5	2.6	2.6	5.2-10.4	20.8	NA	0
Th6	NA[b]	20.8	NA	NA	NA	0

[a]The antibacterial activities of the peptides were measured by the minimum inhibitory concentrations (MICs).
[b]NA: no activity up to the peptide concentration 20.8 μM.

Antibacterial and hemolytic activities of thanatin and its analogues In this study, we tried to evaluate the effect of a number of amino acids between the disulfide loop of thanatin. The positively charged residues of antibacterial peptides are considered to be important to antibacterial activity. We targeted the residues Thr[15] and Gly[16] to make the deletion mutants (Th1, Th2, and Th3) (Table 1). We also designed the insertion mutant peptides with one and two additional alanine residue(s) between Arg[14] and Thr[15] (Th4 and Th5, respectively) (Table 1). The resulting peptides were designed to have 4 to 8 residues between the disulfide bond. To remove the disulfide loop, the mutant (Th6) substituted alanines for cysteines at positions 11 and 18 were synthesized.

Antibacterial and hemolytic activities of thanatin and its analogues are summarized in Table 2. The anti-bacterial activities of the peptides were determined by measuring MICs. Antibacterial activity of thanatin was stronger to the Gram negative bacteria (0.63 to 2.5 μM) than to the Gram positive bacteria (10 to 20 μM) (Table 2). The mutant peptides (Th2-Th5), except Th1 with similar anti-Gram negative activity to thanatin, were found to have lower antibacterial activity than thanatin. The most reduction (12.5% to 25%) was found against *E. coli*. However, those mutations were either less sensitive or not at all sensitive to *S. typhimurum* (Table 2). The mutant peptides elicited moderate reduced activities (25% to 50%) against *P. aerugenosa*. However, the anti-Gram-positive activities of the mutant peptides were different, according to mutation types. The mutant peptides with deletion (Th1, Th2, and Th3) elicited 1 to 2-fold higher activity than thanatin; the mutant peptides with alanine insertion (Th4 and Th5), however, still showed lower activity (Table 2). The mutant substituted alanine for cysteine (Th6) showed no or little antibacterial activities against all of the bacterial strains tested, suggesting that the disulfide bond of thanatin is important for antibacterial activity. All of the peptides that were used did not exhibit hemolytic activity at 200 μg/ml concentration (Table 2). Th1 with the threonine deletion showed the most potent antibacterial activity for all of the peptides tested without hemolytic activity. Even though the chimeric peptide T-B1 that was used for our previous study (Shin *et al.*, 1999b) showed higher anti-Gram positive bacterial activity than thanatin, it elicited lower anti-Gram negative bacterial activity. It was also found to have hemolytic activity at 100 μg/ml peptide concentration. These results suggest that Th1 may be a useful candidate for developing the antibacterial drug without cytotoxicity.

Liposome disruption activities of the peptides The negatively charged liposome has been used frequently for mimicking the negatively charged bacterial membrane. We prepared liposome that is composed of a mixture of PC and PS (4 : 1) entrapped carboxyfluorescene. The liposome preparation was diluted to get approximately 2,500 fluorescence unit at 100% disruption (600-700 fluorescence unit in PBS). Serially diluted peptides (0 to 50 μg/ml peptide concentrations) were used for these assays. Fig. 1 shows the liposome disruption activities of the peptides. Interestingly, Th1 showed the highest liposome disruption at 8 μg/ml. The

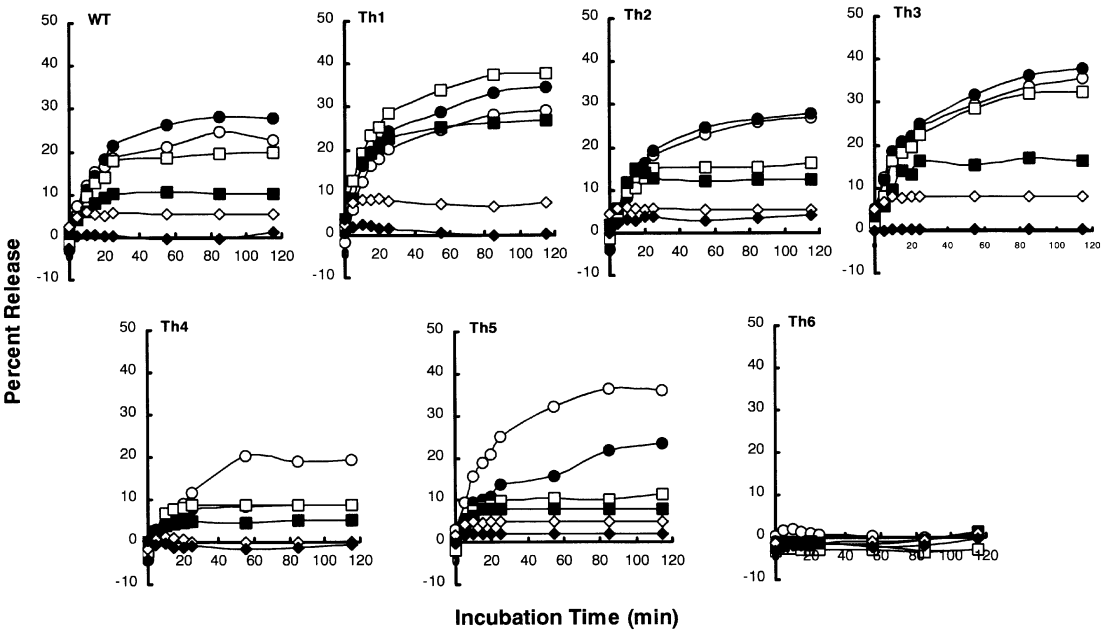

Fig. 1. Liposome disrupting activities of thanatin and its analogues. Serially diluted peptides [final concentrations, 0.512 (◆), 1.28 (◇), 3.2 (■), 8.0 (□), 20 (●), and 50 (○) μg/ml] were added to the carboxyfluorescein entrapped liposome solution. The fluorescence was monitored by a microplate spectrofluorometer at excitation 490 nm and emission 425 nm (415 nm cutoff). For details, see "Materials and Methods".

orders of the Th1 concentration for liposome disruption was 8>20>3.2≅50>1.28>0.512. These experiments were performed two to three times; obtaining similar results. Although we could not explain why there was less liposome disruption activity at the higher peptide concentration, the result was considered to be caused by the peptide-peptide aggregation at the higher peptide concentration. Similar results were also shown when thanatin, Th2, and Th3 were used. The highest activities were at 20 μg/ml. However, liposome disruption activities of Th4 and Th5 with alanine insertion increased, according to the increases of their concentrations. As shown in Fig. 2, at 8 μg/ml the Th1 peptide concentration that had the highest antibacterial activity against all of the bacterial strains also showed the highest liposome disruption activity; Th3 that showed higher activity against Gram positive than thanatin also had stronger activity than thanatin. Th2 showed slightly less liposome disruption activity than thanatin. However, the peptides with insertion (Th4 and Th5) that had a much lower anti-Gram positive activity than thanatin elicited much less activity than thanatin; Th6 (without the disulfide bond) showed no activity. These results indicate that the liposome disruption activities of the peptides that are derived from thanatin are correlated well with the anti-Gram positive activity, but not with the anti-Gram negative activity. The maximum disruptions of all of the peptides were not over 40%, even for more than a 100 min incubation, suggesting that the peptides are not potent for disrupting the artificial membrane. Since there is no outer membrane of Gram-positive bacteria, thanatin and its analogues are considered to interact directly with the Gram positive bacterial membrane.

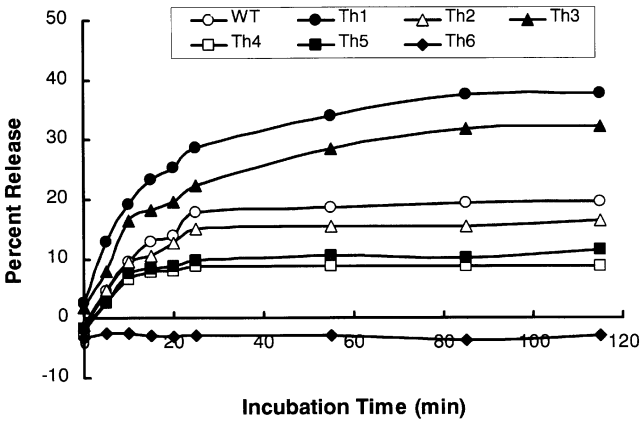

Fig. 2. Comparison of liposome disruption of thanatin and its analogues at the concentration 8 μg/ml. The fluorescence was monitored by the same method as Fig. 1.

The lower activities of the peptides against the Gram-positive bacteria than the Gram negative bacteria was considered to be correlated with the lower membrane disrupting activity of the peptides. This result also suggested that an additional mechanism(s) may be involved in the strong anti-Gram negative bacterial activity.

Secondary structures of the peptides It has been reported that thanatin form a β-sheeted structure in water (Mandard *et al.*, 1998). In this study, we tried to find the structure-activity relationships of the peptides. The secondary structures of thanatin and the deleted peptides were identified in the

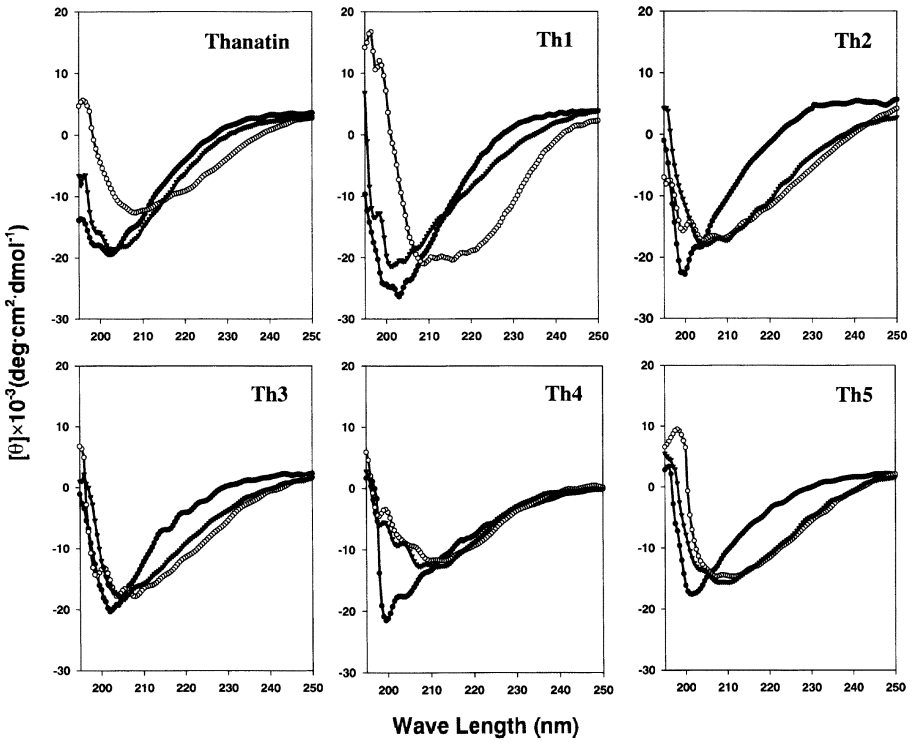

Fig. 3. CD spectra thanatin and its analogues in 10 mM sodium phosphate, pH 7.0, buffer only (●), 30 mM SDS in buffer (○), and 50% TFE in buffer (▼). For details, see "Materials and Methods".

aqueous solution, 30 mM SDS and 50% TFE by CD. SDS was used to mimic the cell membrane condition. Thanatin showed an almost random structure in the aqueous solution (10 mM sodium phosphate, pH 7.0) and elicited a similar structure, even in 50% TFE. However, its secondary structure in 30 mM SDS showed more α-helical propensity than that in 50% TFE (Fig. 3). Th1 also showed an almost random structure in the aqueous solution, and a slightly ordered structure in 50% TFE. However, Th1 in 30 mM SDS strongly adapted to an α-helical structure. Many peptides, especially α-helix forming peptides, have been found to form much more ordered structures in TFE (Shin *et al.*, 1999a). However, the secondary structures of thanatin and Th1 in 50% TFE were slightly changed in our study, while those in 30 mM SDS were much ordered. This indicates that the charged interaction, in addition to the hydrophobic interaction, is also needed to form the ordered structure in this case. Similar patterns of thanatin and Th1 were also observed in the lower SDS concentration (10 mM) (data not shown). Since SDS mimics the bacterial membrane environment, the higher α-helical propensity of Th1 than thanatin was considered to be correlated with the higher anti-Gram positive activity. The number of residues between the disulfide bond of the peptide is important to determine its secondary structure. The peptide with 6 residues within the disulfide bond can stabilize the β-turn and β-sheet conformation (Mandard *et al.*, 1998). However, the 5 residues may not be sufficient to stabilize the structure. Moreover, it has been reported that 5 residues within the disulfide bond stabilize the α-helical structure in brevinins (Kwon *et al.*, 1998). Our result agreed with this observation. Th2 with the glycine deletion within the disulfide bond displayed random structures in the aqueous solution. However, their CD spectra in 50% TFE were much more structural than that in the aqueous solution, and was similar to that in 30 mM SDS, unlike thanatin and Th1 (Fig. 3). Even though both Th1 and Th2 have 5 amino acid residues within the disulfide bond, their structure and biological activities were quite different. This result demonstrates that the flexible glycine residue within the loop of Th1 may be important for the higher activity and inducing α-helicity. However, a nuclear magnetic resonance (NMR) study in the membrane mimicking condition is needed to prove the importance of glycine of Th1. Th3 with two residue (The-Gly) deletions showed a similar pattern with Th2 in TFE and SDS. Th5 with two alanine insertions within the disulfide bond showed similar patterns with Th2 and Th3. However, Th4 with one alanine insertion showed a less conformational structure in TFE and SDS (Fig. 3). Th6 without the disulfide bond showed almost random conformation, even in 50% TFE or 30 mM SDS (data not shown). Since the SDS solution mimics the bacterial membrane structure, the secondary structures of the peptides in 30 mM SDS might be more important for evaluating the anti-bacterial properties of the peptides. Although the 3-dimensional structure of thanatin was conformed in water (Mandard *et al.*, 1998), the 3-dimensional structures of thanatin and its analogues should be tested in the

mimicking condition of the bacterial lipid membrane in order to analyze the role of the disulfide bond and the residues within disulfide bond.

In conclusion, the number of residues within the disulfide bond of thanatin and its analogues affect the antibacterial activities. Interestingly, the deleted peptides (Th1, Th2, and Th3) showed higher anti-Gram positive bacterial activity than thanatin; whereas, the peptides with alanine insertion(s) had lower anti-Gram positive bacterial activity than thanatin (Table 2). Similar patterns also appeared in the liposome disruption study (Figs. 1 and 2). Since the net charges of the peptides are the same, the residue(s) between the positively charges within the loop may affect their activities and structures. Th1 with the deletion of Thr^{15} of thanatin showed the highest activity against all of the bacterial strains tested. Th1 also elicited more potent liposome disruption activity than the other peptides at a 8 μg/ml concentration; this was correlated with higher anti-Gram positive activity rather than anti-Gram negative activity. The α-helical propensity of Th1 in SDS was thought to be important for anti-Gram positive activity and liposome disrupting activity. Th1 will be useful as a model peptide for the development of more potent antibacterial peptide without cytotoxicity.

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