Structure-activity relationships of the intramolecular disulfide bonds in coprisin, a defensin from the dung beetle

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INTRODUCTION

The innate immune system is the first line of defense against bacterial, fungal, and viral pathogens (1). This defense system is essential for host survival, in a world full of potentially dangerous microbes. As invertebrates do not have an adaptive immune system (2), the innate immune system is particularly important in these organisms. For example, insects protect themselves from pathogens through the production of antimicrobial peptides, the so-called defensins. Defensins, which are small cationic molecules produced by organisms as part of their innate immune response, share a common structural scaffold that is stabilized by three disulfide bridges. Coprisin is a 43-amino acid defensin-like peptide from Copris tripartitus. Here, we report the intramolecular disulfide connectivity of cysteine-rich coprisin, and show that it is the same as in other insect defensins. The disulfide bond pairings of coprisin were determined by combining the enzymatic cleavage and mass analysis. We found that the loss of any single disulfide bond in coprisin eliminated all antibacterial, but not antifungal, activity. Circular dichroism (CD) analysis showed that two disulfide bonds, Cys20-Cys39 and Cys24-Cys41, stabilize coprisin’s α-helical region. Moreover, a BLAST search against UniProtKB database revealed that coprisin’s α-helical region is highly homologous to those of other insect defensins. [BMB Reports 2014; 47(11): 625-630]

RESULTS

Synthesis of coprisin and its Cys-to-Ala analogs

Coprisin contains six cysteines that form three disulfide bonds. We employed the standard Fmoc solid-phase peptide method
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Fig. 1. Scheme for identifying the intramolecular disulfide bonds within coprisin. (A) Coprisin was digested with thermolysin, after which the peptide fragments (F1-F4) were purified, using RP-HPLC. The molecular weights of the fragments were measured using MALDI-TOF MS. The theoretical masses (4409.1 Da) of Cop [Ala3,34], Cop [Ala20,39], and Cop [Ala24,41] were also consistent with the measured molecular weights (4409.2 Da).

Conformational studies
The secondary structures of coprisin and the three Cys-to-Ala analogs were analyzed using circular dichroism (CD) spectroscopy, with a membrane-mimicking environment that was achieved through the addition of 50 mM SDS to sodium phosphate buffer (Fig. 1C). Consistent with previously reported CD spectra (10), coprisin’s spectra showed two minima around 208 and 222 nm, which indicate the presence of α-helix. In addition, the 208 nm band was of larger magnitude, and more prominent than the 222 nm band, which is characteristic of the α + β class of proteins, and indicates that coprisin contains α-helix and β-strands (14).

To investigate the structure-function relations of the disulfide bridges, we used three coprisin analogs, in which a single disulfide bond was deleted, by substituting two cysteine residues with alanine (Cop [Ala3,34], Cop [Ala20,39], and Cop [Ala24,41]). When measured under the same conditions, the CD spectrum of Cop [Ala3,34] was similar to that of coprisin. By contrast, the CD spectra of Cop [Ala20,39] and Cop [Ala24,41] revealed more flexible conformations than the wild-type peptide (Fig. 1C). These data suggest that two disulfide bonds (Cys20-Cys39 and Cys24-Cys41) are important for the conformational stability of coprisin.
Cys24-Cys41) play major roles in stabilizing the α-helical structure within coprisin, while the first disulfide bond (Cys3-Cys34) appears to be involved in forming the N-terminal loop. These findings are consistent with the three-dimensional structure of coprisin, which has two disulfide bridges (Cys20-Cys39 and Cys24-Cys41) located in the α-helical region, and one (Cys3-Cys34) located between the N-terminal loop and the β-strand (Fig. 1B).

Functional characterization of coprisin and its Cys-to-Ala analogs
To assess the importance of the disulfide bonds to coprisin function, we examined the antimicrobial and antifungal activities of the wild-type coprisin, and the aforementioned analogs, against a representative set of Gram-negative (Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa) and Gram-positive (Staphylococcus aureus, S. epidermidis, Bacillus subtilis) bacterial strains, as well as four fungal strains (Candida albicans, C. parapsilosis, Malassezia furfur, Trichosporon beigelii). In the liquid growth inhibition assays, the ability of serial dilutions of coprisin, Cop [Ala3,34], Cop [Ala20,39], and Cop [Ala24-41] to inhibit cell growth in vitro was evaluated. The positive control was melittin, a well-known antimicrobial peptide. As summarized in Table 1, coprisin inhibited growth of bacterial strains in the range 0.8 to 3.1 μM, and fungal strains in the range of 5 to 10 μM. These values are similar to previously reported MICs (11, 15). By contrast, Cop [Ala3,34], Cop [Ala20,39], and Cop [Ala24-41] did not inhibit bacterial growth at any concentration up to 100 μM, although antifungal activity was slightly reduced. Thus, the three disulfide bonds appear to be essential for coprisin’s antibacterial activity, but not its antifungal activity.

Table 1. Antimicrobial activities of coprisin and its three Cys-to-Ala analogs

| Microorganism                  | Coprisin | Cop [Ala3,34] | Cop [Ala20,39] | Cop [Ala24,41] | Melittin |
|-------------------------------|----------|--------------|---------------|---------------|----------|
| Gram-negative bacteria        |          |              |               |               |          |
| E. coli                       | 3.1      | >100         | >100          | >100          | 1.6      |
| S. typhimurium                | 3.1      | >100         | >100          | >100          | 1.6      |
| P. aeruginosa                 | 3.1      | >100         | >100          | >100          | 1.6      |
| Gram-positive bacteria        |          |              |               |               |          |
| S. aureus                     | 0.8      | >100         | >100          | >100          | 0.8      |
| S. epidermidis                | 1.6      | >100         | >100          | >100          | 1.6      |
| B. subtilis                   | 1.6      | >100         | >100          | >100          | 0.8      |
| Fungal strains                |          |              |               |               |          |
| C. albicans                   | 10       | 12.5         | 12.5          | 12.5          | 3.1      |
| C. parapsilosis               | 10       | 12.5         | 12.5-25.0     | 12.5          | 6.3      |
| M. furfur                     | 5        | 25.0         | 25.0          | 25.0          | 6.3      |
| T. beigelii                   | 10       | 25.0         | 25.0          | 25.0          | 6.3      |
Location of the active site related to antimicrobial activity

Interestingly, Cop [Ala1,34] had no antibacterial activity, despite having a secondary structure similar to that of coprisin. We therefore sought to identify the active site of coprisin. In an earlier report, it was suggested that the active site is the amphipathic α-helical region of insect defensins (16). To test whether the antimicrobial activity of coprisin originates from the amphipathic α-helical region, we synthesized 18 short peptides that were derived from the α-helical regions of a set of insect defensins, including coprisin (Fig. 2, Table S2). The primary sequence identity and similarity between coprisin and other insect defensins lie in the range from 50% to 78%, and 59% to 90%, respectively. As expected, N1, which was derived from coprisin, showed antimicrobial activity against selected Gram-negative bacteria (MIC = 15 μM), Gram-positive bacteria (MIC = 7.5-15 μM), and drug-resistant bacteria (MIC = 3.8-15 μM). Although the MICs for N1 were about 10-fold higher than those for coprisin, N1 has the potential to serve as an antimicrobial peptide. Most of the other peptides that were tested also showed broad-spectrum antimicrobial activity against the bacterial strains, in the range of 3.8 to 30 μM. Three exceptions were N12, N14, and N15, which at concentrations up to 30 μM, did not show antibacterial activity. Thus 15 of the 18 tested short peptides corresponding to the α-helical region apparently exhibited a meaningful antibacterial activity. This strongly suggests the active site for its antibacterial activity may be the α-helical region of insect defensin.

DISCUSSION

In this study, we investigated the structure-activity relationships of coprisin. To obtain information about its structure, we determined the disulfide bond structure, and analyzed its CD spectrum. Enzymatic digestion and mass measurements revealed that coprisin has the same disulfide bond pairings as other insect defensins (17). Analysis of its CD spectrum showed that the structure of coprisin contains α-helix and β-sheet. The CD spectrum further showed that two disulfide bonds (Cys20-Cys39 and Cys24-Cys41) are important for stabilizing the α-helix, which was lost in Cop [Ala20,39] and Cop [Ala24,41], along with the peptides’ antibacterial activity. On the other hand, Cop [Ala1,34] also did not show antibacterial activity, despite retaining its α-helical region. In Cop [Ala1,34], it was the N-terminal loop that was lost. This raises the question, why does Cop [Ala1,34] not inhibit bacteria growth? It may be that the loss of either the flexible N-terminal loop, or the α-helical region of coprisin, affects oligomerization of the peptide. Coprisin shows a high degree of similarity with sacepin, which is the only insect defensin whose oligomerization mechanism is known. In sacepin, two residues (Asp4 and Arg28) mediate oligomer formation through electrostatic interaction (5). These residues are conserved in coprisin, which prompts us to speculate that coprisin also forms oligomers. If so, loss of the N-terminal loop or the α-helix could reduce the likelihood of electrostatic interaction between Asp4 and Arg28 of coprisin. Alternatively, the N-terminal loop may be important for the activity itself. It was previously reported that even though the α-helical region and all three disulfide bonds were retained, deletion of the N-terminal loop of tenecin 1, an insect defensin from T. molitor, eliminated the peptide’s antimicrobial activity (7). Tenecin 1 and coprisin have the same number of residues in their N-terminal loops, and show >80% sequence homology. It therefore seems likely that the N-terminal loop of coprisin also contributes to its antimicrobial activity.

Interestingly, we found that irrespective of the α-helical content, the antifungal activities of the three Cys-to-Ala analogs were sustained. One reported observation that provides a possible explanation was made in C. albicans, where coprisin induces apoptosis without membrane disruption, after being taken up into the cells via active transport (11). By contrast, when

Table 2. Antimicrobial activities of 9-mer peptides derived from insect defensins

| Microorganism | Minimal inhibitory concentrations (μM) |
|--------------|----------------------------------------|
| Gram-negative bacteria | N1 | N2 | N3 | N4 | N5 | N6 | N7 | N8 | N9 | N10 | N11 | N12 | N13 | N14 | N15 | N16 | N17 | N18 | CopA3 |
| E. coli | 15 | 7.5 | 15 | 15 | 30 | 15 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >7.5 | 15 | 15 | 15 | 7.5 |
| S. typhimurium | 15 | 7.5 | 15 | 15 | 30 | 7.5 | 7.5 | 15 | 7.5 | 30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 15 | 15 | 15 | 15 |
| P. aeruginosa | 15 | 7.5 | 15 | 15 | 30 | 7.5 | 7.5 | 30 | 7.5 | 30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 15 | 15 | 15 | 15 |
| S. aureus | 7.5 | 7.5 | 15 | 15 | 30 | 7.5 | 7.5 | 30 | 15 | 30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 15 | 15 | 15 |
| S. epidermidis | 15 | 7.5 | 7.5 | 15 | 30 | 7.5 | 7.5 | 15 | 7.5 | 30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 |
| B. subtilis | 7.5 | 7.5 | 7.5 | 15 | 7.5 | 7.5 | 30 | 15 | 30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >15 | >15 | 7.5 | >15 |
| E. faecium | 15 | 7.5 | 30 | 15 | >30 | 15 | 3.8 | >30 | >30 | >30 | >30 | 7.5 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 7.5 |
| E. faecalis | 15 | 15 | >30 | 30 | 15 | >30 | >30 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 3.8 |
| Drug resistant bacteria | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| VRE (E. faecium) | 3.8 | 3.8 | 7.5 | 15 | 7.5 | 3.8 | 15 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 7.5 |
| VRE (E. faecalis) | 15 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 7.5 |
| MRSA | 7.5 | 7.5 | 15 | 30 | 7.5 | 7.5 | 30 | 15 | 30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | >30 | 15 |

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applied to bacteria, coprisin induces membrane disruption (10). These findings suggest the mode of action of coprisin differs between bacteria and fungi.

Generally, the primary structures of insect defensins are highly homologous. In addition, structural studies have also demonstrated that these peptides share a common structural feature, the cysteine-stabilized α/β motif. This is composed of a short amphipathic α-helix, followed by C-terminal anti-parallel β sheets that are stabilized by three disulfide bridges (19). In their analyses of the structure-function relationships of these peptides, several groups have shown that short linear fragments corresponding to the α-helical region possess antimicrobial activity (16, 19, 20). Our present findings that were obtained using 18 short peptides derived from the α-helical regions of a set of insect defensins (Table 2) are consistent with those earlier reports. However, three of the short peptides, N12, N14, and N15, did not show antimicrobial activity. Given what we know about the parameters important for activity, and the primary structures of these three peptides, as compared to the active peptides, we suggest that the net positive charge and the number of the hydrophobic residues could be critical parameters that affect antimicrobial activity.

In sum, we determined a disulfide bond pattern of coprisin, and showed that it is the same as in other insect defensins. In addition, mutation studies showed that all three disulfide bonds are essential for antibacterial activity, and that coprisin’s modes of action against bacteria and fungi likely differ. Finally, we determined that the α-helical region is the active site in insect defensins.

MATERIALS AND METHODS

Peptide synthesis and disulfide formation

All peptides were purchased from Anygen (Korean, Gwangju). These peptides were manually synthesized, using the solid-phase peptide synthesis method with Fmoc chemistry. The peptides were cleaved from the resin using trifluoroacetic acid containing various scavengers, and purified using preparative RP-HPLC (Shimadzu, Tokyo, Japan). The purity of the peptides was verified by analytical RP-HPLC, and correct peptide masses were confirmed using MALDI-TOF MS (Shimadzu). For disulfide bond formation, linear coprisin and the Cys-to-Ala mutants, Cop [Ala1-34], Cop [Ala20-39], and Cop [Ala24-39], were dissolved to a concentration of 25 μM in 0.1 mM ammonium acetate solution (pH 7.0), and allowed to react at room temperature for 24 h, with gentle stirring. The course of the reaction was monitored using HPLC. The yields of coprisin and the three cysteine mutants were >90%.

Antimicrobial activity

Using broth microdilution assays, we assessed the antimicrobial activities of each peptide, against six selected organisms obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). These included three Gram-negative (E. coli KCTC 1682, S. typhimurium KCTC 1926, and P. aeruginosa KCTC 1637) and three Gram-positive (S. aureus KCTC 1621, S. epidermidis KCTC 1917, and B. subtilis KCTC 3068) strains. Briefly, single colonies of bacteria were inoculated into medium (LB broth), and cultured overnight at 37°C. An aliquot of the culture was then transferred to 10 ml of fresh medium, and incubated for an additional 3-5 h at 37°C, until the mid-logarithmic phase. Two-fold dilution series of the peptides in 1% peptone was then prepared, after which the serial dilutions (100 μl) were added to 100 μl of cells (5 x 10^5 colony-forming units (CFU/ml)), in 96-well microtiter plates (96 microtiter plates; Nunc, Odense, Denmark), and incubated for 16 h at 37°C. The lowest concentration of peptide that completely inhibited growth was defined as the MIC. MICs were determined as the average of triplicate measurements, in three independent assays.

Fungal strains and antifungal susceptibility test

Candida albicans (ATCC 90028) and C. parapsilosis (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Malassezia furfur (KCTC 7744) and Trichosporon beigelii (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). With the exception of M. furfur, the fungal strains were cultured in YPD broth (Difco), with aeration at 28°C. Malassezia furfur was cultured at 32°C in a modified YM broth (Difco), containing 1% olive oil. The cell suspensions were adjusted to obtain standardized populations, by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). Fungal cells at log phase (2 × 10^5/ml) were inoculated into 100 μl of YPD or YM broth well, in microtiter plates. The MIC values were then determined in three independent assays.

Determination of disulfide bond pairings

Intramolecular disulfide bond pairings within coprisin were identified through enzymatic cleavage, using thermolysin and MALDI-TOF MS measurements. Coprisin was digested with thermolysin (Promega, USA) in 100 mM ammonium acetate (pH 6.2) with 2 mM CaCl₂, for 1 h at 60°C. The ratio of coprisin to thermolysin was 10:1 (w/w). The resultant mixture was subjected to RP-HPLC separation, after which the collected fragments were analyzed, using MALDI-TOF MS.

CD analysis

The CD spectra of the peptides were recorded, using a Jasco J-710 CD spectrophotometer (Jasco, Tokyo, Japan), with a 1 mm path-length cell. Wavelengths were measured from 190 nm to 240 nm (bandwidth, 1 nm; step resolution, 0.1 nm; speed, 50 nm/min; response time, 0.5 s). The CD spectra were collected for the peptides in the presence of 50 mM SDS micelles (pH 7.4) at 25°C. The spectra were averaged over 4 scans, and were
expressed as molar ellipticity $[\theta]$ versus wavelength.

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REFERENCES

1. Iwanaga, S. and Lee, B. L. (2005) Recent advances in the innate immunity of invertebrate animals. *J. Biochem. Mol. Biol.* **38**, 128–150.

2. Satake, H. and Sekiguchi, T. (2012) Toll-like receptors of deuterostome invertebrates. *Front Immunol.* **3**, 34.

3. Bulet, P., Stöcklin, R. and Menin, L. (2004) Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev.* **198**, 169-184.

4. Bulet, P., Hetru, C., Dimarcq, J. L. and Hoffmann, D. (1999) Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.* **23**, 329-344.

5. Takeuchi, K., Takahashi, H., Vogai, M., Iwai, H., Kohno, T., Sekimizu, K., Natori, S. and Shimada, I. (2004) Channel-forming membrane permeabilization by an antibacterial protein, sapecin: determination of membrane-buried and oligomerization surfaces by NMR. *J. Biol. Chem.* **279**, 4981-4987.

6. Ahn, H. S., Cho, W., Kang, S. H., Ko, S. S., Park, M. S., Cho, H. and Lee, K. H. (2006) Design and synthesis of novel antimicrobial peptides on the basis of alpha helical domain of Tenecin 1, an insect defense protein, and structure-activity relationship study. *Peptides* **27**, 640-648.

7. Lee, K. H., Hong, S. Y. and Oh, J. E. (1998) Synthesis and structure-function study about tenecin 1, an antibacterial protein from larvae of *Tenebrio molitor*. *FEBS Lett.* **439**, 41-45.

8. Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W. F., Hetru, C. and Hoffmann, J. A. (1994) Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J. Biol. Chem.* **269**, 33159-33163.

9. Hwang, J. S., Lee, J., Kim, Y. J., Bang, H. S., Yun, E. Y., Kim, S. R., Suh, H. J., Kang, B. R., Nam, S. H., Jeon, J. P., Kim, I. and Lee, D. G. (2009) Isolation and characterization of a defensin-like peptide (*Coprisin*) from the dung beetle, *Copris tripartitus*. *Int J Pept.*, 1-5.

10. Lee, E., Kim, J. K., Shin, S., Jeong, K. W., Shin, A., Lee, J., Lee, D. G., Hwang, J. S. and Kim, Y. (2013) Insight into the antimicrobial activities of coprisin isolated from the dung beetle, *Copris tripartitus*, revealed by structure-activity relationships. *Biochim. Biophys. Acta.* **1828**, 271-283.

11. Lee, J., Hwang, J. S., Hwang, I. S., Cho, J., Lee, E., Kim, Y. and Lee, D. G. (2012) Coprisin-induced antifungal effects in *Candida albicans* correlate with apoptotic mechanisms. *Free Radic. Biol. Med.* **52**, 2302-2311.

12. Kang, B. R., Kim, H., Nam, S. H., Yun, E. Y., Kim, S. R., Ahn, M. Y., Chang, J. S. and Hwang, J. S. (2012) CopA3 peptide from *Copris tripartitus* induces apoptosis in human leukemia cells via a caspase-independent pathway. *BMB Rep.* **45**, 85-90.

13. Kim, J. I., Iwai, H., Kurata, S., Takahashi, M., Masuda, K., Shimada, I., Natori, S., Arata, Y. and Sato, K. (1994) Synthesis and characterization of sapecin and sapecin B. *FEBS Lett.* **342**, 189-192.

14. Manavalan, P. and Johnson, W. C. (1983) Sensitivity of circular dichroism to protein tertiary structure class. *Nature* **305**, 831-832.

15. Hwang, I. S., Hwang, J. S., Hwang, J. H., Choi, H., Lee, E., Kim, Y. and Lee, D. G. (2013) Synergistic effect and antibiotic activity between the antimicrobial peptide coprisin and conventional antibiotics against opportunistic bacteria. *Curr. Microbiol.* **66**, 56-60.

16. Lee, K. H. (2002) Development of short antimicrobial peptides derived from host defense peptides or by combinatorial libraries. *Curr. Pharm. Des.* **8**, 795-813.

17. Lepage, P., Bitsch, F., Roecklin, D., Keppi, E., Dimarcq, J. L., Reichhart, J. M., Hoffmann, J. A., Roitsch, C. and Van Dorseeaer, A. (1991) Determination of disulfide bridges in natural and recombinant insect defensins *A. Eur. J. Biochem.* **196**, 735-742.

18. Bulet, P. and Stöcklin, R. (2005) Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept. Lett.* **12**, 3-11.

19. Yamada, K. and Natori, S. (1994) Characterization of the antimicrobial peptide derived from sapecin B, an anti-bacterial protein of *Sarcophaga peregrina* (flesh fly). *Biochem. J.* **298**, 623-628.

20. Saito-Sakanaka, H., Ishishishi, J., Sagisaka, A., Momotani, E. and Yamakawa, M. (1999) Synthesis and characterization of bactericidal oligopeptides designed on the basis of an insect anti-bacterial peptide. *Biochem. J.* **338**, 29-33.