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[Note]

Antibacterial effects of two cecropin type peptides isolated from the silkworm against *Salmonella* species

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Abstract

In insect defense system, antimicrobial peptides (AMPs) are one of important biological molecules to survive in a variety of environments. Insect can synthesize AMPs to protect against invading pathogens in humoral immune response. Taking more advantage of biological antimicrobial molecules, we report antibacterial activity of two cecropin type peptides, cecropin and moricin, isolated from the silkworm against four *salmonella* species. In this work, we purified antimicrobial candidate peptides (AMCP) from the extracts of immune challenged silkworm larval hemolymph by two-step chromatographic purification procedure, cation exchange and gel permeation chromatography. The molecular weights of purified peptides were estimated to be about 4 ~ 5 kDa by Tricin SDS-PAGE analysis, and identified as silkworm cecropin and moricin by NCBI BLAST homology search with their N-terminal amino acid sequences. As antibacterial activity assay, the purified peptides showed stronger antibacterial activity against *Salmonella* pathogens with an MIC value of 1 ~ 4 μg/mL. Therefore two cecropin type peptides purified from the silkworm will be valuable potential materials for development of new natural antibiotics.

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Introduction

Insects produce peptides/proteins, which have a stronger antimicrobial activity, to effectively self-defense against invading pathogens in humoral immune response (Hoffman *et al.,* 1999; Kim *et al*., 2016). Insect antimicrobial peptide/protein (AMP) was first purified from bacteria-challenged pupal hemolymph of the giant silk moths *Hyalophora cecropia* (Steiner *et al*., 1981), and since then a large number of AMPs (over 200) have been revealed from various insect species. Most of these AMPs are small, cationic (net charge of +2 to +9) and amphipathic properties (Jenssen *et al*., 2006). It has been reported that insect rapidly synthesize AMP as inactive precursor protein in the specific tissues such as fat bodies after immune challenge, and generate active peptides or proteins by limited proteolysis to act against pathogens (Hoffman *et al*., 1999; Yi *et al*., 2014). Insect AMPs can be divided into four groups on the certain structures and unique amino acid sequences: α-helical cecropin type peptides such as cecropin and moricin, cystine-stabilized β-sheet peptides such as defensing and drosomycin, proline-rich peptides such as apidaecin and lebocin, and glycine-rich proteins such as attacin and gloverin (Bulet and Stocklin, 2005).

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Cecropin type peptides are a well-studied antimicrobial peptide in lepidopteran immunity. Cecropin is synthesized as secreted cationic/basic peptide of 31 to 39 residues in length, and form α-helical structure with two amphipathic α-helixes in the hydrophobic environment (Yi *et al*., 2014). Moricin is produced as a highly basic 42 residue peptide in silkworm, *Bombyx mori* (Hara and Yamakawa, 1995), and showed a long α-helix with 8 turns along in tertiary solution structures (Dai *et al*., 2008). These α-helical cecropin type peptides have a broad spectrum of activity against Gram-negative and Gram-positive bacteria, as well as filamentous fungi and yeast (Dai *et al*., 2008; Kim *et al*., 2010). In addition, cecropin is also active against HIV-1 virus (Wachinger *et al*., 1998) and cancer cells (Suttmann *et al*., 2008). In the previous study, we identified papiliocin, a 37-residue cecropin type antimicrobial peptide, from the swallowtail butterfly *Papilio Xuthus* (Kim *et al*., 2010). The papiliocin is very stronger antimicrobial peptide with a broad-spectrum of activities against several human pathogenic bacterial strains, and also has anti-inflammatory activity (Kim *et al*., 2011). To more evidence of biological antimicrobial molecules, we purified and identified cecropin type peptides from the immunized larvae of silkworm, *B. mori* by two-step chromatographic purification procedure, cation exchange and gel permeation chromatography. Furthermore, we described antibacterial effects of purified peptides against four *Salmonella* pathogens.

Material and Methods

Immunization and hemolymph protein extraction

Day 5 of the last instar *B. mori* (F_1 hybrid between Jam123 and Jam124 of the Korean silkworm strain) larvae were used for immune challenge. A volume of 50 uL of *Lactobacillus* cell wall extracts dissolved in PBS was injected into the hemocoel using 1 mL disposable syringes. Hemolymph samples were directly collected from the last instar larvae at 18 h post-injection or untreated larvae, and then were centrifuged at 13,000 rpm for 20 min at 4ºC. To facilitate the extraction of hemolymph protein, the supernatant was mixed with the same volume of extracting solution (90% methanol/1% acetic acid) with stirring at room temperature for two hours and then was centrifuged at 13,000 rpm for 20 min at 4ºC. After the extracting process, low molecular weight proteins were separated and concentered with centricon centrifugal filter device (20 kDa cut off). The final solution was stored at -20 ºC before used.

Purification and indeification of antimicrobial peptide

The target antimicrobial candidate peptide (AMCP) was purified by cation exchange chromatography (HiTrap SP HP column, GE Healthcare, USA) as the first step purification and gel permeation chromatography (Superdex peptide 10/300 GL column, GE Healthcare, USA) as the final purification according to the manufacturer instruction. Purification process was performed on AKTA P-910 FPLC system (Amersham Pharmacia, USA). After final purification process, the eluted fractions containing AMCPs were determined by Tricin SDS-PAGE analysis and agar well diffusion assay. Finally, the purified AMCPs were concentrated by Amicon Ultra-10K (Millipore, USA) and then subjected to N-terminal sequencing analysis and antimicrobial activity assay. The N-terminal sequencing of purified peptide was performed by the automated Edman degradation method on ABI 491 liquid-phase sequencer (Applied Biosystems, USA).

Antibacterial activity assay

The antibacterial activity of eluted fractions was performed against *E. coli* ML35 by agar well diffusion assay as described previously (Kim *et al.*, 2015). In addition, MICs (minimum inhibitory concentration) of the purified peptides were also determined by a broth microdilution assay as described previously (Kim *et al*., 2010). Those MIC values were determined against *E. coli* ML35 and four *Salmonella* stains, *S. pullorm*, *S. typhimurium*, *S. enteritidis*, and *S. gallinarum*.

Results and discussion

As previous research, silkworm antimicrobial peptides (AMPs) were synthesized by fat body, which is a major immune responsive tissue, when bacteria or bacterial cell membrane components such as LPS were injected into the body cavity (Hara and Yamakawa, 1995). In this work, we were injected *lactobacillus* extracts containing peptidoglycan into the hemocoel of silkworm larvae to up-expressed the

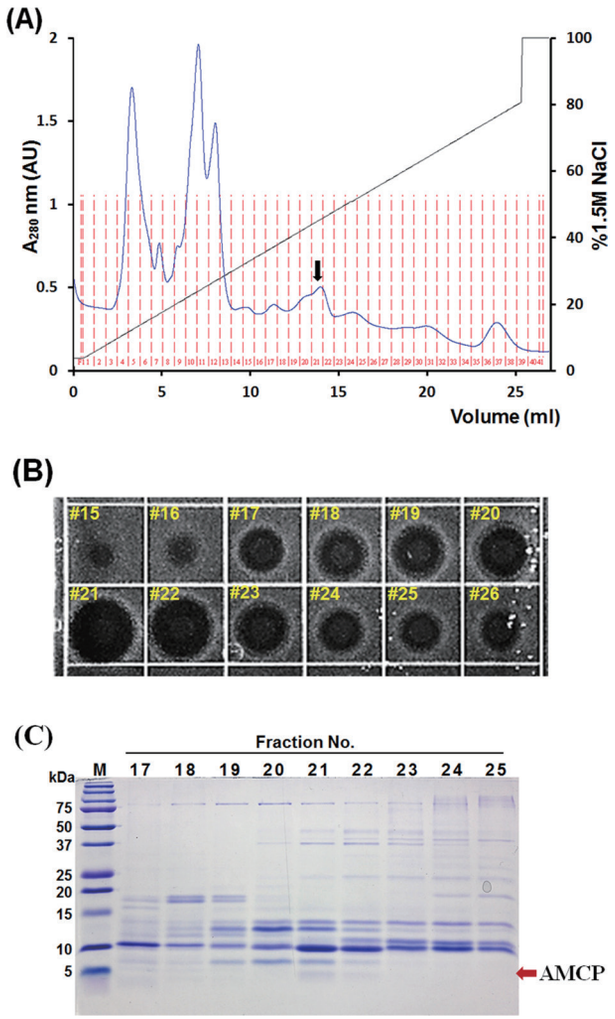

Fig. 1. Fractionation of hemolymph extracts from the immunized larvae by cation exchange chromatography. (A) Profile of cation exchange chromatography using HiTrap SP HP column with linear gradient of 0 - 80% 1.5M NaCl as the first step purification. The peak containing antibacterial activity is indicated with an arrow. (B) Antibacterial activity of fractions eluted form the cation exchange column by agar well diffusion assay (C) Tricine SDS-PAGE analysis of eluted fractions containing antibacterial activity.

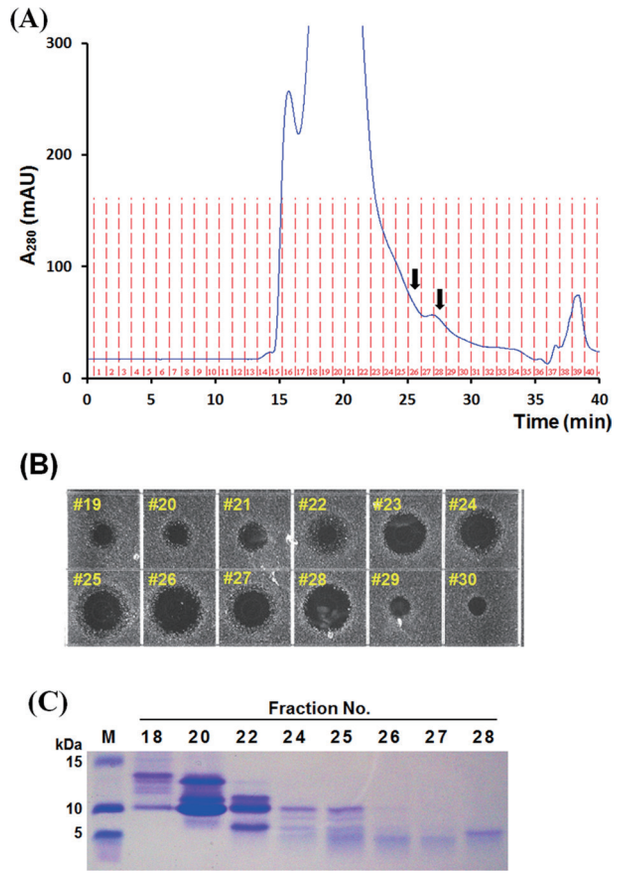

Fig. 2. Final purification of antimicrobial peptides by gel permeation chromatography. (A) Profile of superdex peptide gel permeation chromatography as the final purification. The peaks containing strongest antibacterial activity are indicated with arrows. (B) Agar well diffusion assay of fractions 19 ~30 collected form the superdex peptide column. (C) Tricine SDS-PAGE analysis of fractions 18 ~ 28 containing antibacterial activity.

antimicrobial peptides. As expected, immune challenged larval hemolymph by *lactobacillus* extracts has higher antibacterial activity than non-immune larval hemolymph (data not shown). To identify of antimicrobial peptides produced from immune challenged silkworm larvae, we purified peptides from the extracts of immune challenged larval hemolymph by a two-step chromatographic purification procedure, cation exchange and gel permeation chromatography, on AKTA FPLC system. After cation exchange chromatography, the fractions No. 18 ~ 23 that showed the strong antibacterial activity against *E. coli* by agar well diffusion assay (Fig. 1B) were eluted at 0.8 ~ 0.9 M NaCl concentration (Fig. 1A). As a result of SDS-PAGE analysis, all fractions contained various proteins, but a single peptide band with about 4 ~ 5 kDa was detected in fraction No. 21, which has strongest antibacterial activity against *E. coli* (Fig. 1C). This peptide band is designated as antimicrobial candidate peptide (AMCP). The eluted fractions (No.18 ~23) were collected and fractionated by gel permeation chromatography on Superdex peptide column (Fig. 2A). In the final purification step, we separated peaks (fractions No. 22 ~ 28) that exhibited antibacterial activity against *E. coli* as shown in Fig. 2B, and detected two peptides (named as AMCP-1 and AMCP-2) with about 4 ~ 5 kDa in fractions No. 26 and 28 by tricine SDS-PAGE

Table 1. N-terminal amino acid sequences of purified peptides

Peptide	N-terminal sequence	Identified peptide	Calc. PI	Calc. mass (Da)
AMCP-1	AKIPIKAIKT	Moricin	11.36	4543.52
AMCP-2	RWKIFKKI	Cecropin	10.64	3894.73

Table 2. Antimicrobial activity of purified cecropin and moricin

Bacteria strains	Minimum inhibitory concentration (MIC, μg/mL)		
	Cecropin	Moricin	Melittin
E. coli ML35	2	4	8
Salmonella pullorum	2	4	8
Salmonella typhimurium	2	4	16
Salmonella enteritidis	2	4	16
Salmonella gallinarum	1	2	8

analysis (Fig. 2C). The purified AMCPs were determined N-terminal sequence by the Edman degradation method as shown in Table 1. Based on partially amino acid sequences, two peptides AMCP-1 and AMCP-2 were identified as moricin and cecropin, respectively (Table 1). Table 2 shows the minimum inhibitory concentration (MIC) values of purified moricin and cecropin against several Gram-negative bacteria, *E. coli* and Salmonella stains. As expected, purified cecropin proved to be active against all *Salmonella* stains tested. Our result showed higher growth inhibitory effect on *Salmonella* cells (MIC value of 1 ~ 2 μg/mL). It is in a good agreement with previous studies on cecropin like peptide from swallowtail butterfly (Kim *et al*, 2015). Purified moricin also showed stronger antibacterial activity against *Salmonella* stains with an MIC value of 2 ~ 4 μg/mL. These antibacterial effects of cecropin and moricin were stronger than melittin (MIC value of 8 ~ 16 μg/mL) used as control. The previously study suggested that moricin has higher antibacterial activity against Gram-positive bacteria than cecropin, a major antibacterial peptide of silkworm (Hara and Yamakawa, 1995). However, our results showed that cecropin has higher activity against salmonella stains tested than moricin.

In conclusion, we purified cecropin type antimicrobial peptides cecropin and moricin with molecular mass at 4 ~ 5 kDa from immune challenged silkworm larvae. These peptides have stronger antibacterial activity against clinically important *Salmonella* stains. The current study suggests that cecropin and moricin are essential molecules on immune reaction of silkworm, and it also may be potential as antibacterial material for development of new natural antibiotic.

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