# Antibacterial Activity of Peptides Synthesized Based on the *Bombus ignitus* abaecin, A Novel Proline-Rich Antimicrobial Peptide

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Abaecin is a largest member of the proline-rich antimicrobial peptide family found only in the hymenopterans. A cDNA of abaecin was previously isolated and cloned from *Bombus ignitus*: the mature peptide of *Bombus ignitus* abaecin was composed of 39 amino acid residues. In the present study, we determined the antibacterial effect of *B. ignitus* abaecin synthesized at several lengths against several bacteria by radial diffusion assay. The 37-mer peptide (Ab37) inhibited the growth of Gram-negative bacteria *Escherichia coli* ML-35, *Pseudomonas aeruginosa* and *Salmonela typhimurium*, but showed limited inhibitory activity toward Gram-positive bacteria, except for *Micrococcus luteus*. The truncated 26-mer peptide (Ab26), which was synthesized after truncating some amino acid residues at both N-terminus and C-terminus from the Ab37 peptide, still showed equivalent antibacterial activity to the Ab37. On the other hand, several further truncated peptides exhibited lower activity then did Ab37 peptide.

Key words: Abaecin, Antimicrobial peptide, *Bombus ignitus*, Antibacterial activity

## Introduction

The antimicrobial peptides (AMPs) play important role in

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innate immune responses of insects. When infected by pathogens and parasites, insect produces small, extracellularly secreted AMPs in fat body tissue and certain blood cell (Bulet *et al.*, 1999). These peptides disrupt microbial membranes via a peptide-lipid interaction or a receptor-mediated recognition process (Boman, 1995; Bulet *et al.*, 1999). Most of AMPs have a positive net charge due to the presence of a higher content of positively charged residues, such as arginine and lysine. This cationic character of the AMPs is allowed for insertion into the anionic cell wells and phospholipid membranes of microorganisms (Bulet *et al.*, 2004). After the first isolation and full characterization of cecropin from bacteria challenged *Hyalophora cecropia* pupa (Steiner *et al.*, 1981), more then 200 AMPs have been found in insects (Boman, 1995; Bulet *et al.*, 1999). In general, the insect AMPs have a low molecular weight (below 5-10 kDa), a broad range of antimicrobial activity and an amphipathic structure of α-helix, β-hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed, and are not cytotoxin (Bulet *et al.*, 1999).

Several proline-rich peptides, one of AMPs families, have been pound in the hymenopterans such as *Apis mellifera* (apidaecins and abaecin: Casteels *et al.*, 1989, 1990), *Bombus terrestris* (apidaecin: Casteels *et al.*, 1994) and *Bombus pascuorum* (apidaecin and abaecin: Rees *et al.*, 1997), in the dipteran such as *Drosophila mellanogaster* (drosocin and metchnikowin: Bulet *et al.*, 1993; Levashina *et al.*, 1995), in the lepidopteran such as *Bombyx mori* (lebocins: Hara *et al.*, 1995), and in the hemipteran auch as *Pyrrhocoris apterus* (pyrrhocoricin: Cociancich *et al.*, 1994). In addition, metalnikowins similar to *P. apterus* pyrrhocoricin was isolated from two true bugs, *Palomena prasina* (Chernysh *et al.*, 1996) and *Podisus maculiventris* (Fehlbaum *et al.*, 1996). These pro-

**Table 1.** Sequences and mass of synthetic abaecin congeners used in this study

| Peptide | Amino acid Sequence | Position | Determined mass (Calculated mass) |
|---------|--------------------|----------|-----------------------------------|
| Ab37 | FVPYNPPRPGQSKPFP<u>TFP</u><u>GHG</u>PFNPKIQWPYPLPNP-NH₂ | 1-37 | 4213.32 (4218.82) |
| Ab26 | PPRPGQSKPFP<u>TFP</u><u>GHG</u>PFNPKIQWP-NH₂ | 6-31 | 2911.34 (2916.34) |
| Ab19-N | FVPYNPPRPGQSKPFP<u>TFP</u>-NH₂ | 1-19 | 2172.52 (2275.49) |
| Ab16-N | FVPYNPPRPGQSKPFP-NH₂ | 1-16 | 1825.96 (1830.09) |
| Ab14-N | PPRPGQSKPFP<u>TFP</u>-NH₂ | 6-19 | 1550.84 (1554.79) |
| Ab18-C | <u>GHG</u>PFNPKIQWPYPLPNP-NH₂ | 20-37 | 2058.39 (2061.34) |
| Ab15-C | PFNPKIQWPYPLPNP-NH₂ | 23-37 | 1805.96 (1809.09) |
| Ab12-C | <u>GHG</u>PFNPKIQWP-NH₂ | 20-31 | 1375.71 (1379.57) |

N and C respectively indicate N-terminus C-terminus parts of abaecin. Thr-Phe-Pro and Gly-His-Gly sites are underlined.

line-rich peptides are subdivided into two groups (Bulet *et al.*, 1999), which are the unsubstituted proline-rich peptides (including apidaecin, abaecin and metchnikowin) and the O-glycosylated proline-rich peptides (including drososin, pyrrchocoricin and lebocin).

A cDNA of abaecin was previously isolated and cloned from *Bombus ignitus*: the mature peptide of *Bombus ignitus* abaecin was composed of 39 amino acid residues. In this paper, we have synthesized a full-sized 37-mer peptide (Ab37, 1F-37P-NH2) and 26-mer peptide (Ab26, 6P-31P-NH2) from deduced amino acid sequence of *B. ignitus* abaecine, and determined antibacterial activities of these synthetic peptides against gram-positive and gram-negative bacteria. We also synthesized further truncated peptide fragments based on Ab37 peptide to determine active site of abaecin, and examined their antibacterial activities.

## Materials and Methods

### Peptide synthesis

Peptides (Table 1) were chemically synthesized at a peptide synthesis facility, PepTron Inc. (Daejeon, Korea). These synthetic peptides were purified by the reverse phage HPLC using a Shiseido Capcell Pak C18 column. Elution was performed with a water-acetonitrile linear gradient (0~80% of acetonitrile) containing 0.1% (v/v) trifluroacetic acid (TFA). The synthesized peptides were confirmed by ESI mass spectrometer (Plaform II, Micromass, Man chester, UK) and MALDI-TOF mass spectrometer (Voyager-DESTR, Applied Biosystem).

### Bacterial strains

The bacterial strains used to identify antibacterial activity of *B. ignitus* abaecin are as follows: *Staphylocccus aureus*, Methicillin resistant *Staphylococcu aureus* (MRSA), *Enterococcus faecalis*, *E. hirae*, Vanocomycin resistant

*enterococci* (VRE), *Micrococcus luteus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Escherichia coli* ML-35, and *Salmonela typhimurium*, *Pseudomonas aeruginosa*.

### Antibacterial assay

The antibacterial activity of each synthetic peptide was examined by the redial diffusion assay described in Lehrer *et al.* (1991). Briefly, bacteria were grown overnight in tryptic soy broth (TSB; Difco) at 200 rpm and 37°C to stationary phase. The cultures were diluted in fresh TSB and were incubated at 37°C until the optical density reached 0.4 at 620 nm. The cultured bacteria were centrifuged at 3000 rpm for 10 min at 4°C, washed twice in cold 10 mM sodium phosphate buffer (SPB; pH 7.4) and resuspended in cold SPB. A volume containing $4 \times 10^6$ CFU bacteria was added to 10 m*l* of warm (40 to 50°C) citrate phosphate buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 7.4) containing 1% (w/v) low- electroendosmosis -type agarose (Sigma) and 0.03% TSB. Three-millimeter diameter holes were punched in the set agarose and filled with 5 μ*l* of test peptides. After allowing 3 hrs for diffusion of the peptides, a 10 m*l* nutrient-rich overlay gel containing 6% TSB and 1% (w/v) agarose was overlaid and was then incubated overnight at 37°C. The diameters of clear zones surrounding each well was measured and expressed in activity units (1 mm = 10 units). Alternatively, to assess the dose-dependent antibacterial activity of a full-sized synthetic abaecin, a stock peptide solutions were prepared at 200 μ*l*/m*l* in 0.01% acetic acid and serially diluted to 3.12 μ*l*/m*l*. Mean values of activity units obtained from experiments repeated at least three times were plotted against the $\log_{10}$ of the peptide concentration.

## Results and Discussion

### Chemical synthesis of peptides from abaecin

To determine the antibacterial effect of *B. ignitus* abaecin

**Table 2.** Antibacterial activity of synthetic Ab37 (1 μg) against the several bacteria by using radial diffusion assay

| Bacteria | | Antibacterial activity |
|---|---|---|
| Gram-negative bacteria | *Escherichia coli* | ++++ |
| | *Salmonella typhimurium* | ++ |
| | *Pseudomonas aeruginosa* | ++ |
| Gram-positive bacteria | *Micrococcus luteus* | + |
| | *Staphylococcus aureus* | − |
| | *Listeria monocytogenes* | − |
| | *Bacillus subtilis* | − |
| | *Enterococcus faecalis* | − |
| | *Enterococcus hirae* | − |
| Resistant bacteria | MRSA | − |
| | VRE | − |

Peptide was applied in 3 mm well, 1 μg/well. Diameter of inhibition zones: +, 5−7 mm; ++, 7.5−9.5 mm; +++, 10−12 mm; ++++, 13 or more mm. - denotes that no inhibition zone was observed.

against several bacteria, a full-sized abaecin (37-mer peptide, 1F-37P-NH2) was synthesized by the Fmoc method. The synthetic abaecin was identified by ESI mass spectrometer and MALDI-TOF mass spectrometer (data not shown). We also synthesized several truncated peptides including 6P-31P-NH2 fragment based on the deduced amino acid sequence of *B. ignitus* abaecin. The amino acid sequences and molecular mass of synthetic oligopeptides are provided in Table 1.

**Antibacterial activity of a full-size synthetic abaecin**
We first assessed antibacterial activities of a full-sized 37-mer synthetic abaecin (Ab37, 1F-37P-NH2) and truncated 26-mer peptide (Ab26, 6P-31P-NH2), which was synthesized after removing some amino acids at both the N-terminus and C-terminus by radial diffusion assay (Table 2; Fig. 1). The activity of Ab37 against *E. coli* (ML-35), *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *L. monocytogenes*, *B. subtilis*, *E. faecalis*, *M. luteus*, MRSA and VRE was examined (Table 2). As we expected, the Ab37 peptide inhibited the growth of Gram-negative bacteria *E. coli*, *P. aeruginosa* and *S. typhimurium*, but exhibited limited inhibitory activity toward Gram-positive bacteria. Also, the peptide showed activity against Gram-positive *M. luteus*, but no zones of bacterial growth inhibition were detected for other Gram-positive bacteria and resistant bacteria. Interestingly, the truncated Ab26 peptide showed higher activity then Ab37 peptide in *E. coli*, but it was less then cecropin A (Fig. 1A). However, the activities of Ab37 and Ab26 against *S. typhimurium* were similar to cecropin A (Fig. 1B). The present antibacterial spectrum data are in agreement with the activity data reported for *B. pascuorum* and *A. melifera* abaecin (Casteels *et al.*, 1990; Rees *et al.*, 1997).

**Effect of truncated peptides on the growth of *E. coli***
To identify the active site of abaecin, we have synthesized several truncated peptide fragments based on the hydrophobicity of amino acid sequence of *B. ignitus* mature abaecin (Table 1). The result of hydrophobicity indicated that mature abaecin has two different characteristic region: N-terminal hydrophilic peptide region and C-terminal hydrophobic peptide region (data not shown). This characteristic is one of the important factors that affect antimicrobial activity and specificity by enabling water-soluble antimicrobial peptides to partition into the membrane lipid bi-layer (Brogden, 2005). Thus, we have synthesized Ab19-N (1F-19P-NH2), Ab16-N (1F-16P-NH2) and Ab14-N (6P-19P-NH2) fragments based on the hydrophilic peptide region at N-terminal of abaecin, and also synthesized Ab18-C (20G-37P-NH2), Ab15-C (23P-37P-NH2) and Ab12-C (20G-31P-NH2) fragments based
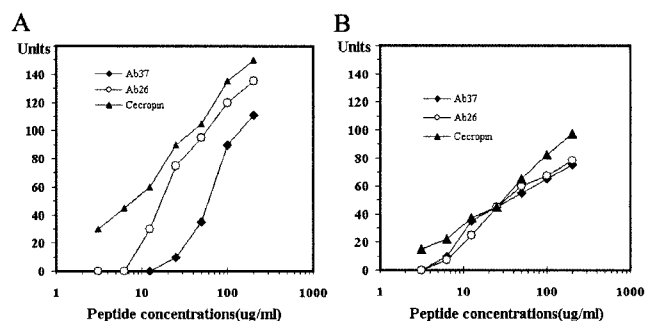


**Fig. 1.** Antibacterial activities of synthetic Ab37 and Ab26 peptides against *E. coli* (A) and *Salmonela typhimurium* (B) by using radial diffusion assay. Ab 37 indicates the synthetic 1F-37P-NH2 fragment of *B. ignitus* abeacin. Ab26 is truncated into 26-mer peptide, 6P-31P-NH2. For the control the cecropin (Sigma) was used. Diameters of clearing zone have been expressed in units (1 mm = 10 U).
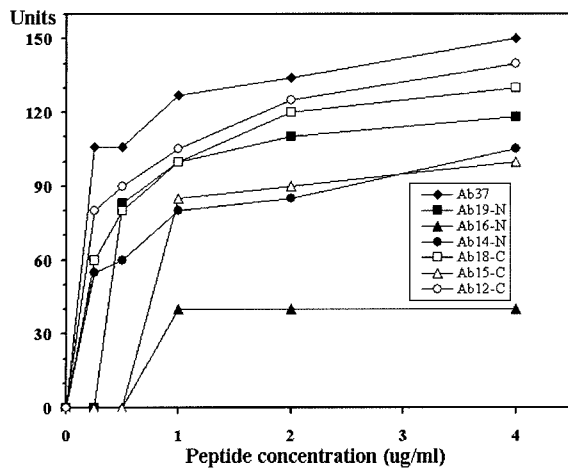
Fig. 2. Antibacterial effects of synthetic abaecin congeners against *E. coli* by radial diffusion assay. Numbers represent the concentration (mg/ml) of peptides applied in each well. Diameters of clearing zone have been expressed in units (1 mm = 10 U). Ab 37, synthetic 1F-37P-NH2 fragment; Ab19-N, 1F-19P-NH2 fragment; Ab16-N, 1F-16P-NH2 fragment; Ab14-N, 6P-19P-NH2 fragment; Ab18-C, 20G-37P-NH2 fragment; Ab15-C, 23P-37P-NH2 fragment; and Ab12-C, 20G-31P-NH2 fragment.

on the hydrophobic peptide region at C-terminal of abaecin. We then determined antibacterial activity of the truncated peptide fragments against *E. coli*. These all synthetic truncated peptides showed lower activity then did a full-sized peptide, Ab37 (Fig 2.). Furthermore, the Ab16-N (1F-16P-NH2) and Ab15-C (23P-37P-NH2) fragments dramatically reduced antibacterial activity. These results suggest that the 17Pro-Gly22 site, which is mainly hydrophobic region of *B. ignitus* abaecin (data not shown) are important region for exerting antibacterial activity. We assumed, therefore, that this region may be aligned with the lipid core region of bacterial cell well components such as lipopolysaccharide present only in the Gram-negative bacteria.

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