Int. J. Indust. Entomol. Vol. 17, No. 1, 2008, pp. 137~141

International Journal of
Industrial Entomology

Expression of Active Antibacterial Bumblebee Abaecin in *Escherichia coli* Cells

Seong Ryul Kim, Jae Sam Hwang, Hyung Joo Yoon, Kwan Ho Park, Mee Yeon Hong[1], Kee Young Kim, Byung-Rae Jin[2] and Iksoo Kim[1,*]

Department of Agricultural Biology, NIAST, Rural Development Administration, Suwon 441 - 100, Korea
[1]College of Agriculture & Life Sciences, Chonnam National University, Gwangju 500 - 757, Korea
[2]College of Natural Resources and Life Science, Dong-A University, Busan 604 - 714, Korea

(Received 30 July 2008; Accepted 16 August 2008)

We previously isolated and cloned a cDNA of abaecin from the *Bombus ignitus*. In an effort to produce a large amount of soluble abaecin at low cost, we successfully expressed the peptide in *Escherichia coli* that are highly sensitive to its mature form. For this, we fused the peptide encoding 39 amino acids of mature *B. ignitus* abaecin to the thioredoxin gene together with a C-terminal 6xHis tag. An enterokinase cleavage site was introduced between the 6xHis tag and mature abaecin to allow final release of the recombinant peptide. A high yield of 9.6 mg soluble fusion protein from 200 ml of bacterial culture was purified by Ni^{2+}-charged His-Bind resin affinity column, and 1.4 mg of pure active recombinant abaecin was readily obtained by enterokinase cleavage, followed by affinity chromatograph. The molecular mass of recombinant abaecin peptide was determined by Tricin-SDS-PAGE analysis. The recombinant abaecin exhibited antibacterial activity against Gram-negative bacteria.

Key words: *Bombus ignitus,* Expression, Recombinant abaecin, Antibacterial activity.

Introduction

Abaecin belongs to a family of proline-rich antibacterial peptide that presents high activity against Gram-negative bacteria in the hymenopterans, *Apis mellifera* (Cateels *et al*., 1990), *Bombus pascuorum* (Rees *et al*., 1997) and *B. ignitus* (Choi *et al*., 2008). They are rapidly produced in fat body after septic body injury or immune challenge, and then released into hemolymph to act against microorganisms (Cateels *et al*., 1990; Choi *et al*., 2008). In bumblebee, the mature form of abaecin peptide is composed of 39 amino acid residues. In our previous work, the mature abaecin peptide was chemically synthesized, and the 37-mer synthetic abaecin was highly effective against Gram-negative bacteria (Kim *et al*., 2007). However, as a potential for medical use, chemical synthesis of the peptide is economically disadvantageous because of high cost for production. In recent years, several approaches have been proposed to produce large quantities of active antimicrobial peptides in bacterial expression system, and a good few of small antimicrobial peptides were successfully produced (Li *et al*., 2006; Xu *et al*., 2007; Shlyapnikov *et al*., 2008; Vassilevski *et al*., 2008). In this paper, we report successful production of abaecin peptide as soluble fusion protein efficiently in bacterial expression system. To increase productivity and solubility, abaecin was fused with thioredoxin (Trx) and expressed in *E. coli* cells. The conditions for culture and induction were also optimized. Finally, the recombinent abaecin was examined for antibacterial activity.

*To whom the correspondence addressed
College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500 - 757, Korea. Tel: +82-62-530-2073; Fax: +82-62-530-2079; E-mail: ikkim81@chonnam.ac.kr

Materials and Methods

Materials

pGEM-T easy vector (Promega, USA) and pET-32b (Novagen, Germany) were used to clone and construct expression vector. The restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase (Takara, Japan) were used. Reagents for RNA isolation and first-strand cDNA

synthesis were used (Invitrogen, USA). Low-electroendosmosis type agarose, Tris, acrylamide, TEMED, ammonium persulfate, and other chemicals were purchased from Sigma. *Escherichia coli* DH5á (Invitrogen) and *E. coli* BL21 (DE3) (Novagen) were used as the host for maintenance of recombinant plasmid DNA and 6x His-tagged fusion protein expression, respectively. *E. coli* ML-35, Psudomonas aeruginosa, Salmonella typhimurium and Micrococcus luteus were used in the antimicrobial activity assay. Luria-Bertani (LB) medium and Tryptic soy broth (TSB) were from Difco (USA).

RNA isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) from *B. ignitus* workers immunized with Lipopolysaccharide (LPS) and then treated for 15 min with DNase I at 37°C to remove any residual genomic DNA. Isolated RNA sample (1 ug) was used for cDNA synthesis with oligo (dt) primer and Superscript III reverse transcriptase (Invitrogen). To obtain a cDNA fragment encoding the mature *B. ignitus* abaecin, PCR amplification was performed using the first strand cDNA as a template and a pair of specific primers (5'-GGTACCGACGACGACGACA AGTTTGTACCATATAATCCG and 5'-CTCGAGTTAATGACCAGGGTTTGGTAA) containing *Kpn*I and *Xho*I restriction site (underlined nucleotides), respectively. Specific primers containing enterokinase cleavage site at the end of the forward primer were designed based on the nucleotide sequence of mature *B. ignitus* abaecin (Genbank Accession No. AAQ90411). PCR product was inserted into a pGEM-T easy vector (Promega) to construct the cloning vector pGEM-Biabaecin, and then nuclotide sequence of the *B. ignitus* mature abaecin was confirmed by sequencing.

Construction of the expression vector

The mature Biabaecin cDNA containing enterokinase cleavage site was isolated from pGEM-Biabaecin plasmide by digestion with *Kpn*I and *Xho*I enzymes and ligated into the *Kpn*I/*Xho*I-digested pET-32b vector to the carboxyl-terminus of the fusion partner thioredoxin (Trx). This constructed plasmid (pTrx-Biabaecin) was transformed into *E. coli* DH5á competent cells, purified, and confirmed by restriction endonucleases digestion and DNA sequence analysis. The resulting plasmid was transformed into *E. coli* BL21 (DE3) competent cells for fusion expression.

Expression of fusion protein

A single colony of *E. coli* BL21 (DE3) cells transformed with the pTrx-Biabaecin plasmide was cultured in LB medium containing 50 ul/ml ampicillin for overnight at 37°C. The overnight culture of transformed colony were inoculated into 200 ml LB medium with the ratio of 5% (v/v,) and grown at 37°C with vigorous shaking until an optical density (OD_{600}) reached 0.6 - 0.7. Expression was then induced by adding IPTG to a concentration of 0.2 mM for 6 hrs at 37°C. The culture was centrifuged at 4000 g for 10 min and resuspended in 20 mM Tris-HCl (pH 7.4), and then lyzed by sonication. After centrifuged at 13000 g for 20 min at 4°C, the supernatant was taken out as the soluble protein sample. The pellet was resuspended in 1% SDS solution as the insoluble sample. Proteins were separated on 12.5% SDS-PAGE gel and visualized with commassie blue staining.

Purification of recombinant abaecin

The target fusion protein (Trx-Biabaecin) was purified by Ni^{2+}-charged His-Bind resin affinity column (Novagen). After sample loading and equilibration, the recombinant fusion protein was eluted with the elution buffer (20 mM Na_3PO_4, 500 mM NaCl, and 200 mM Imidazole, pH 7.4) at 1 ml/min. The eluted fractions were applied to SDS-PAGE analysis, while the concentration of eluted Trx-Biabaecin protein was determined by Bradford protein assay. To relase recombinant abaecin from the fusion protein, enterokinase digestion was performed in a reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, and 50 mM NaCl) at 20°C for 18 hrs. The digested sample was applied to Ni^{2+}-charged His-Bind resin affinity column again to remove the Trx and undigested fusion proteins. Finally, The released recombinant peptide was concentrated using the Amicon ultrafilteration (Amicon Ultra-3 K, Millipore) and determined by Tricin-SDS-PAGE analysis.

Antibacterial activity assay

Lyophilized recombinant abaecin peptide was dissolved with 20 mM Tris-HCl (pH 7.4) to a final concentration of 100 ul/ml. The antibacterial activity of recombinant abaecin solution (5 ul per well) was examined against several Gram-negative bacteria by agar well diffusion assay described in Lehrer *et al.* (1991) and Kim *et al.* (2007). The bioactivity of recombinant peptide was further measured by inhibitory zone.

Results

Cloning and expression vector construction

The complete enconding sequence of mature abaecin peptide containing enterokinase cleavage site was amplified by RT-PCR. After cloning and sequence verification, the

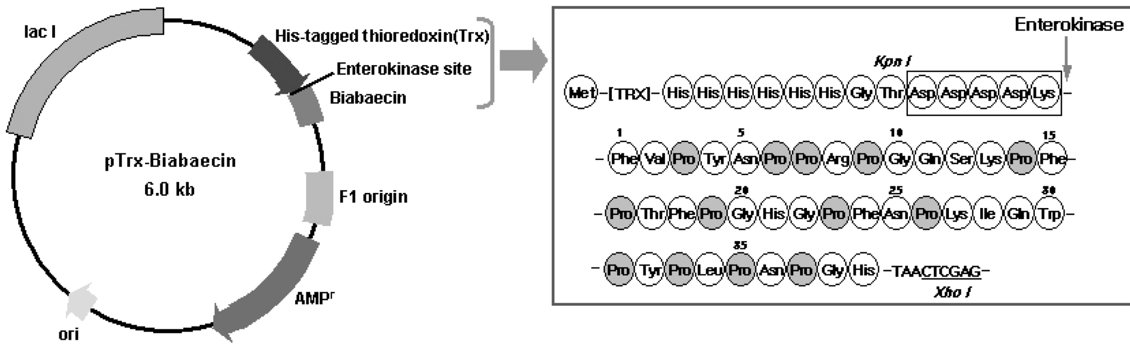

Fig. 1. Construction of recombinant plasmid pTrx-Biabaecin. The cDNA fragment encoding *B. ignitus* muture abaecin with an enterokinase cleavage site at the amino-terminus of abaecin was cloned into pET-32b to the carboxyl-terminus of the fusion partner thioredoxin (Trx). The enterokinase recognition sequence is boxed and proline residues are shown as gray circles.

abacein cDNA fragment with enterokinase cleavage site sequence was inserted into down-stream of thioredoxin gene of pET-32b to construct the expression plasmid pTrx-Biabaecin (Fig. 1). The correct orientation was confirmed by digestion with restriction endonucleases and automated cDNA sequencing (data not shown).

Expression of recombinant Trx-Biabaecin fusion protein

The correct recombinant plasmid pTrx-Biabaecin to express a recombinant fusion protein was transformed into the expression bacterial strain BL21 (DE3) cells. Upon IPTG induction, the target protein (Trx-Biabaecin) was predominantly expressed as soluble protein of about 22 kDa, with a high solubility (Fig. 2). The recombinant protein was not detectable before IPTG induction. In order to optimize the conditions for expression, we employed several one-factor experiments (data not shown). The optimum conditions to obtain high yield of soluble fusion protein in the culture was 0.6 - 0.7 at OD_{600}, with 0.2 mM of IPTG at 37°C for 6 hrs. The recombinant fusion protein was easily purified by Ni^{2+}-charged His-Bind resin affinity column, and was applied to SDS-PAGE analysis (Fig. 2). The result revealed that the purified 22 kDa fusion protein to have the puriy of more than 90%. A high yield of 9.6 mg soluble fusion protein was purified from 200 ml bacterial culture (Table 1).

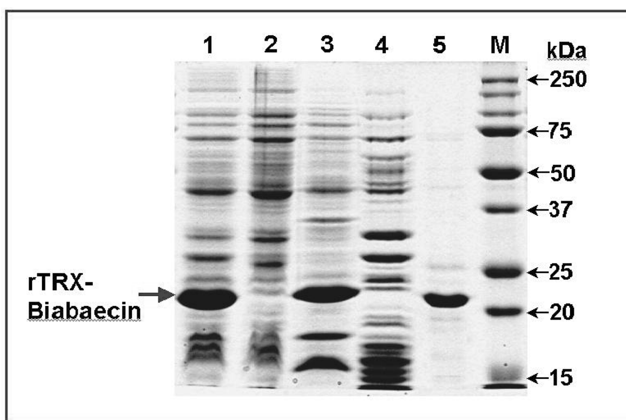

Fig. 2. Analysis of expressed and purified fusion protein by SDS-polyacrylamide gel electrophoresis. The recombinant Trx-Biabaecin was expressed in *E. coli* BL21. Lane 1, induced cell extract; lane 2, non-induced cell extract; lane 3, soluble fraction of cell extract; lane 4, insoluble fraction of cell extract; and lane 5, purified fusion protein (Trx-Biabaecin). M indicate protein molecular weight marker.

Purification of recombinant abaecin peptide

To generate mature *B. ignitus* abaecin peptide, the purified fusion protein was subjected to a three-step process

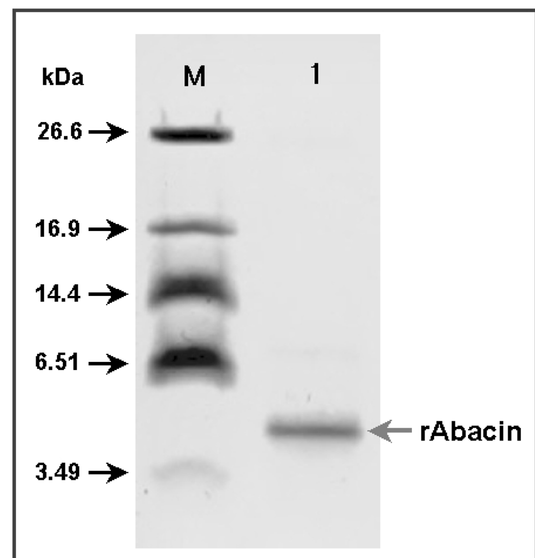

Fig. 3. Analysis of purified recombinant *B. ignitus* abaecin by Tricine-SDS-PAGE. Purified rAbaecin (0.1 ug, lane 1) was analyzed on 16.5% SDS-PAGE in Tricine buffer and visualized by Coomassie brilliant blue. M indicate polypeptide molecular weight standards.

Table 1. Purification of recombinant abaecin peptide*

Purification steps	Volume (ml)	Total protein (mg)	Yield (%)
Cell lysate	50	64.2	100
Soluble protein fraction	46	59.3	92.4
Affinity purification of Trx-Biabaecin	10	9.6	15.0
Purification after cleavage of Trx	2	1.4	2.1

*About 200 ml of bacteria culture corresponds to about 0.8 g (wet weight) of cell pellet.

including enterokinase digestion, desalting, and affinity chromatography. After effectively cleave with enterokinase, the recombinant abaecin peptide was relased from fusion protein by Ni^{2+} fusion protein -charged His-Bind resin affinity column. Analysis of purified recombinant peptide on Tricin-SDS-PAGE revealed that the molecular mass of recombinant abaecin was about 4.4 kDa (Fig. 3). To prevent the negative salt effect on the antibacterial activity, the purified recombinant abaecin was desalted by ultrafilteration (Amicon Ultra-3 K, Millipore) and then freeze-dried. The final yield of pure recombinent abaecin was about 1.4 mg per 200 ml bacterial culture (Table 1).

Antibacterial activity assay

Antibacterial activity of recombinant abaecin was examined against *E. coli* ML- 35 by agar well diffusion assay. As shown in Fig. 4, the fusion partner thioredoxin (Trx) and fusion protein (Trx-Biabaecin) with a high concentration (1 mg/ml) did not exhibit inhibition zones, while the purified recombinent abaecin (100 ug/ml) obviously inhibited the growth of *E. coli*. Moreover, antibaterial activity of recombinent abaecin against various bacterial strains was examined (Table 2). As we expected, the recombinent abaecin was highly effective against Gram-negative bacteria *E. coli*, *Psudomonas aeruginosa* and *Salminella typhimurium*, but had a low inhibitory activity toward Gram-positive bacteria *Micrococcus luteus*.

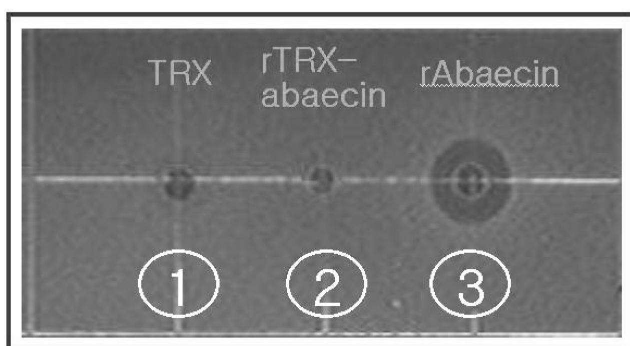

Fig. 4. Antibacterial activity of rAbaecin by agar well diffusion assay against *E. coli*. The analyzed samples were 1 mg/ml thioredoxin (Trx, 1), 1 mg/ml recombinant fusion protein (rTrx-Biabaecin, 2) and 0.1 mg/ml purified recombinant abaecin (rAbaecin, 3). Bacteria were grown overnight at 37°C.

Discussion

A number of approaches for the production of recombinant antibiotic peptide in *E. coli* cells has been proposed (Shlyapnikov *et al.*, 2008; Vassilevski *et al.*, 2008). These include fusion with a partner protein or with anionic properties to prevent the intrinsic antibacterial activity to the host cells and proteolytic degradation. Although a number of partner proteins suggested (Zhang *et al.*, 1998; Hwang *et al.*, 2001; Skosyrev *et al.*, 2003), thioredoxin (Trx, 12 kDa) has been utilized as a useful partner for high soluble expression and probe antibiotic peptide production (Li *et al.*, 2006; Xu *et al.*, 2007). For that reason, we applied a similar expression system to product soluble abaecin peptide in *E. coli* cells. In the present study, we cloned the synthetic mature abaecin gene from *B. ignitus* into pET-32b, which is the vector that was commercially designed for Trx-fused protein and peptide production. We purified 9.6 mg fusion protein with a high solubility from 200 ml of bacterial cell culture, and obtained 1.4 mg recombinant abaecin peptide by enterokinase digestion. The present study clearly shows that thioredoxin (Trx) serves a perfect fusion partner for high-level production of soluble abaecin in *E. coli* cells, without any toxic effects on the host culture. In this work, soluble fusion protein (Trx-Biabaecin) did not show any antibacterial activity against *E. coli*, while the released recombinant abaecin was fully effective. More importantly, the recombinant abaecin displayed high antibacterial activity against Gram-negative bacteria, and the aspect is simiar to the activity of synthetic abaecin

Table 2. Antibacterial specificity of recombinant abaecin peptide by agar well diffusion assay

Bacteria	Inhibition zone size (mm)
Escherichia coli	14.6
Psudomonas aeruginosa	7.8
Salmonella typhimurium	8.4
Micrococcus luteus	5.6

Peptide was applied in 3 mm well, 0.1 mg/ml

(Kim *et al*., 2007).

In conclusion, we have described the successful bacterial production of a potent abaecin peptide form *B. ignites* for the first time. A method using thioredoxin fusion expression system in *E. coli* led to production of soluble Trx-Biabaecin fusion protein, which has non-toxic effect to the host. We also were able to obtain considerable yield of purified pure active recombinant abaecin. The availability of high purity abaecin peptide with low costs of production at a large scale allows us to apply it for a diverse purpose in the future, and provides the ground for further in-depth structural and functional investigations.

Acknowledgments

This work was supported by a grant (Code 20080401-034-017) from BioGreen 21 Program from Rural Development Administration, Republic of Korea.

References

Casteels, P., C. Ampe, L. Riviere, J. V. Damme, C. Elicone, M. Fleming, F. Jacobs and P. Tempst (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). *Eur. J. Biochem*. **187**, 381-386.

Choi, Y. S., Y. M. Choo, K. S. Lee, H. J. Yoon, I. Kim, Y. H. Je, H. D. Shon and B. R. Jin (2008) Cloning and expression profiling of four antibacterial peptide genes form the bumblebee *Bombus ignitus. Comp. Biochem. Physiol. B* **150**, 141-146.

Hwang, S. W., J. H. Lee, H. B. Park, S. H. Pyo, J. E. So, H. S. Lee, S. S. Hong and J. H. Kim (2001) A simple method for the purification of an antimicrobial peptide in recombinant *Escherichia coli. Mol. Biotechnol.* **18**, 193-198.

Kim, S. R., E. M. Lee, H. J. Yoon, Y. S. Choi, E. Y. Yun, J. S. Hwang, B. R. Jin, I. H. Lee and I. Kim (2007) Antibacterial activity of peptides synthesized based on the *Bombus ignitus* abaecin, a novel proline-rich antimicrobial peptide. *Int. J. Indust. Entomol.* **14**, 147-150.

Lehrer, R. I., M. Rosenman, S. S. Harwig, R. Jackson and P. Eisenhauer (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* **137**, 167-173.

Li, Y., X. Li and G. Wang (2006) Cloning, expression, isotope labeling, and purification of human antimicrobial peptide LL-37 in *Escherichia coli* for NMR studies. *Protein Expr. Purif.* **47**, 498-505.

Rees, J. A., M. Moniatte and P. Bulet (1997) Novel antibacterial peptides isolated from a European bumblebee, Bombus pascuorum (Hymenoptera, Apoidea). *Insect Biochem. Mol. Biol.* **27**, 413-422.

Shlyapnikov, Y. M., Y. A. Andreev, S. A. Kozlov, A. A. Alexander and A. Vassilevski (2008) Bacterial production of latarcin 2a, a potent antimicrobial peptide form spider venom. *Protein Expr. Purif.* **60**, 89-95.

Skosyrev, V. S., N. V. Rudenko, A. V. Yakhnin, V. E. Zagranichny, L. I. Popova, M. V. Zakharov, A. Y. Gorokhovatsky and L. M. Vinokurov (2003) EGFP as a fusion partner for the expression and organic extraction of small polypeptides. *Protein Expr. Purif.* **27**, 55-62.

Vassilevski, A. A., S. A. Kozlov and E. V. Grishin (2008) Antimicrobial peptide precursor structures suggest effective production strategies. *Recent Patents Inflamm. Allergy Drug Discov.* **2**, 58-63.

Xu, X., F. Jin, X. Yu, S. Ji, J. Wang, H. Cheng, C. Wang and W. Zhang (2007) Expression and purification of a recombinant antibacterial peptide, cecropin, from *Escherichia coli*. *Protein Expr. Purif.* **53**, 55-62.

Zhang, L., T. Falla, M. Wu, S. Fidai, J. Burian, W. Kay and R. E. Hancock (1998) Determinants of recombinant production of antimicrobial cationic peptides and creation of peptide variants in bacteria. *Biochem. Biophys. Res. Commun*. **247**, 674-680.