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Biologically Active and C-Amidated HinnavinII-38-Asn Produced from a Trx Fusion Construct in *Escherichia coli*

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The cabbage butterfly (*Artogeia rapae*) antimicrobial peptide hinnavinII as a member of cecropin family is synthesized as 37 residues in size with an amidated lysine at C-terminus and shows the humoral immune response to a bacterial invasion. In this work, a synthetic gene for hinnavinII-38-Asn (HIN) with an additional amino acid asparagine residue containing amide group at C-terminus was cloned into pET-32a(+) vector to allow expression of HIN as a Trx fusion protein in *Escherichia coli* strain BL21 (DE3) pLysS. The resulting expression level of the fusion protein Trx-HIN could reach 15~20% of the total cell proteins and more than 70% of the target proteins were in soluble form. The fusion protein could be purified successfully by HiTrap Chelating HP column and a high yield of 15 mg purified fusion protein was obtained from 80 ml *E. coli* culture. Recombinant HIN was readily obtained by enterokinase cleavage of the fusion protein followed by FPLC chromatography, and 3.18 mg pure active recombinant HIN was obtained from 80 ml culture. The molecular mass of recombinant HIN determined by MALDI-TOF mass spectrometer is 4252.084 Da which matches the theoretical mass (4252.0 Da) of HIN. Comparing the antimicrobial activities of the recombinant hinnavinII with C-amidated terminus to that without an amidated C-terminus, we found that the amide of asparagine at C-terminus of hinnavinII improved its potency on certain microorganism such as *E. coli*, *Enterobacter cloacae*, *Bacillus megaterium*, and *Staphylococcus aureus*.

Keywords: *Artogeia rapae*, C-amidated terminus, hinnavinII, antimicrobial peptide, recombinant protein expression, *Escherichia coli*

Antimicrobial peptides (AMPs) are produced by all species of life and play one of the major roles in primary host defense against infections of pathogenic microorganisms (Dhople *et al*., 2006; Dürr *et al*., 2006). The amphipathic α-helical AMPs of insect origin are 29~42 residues in size, linear and devoid of cystein residues (Bulet and Stöcklin, 2005). Cecropins were the first animal inducible AMPs to be isolated and fully characterized. Since the first insect cecropin was isolated from the moth *Hyalophora cecropia* (Steiner *et al*., 1981), expression of cecropin-like peptides has been documented in several other insect species, all of which belong to phylogenetically higher insect order of Diptera and Lepidoptera (Hetru *et al*., 2002; Bulet *et al*., 2003). The cabbage butterfly *Artogeia rapae* hinnavinII, which belongs to cecropin family, consists of 37 amino acid residues with an amidated C-terminus and is more active against Gram-negative than against Gram-positive bacteria (Yoe *et al*., 2006). HinnavinII also showed a powerful synergistic effect on the inhibition of bacterial growth with lysozyme. With the exception of the *Aedes* cecropins (Lowenberger *et al*., 1999) and *Bombyx mori* cecropin D (Hara *et al*., 1994), all other insect cecropins characterized so far share a common feature of posttranslational amidation. This post-translational modification is considered necessary for the full antimicrobial activity of the molecule (Li *et al*., 1988; Hara *et al*., 1994), and may protect the peptide from carboxypeptidase digestion (Callaway *et al*., 1993).

Before any one AMP can assume a role as a therapeutant, however, a cost-effective and scalable method to produce large quantities of active AMPs is required, and this has been improved by introducing numerous biological expression systems (Ingham and Moore, 2007). Because of their natural destructive behavior toward microorganisms and relative sensitivity to proteolytic degradation, AMPs are often produced by fusing AMPs to a fusion partner in heterologous hosts to neutralize their innate toxic activity and increase their expression levels (Skosyrev *et al*., 2003; Kim *et al*., 2008). *Escherichia coli* thioredoxin (Trx) was employed as a fusion partner because of its high expression of soluble fusion proteins (Tenno *et al*., 2004). The commercial pET-32 series is designed for cloning and high-level expression of peptide sequences fused with Trx protein (LaVallie *et al*., 1993; Symersky *et al*., 2000). This expression vector carrying Trx gene and T7 promoter contains a 6×histidine-tag to facilitate purification of fusion proteins.

The purpose of this study was to produce recombinant hinnavinII as a natural form from *E. coli* expression system that lacks the function of posttranslational amidation. Since it is possible that hinnavinII is naturally amidated, we converted the C-terminal glycine residue to asparagine to par-

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tially mimic the amidated lysine residue by adding the C-terminal amide. The DNA fragment encoding mature hinnavinII-38-Asn (HIN) was then subcloned into the pET-32a(+) expression vector, which contains T7 promoter and Trx as a fusion partner and is expressed highly in *E. coli* BL21 (DE3) pLysS with IPTG induction. Moreover, we compared various antimicrobial activities between the recombinant hinnavinII with and without amidates C-terminus.

Materials and Methods

Bacterial strains and materials

E. coli strain DH5α and BL21 (DE3) pLysS (Novagen, USA) were used as a host for cloning and expression of fusion protein, respectively. Plasmid pET-32a(+), an expression vector for producing fusion protein with the Trx, was purchased from Novagen. In order to assess the antibacterial activities of recombinant hinnavinII, the following bacteria were used: *E. coli* K12 KCTC 1467, *Enterobacter cloacae* KCTC 2361, *Bacillus megaterium* KCTC 1098, and *Staphylococcus aureus* KCTC 1927 from the Korean Collection for Type Cultures (KCTC); *E. coli* BL21 (DE3) and *Bacillus subtilis* from the Laboratory of Microbiology of Soonchunhyang University. PCR reagents, restriction enzymes, and T4 DNA ligase were purchased from Promega (USA). Plasmid extraction kits and PCR production purification kits were from Bioneer (USA). Enterokinase was purchased from Novagen. HiTrap™ Chelating HP column and RESOUC RPC column was obtained from Amersham Biosciences (Sweden). Other chemical reagents were of analytical grade.

Construction of expression vector

The cloning vector pGEM-hinavinII (Yoe *et al*., 2006) containing the cDNA encoding the hinnavinII was utilized as template for construction of expression vector by means of PCR amplification. For C-terminus amidated form of the hinnavinII, the HIN gene was amplified with forward primer; GG GGT ACC GAC GAC GAC GAC AAG AAA TGG AAG ATT TTC AAG AAA ATT and reversed primer; CG GAA TTC TTA (A/G)TT CTT ATA GAT GGT AGC G. Therefore, a *Kpn*I site and an enterokinase cleavage sequence were introduced into the upper stream of the HIN gene, while an *Eco*RI site, a translational termination and an additional asparagine codon into the down stream of it. PCR was performed by running 30 cycles with a temperature profile of 30 sec at 95°C, 30 sec at 50°C, and 15 sec at 72°C. The amplified PCR product was digested with *Kpn*I and *Eco*RI, and ligated into the pET-32a(+) plasmid at the corresponding restriction sites. The resultant recombinant expression vector is referred to as pET32-HIN.

Expression of fusion protein

The resulting plasmid was transformed into competent cells of *E. coli* BL21 (DE3) pLysS and each selected transformant was cultured in LB medium containing 100 μg/ml ampicillin at 37°C. When OD_{600} reached to 0.9, the cultures were induced by adding IPTG to a final concentration of 1 mM. After an additional 4 h after cultivation, cells were harvested by centrifugation at 8,000×g for 10 min at 4°C. The harvested cells (2 g wet weight from 800 ml culture) were resuspened in 40 ml of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, and 1% Triton X-100, pH 8.0) and lysed by sonication at 400 W for 150 cycles (3 sec working, 3 sec free) in ice-water bath. The whole cell lysate was then centrifuged at 10,000×g for 10 min at 4°C to separate soluble and insoluble portions. After decanting the soluble portion, the insoluble portion was solubilized in 20 ml of lysis buffer containing 100 mM β-mercaptoethanol and 8 M urea by stiring for 2 h at 28°C. Subsequently, the soluble and insoluble portions were analyzed by 15% SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin *et al*., 1979).

Purification of fusion protein

The soluble portion containing the fusion protein Trx-HIN was applied to a HiTrap Chelating HP column which had been previously equilibrated with lysis buffer containing 30 mM imidazole instead of EDTA. After washing to baseline absorbance with the same buffer, the bound fraction was eluted with 100 mM and 300 mM of imidazole in turn using AKTA FPLC system (Amersham-Pharmacia Biotech, Sweden) at a flowrate of 1 ml/min. The fractions were collected and applied to SDS-PAGE. The purified fusion protein was pooled and dialyzed against cleavage buffer (20 mM Tris-HCl, 50 mM NaCl, and 2 mM $CaCl_2$, pH 7.4) with three changes within 24 h and then lyophilized.

Release and purification of recombinant HIN

Lyophilized Trx-HIN was resuspended in cleavage buffer to give a final fusion protein concentration of 0.5 mg/ml. Enterokinase stock (0.1 U/μl) was added to the protein pool giving 0.1 U enzyme per 0.1 mg fusion protein. After 12 h of incubation at 22°C, the reaction mixture was loaded to the HiTrap Chelating HP column again. The flow-through, containing recombinant HIN, was subjected to reversed-phase FPLC on a RESOUC RPC column equilibrated with 0.1% TFA. Elution was performed with a linear gradient of 25~50% acetonitrile in 0.1% TFA. Each peak monitored at 214 nm was collected and tested for antimicrobial activity after lyophilization and analyzed by Tricine SDS-PAGE (Schägger and Von Jagow, 1987). Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Mass spectrometry

The molecular weight and homogeneity of the recombinant HIN were analysis by mass spectrometry on an Applied Biosystems Voyager MALDI-TOF mass spectrometer at Korea Basic Science Institute (KBSI).

Antibacterial activity

Antibacterial activities were assessed by radial diffusion assays (Lehrer *et al*., 1991). Twenty-five micromole (3 μl) of the samples were loaded into wells of 3 mm in diameter that had been punched in underlay gels. These underlays contained a uniform dispersion of washed, mid-logarithmic bacteria (4×10^6 CFU/10 ml) immobilized in a 1% agarose matrix that contained buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 7.4) and 0.3 mg/ml of Tryptic Soy Broth (TSB) powder. After incubation at 37°C for 3 h, an 8 ml overlay gel of 1% agarose and 6% TSB was poured

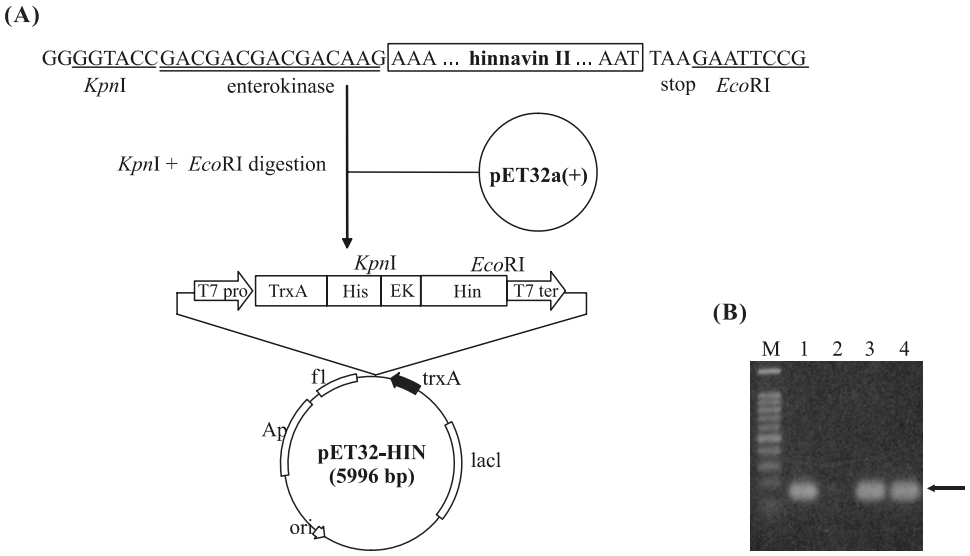

Fig. 1. (A) Schematic representation of expression vector, pET32-hinnavin II-38-Asn (pET32-HIN), with a fusion partner of Trx. To construct hinnavin II-38-Asn, additional DNA sequences (AAT) were added to C-terminal of hinnavin II. The *Kpn*I-*Eco*RI fragment (144 bp) containing hinnavinII-38-Asn cDNA was synthesized, annealed, and then ligated into a commercial vector pET-32a(+). (B) PCR screening of pET32-HIN. Lanes 1 and 2, PCR products of pGEM-hinnavinII and negative control; lanes 3 and 4, PCR products of pET32-HIN; and M, DNA size markers.

on the underlay agar. After the plates were incubated for 24 h at 37°C, the clear zone squares were recorded.

Results and Discussion

Construction of pET32-HIN

The HIN gene was amplified by PCR with a pair of primers templated by pGEM-hinnavinII. As shown in Fig. 1A, the PCR product coded for HIN with an additional asparagine residue at its C-terminus and an enterokinase cleavage site at its N-terminus which facilitated the release of active peptide with native N-terminus from fusion protein by enterokinase. One pair of endonuclease restriction sites *Kpn*I and *Eco*RI flanked the upper and down stream of the PCR product (Fig. 1B), respectively. To construct an effective expression system for HIN, Trx·6×His-tag under the control of T7 promoter was selected as the fusion partner and therefore the plasmid pET-32a(+) was used as an expression vector. The Trx is a soluble cytoplasmic protein, which has been well established as a useful partner for high expression of soluble and stable fusion proteins, including many biologically active mammalian proteins, in the cytoplasm of *E. coli* (Jiang *et al*., 2003; Liu *et al*., 2003; Xu *et al*., 2007). The existence of the 6×His-tag between the Trx and HIN coding sequence is beneficial to facilitate purification of the fusion protein Trx-HIN by metal affinity chromatography. An enterokinase cleavage site immediately upstream to HIN was also created to facilitate enzymatic cleavage of the fusion protein for HIN peptide release. After the digestion with *Kpn*I and *Eco*RI, the PCR fragment was ligated into pET-32a(+) digested with the same enzymes, generating pET32-HIN. Figure 2 shows the complete HIN nucleotide sequence and predicted amino acid sequence in pET32-HIN.

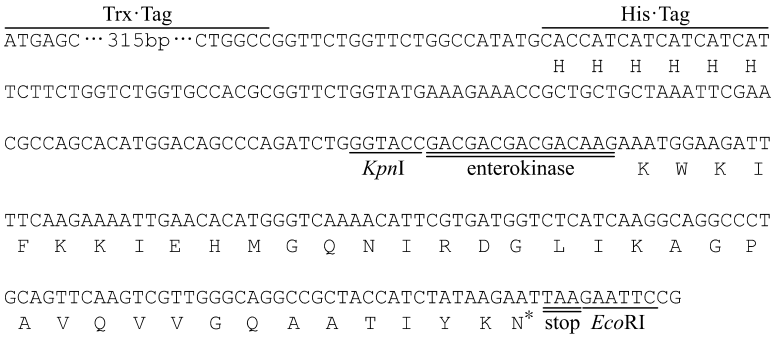

Fig. 2. Partial pET32a(+) and complete hinnavin II-38-Asn nucleotide sequence and predicted amino acid sequence. Amino acid sequence is indicated by the one-letter code written below the second nucleotide of each codon. The *Kpn*I site *Eco*RI site are underline. The enterokinase site and termination codon are double underline. Asparagine residue instead of glycine residue is mark by an asterisk.

Expression and purification of fusion protein

The confirmed construct containing the fusion gene was transformed into the expression host *E. coli* BL21 (DE3) pLysS. After optimization and IPTG induction, the fusion protein expression was analyzed by using SDS-PAGE and Western blotting (Fig. 3A and B). A protein with an apparent molecular weight of 21 KDa was expressed upon induction, which is consistent with the expected molecular weight of Trx-HIN. It has been also found that the resulting expression level of Trx-HIN could reach 15~20% of the total proteins and more than 70% of the target proteins were in a soluble form. Moreover, the existence of the 6×His-tag on the carrier protein provides an effective one-step purification of the fusion protein by Ni^{2+}-chelating chromatography. SDS-PAGE analysis revealed the effectiveness of this purification step (Fig. 4). The bacterial protein was successfully eliminated by 30 mM imidazole wash and most of the fusion

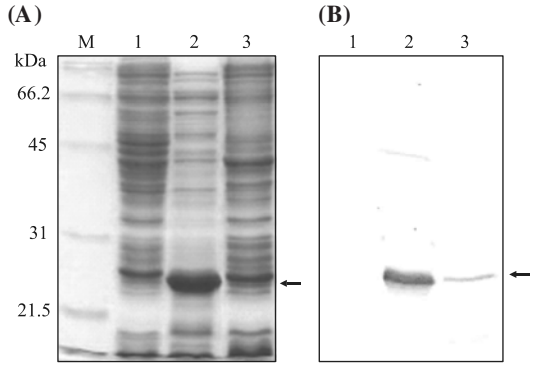

Fig. 3. SDS-PAGE (A) and Western blotting (B) of fusion protein Trx-HIN expression in *E. coli* BL21 (DE3) pLysS. Lane 1, uninduced control; lane 2 and 3, soluble and insoluble form 4 h after induction with IPTG; and M, protein size markers.

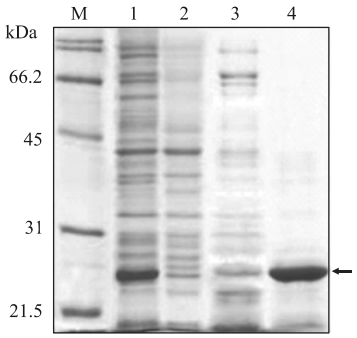

Fig. 4. Purification of the fusion protein on Ni^{2+}-chromatography. Lane 1, supernatant of cell lysates; 2, column flowthrough; 3, 30 mM imidazole wash; 4, 150 mM imidazole wash; and M, protein size markers.

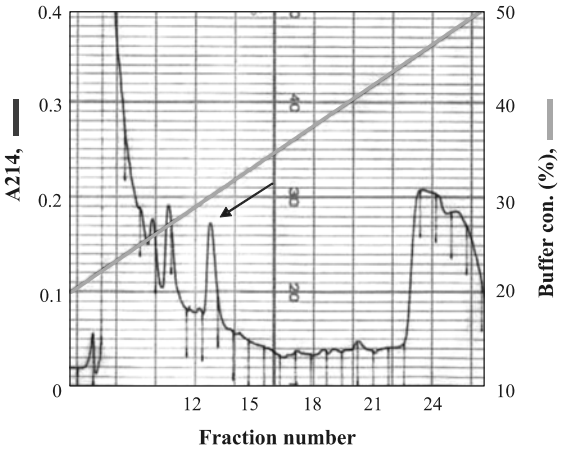

Fig. 5. Purification profile of recombinant hinnavin II by RESOURCE RPC column of eluted fraction from Ni^{2+}-chromatography.

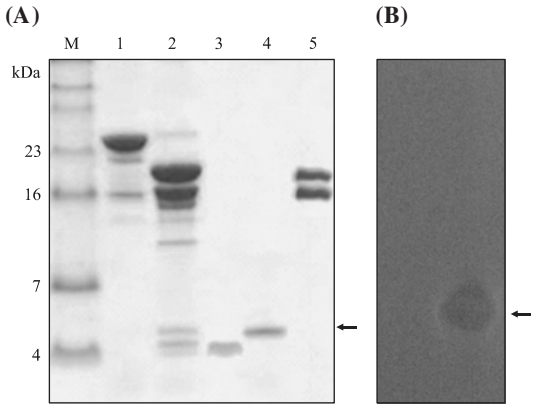

Fig. 6. (A) Tricine SDS-PAGE analysis of recombinant hinnavin II. Lane 1, Trx-HIN fusion protein purified by Ni^{2+}-chelating column; 2, Trx-HIN fusion protein cleaved by enterokinase; 3, recombinant hinnavin II purified by RESOURCE RPC column; and M, low protein size markers. (B) Gel overlay assay on AU-PAGE. Purified recombinant HIN was overlaid with viable *E. coli* K12 to detect antibacterial activity.

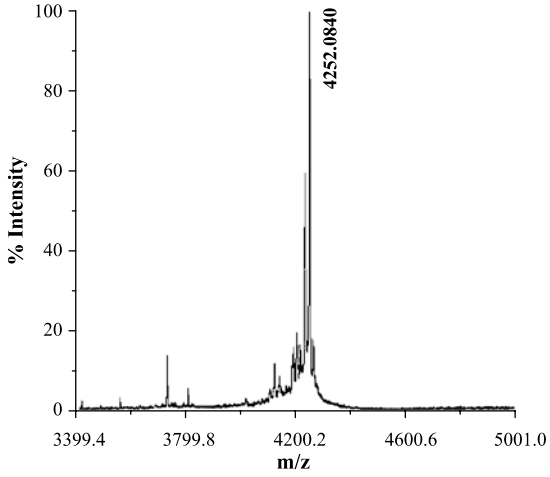

Fig. 7. Mass spectrum of purified recombinant HIN recorded on an applied biosystems voyager MALDI-TOF mass spectrometry. The molecular weight determined for the recombinant hinnavin II-38-Asn was 4252.084 Da (calculate 4252.0 Da).

Table 1. Purification of recombinant HIN from *E. coli*

Purification step	Total protein (mg)[a]	Protein of interest (mg)[b]	Purity (%)	Yield (%)[c]
Crude extracts[d]	220	39	18	100
Ni^{2+}-chelating column	25	15	76	48.7
RESOURCE RPC	3.25	3.18	98	8.2

[a] Total protein concentration was determined by Bradford protein assay, using bovine serum albumin as a standard.
[b] The amount of protein of interest was determined by quantifying the amount in each gel lane by densitometry.
[c] The purification yield is calculated based on the amount of protein of interest.
[d] The starting material was crude extracts from the lysis of 2 g (from 800 ml culture) bacterial of *E. coli* BL21(DE3).

protein was eluted by 150 mM imidazole. The resulting purity of the fusion protein could reach 76% or more.

Cleavage and purification of recombinant HIN

To generate recombinant HIN, the desalted fusion protein was treated with enterokinase at 22°C and pH 7.4. Complete digestion was achieved after 12 h of incubation of 0.5 mg fusion protein per unit enterokinase. After removing contaminating Trx · 6×His-tag and undigested fusion protein by Ni^{2+}-chelating column, recombinant HIN was purified to homogeneity by the RESOURCE RPC column (Fig. 5). Each of 2 peaks eluted at around 27 and 30% acetonitrile was pooled, analyzed by Tricine-SDS-PAGE (Fig. 6A), and its antimicrobial activity was tested by gel overlay assay (Fig. 6B). The fraction eluted at around 30% acetonitrile showed distinct antibacterial activity by gel overlay assay. Analysis of the fraction from MALDI-TOF MS confirmed that the molecular mass is 4252.0840 Da (Fig. 7), which are consistent with the theoretical molecular mass (4252.0 Da) of recombinant HIN. Table 1 summarizes the yields and purity of the fusion protein and recombinant HIN at some key steps of the purification. Finally, a purified recombinant protein was produced at a yield of 3.18 mg/800 ml, the purity was nearly 98%.

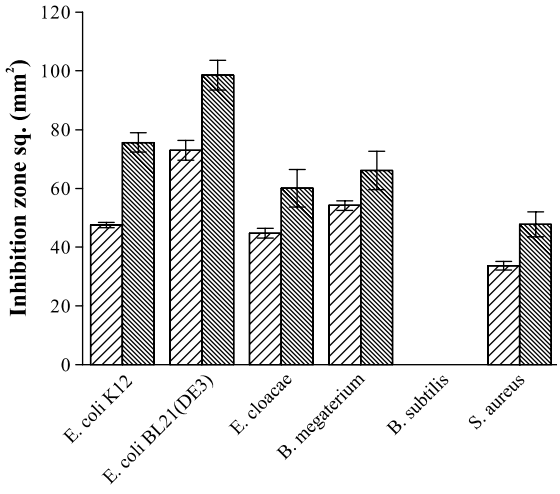

Fig. 8. Comparison of the antibacterial activities between recombinant hinnavinII with (hinnavinII-38-Asn, HIN; dense) and without (hinnavinII-38-Gly, hin; square) amidated C-terminus by radial diffusion assay. Twenty-five micromole (3 μl) of recombinant hinnavinII was placed into each well of 3 mm diameter and plate incubated overnight at 37°C. Inhibition zone squares recorded an average of three independent experiments (the values of the standard deviations are indicated by the vertical bar).

Antimicrobial activity of recombinant hinnavinII

In a previously study, we constructed an expression vector pET32-hinII with *A. rapae* glycine-extended hinnavinII (hinnavinII-38-Gly) gene which had codon for C-terminal glycine residue as non-amidated form (Kang *et al*., 2008). To investigate the role of amidation in *A. rapae* hinnavinII on its activity, we compared the antibacterial activities between the recombinant hinnavinII with (hinnavinII-38-Asn, HIN) and without (hinnavinII-38-Gly, hin) amidated C-terminus. Antibacterial activities of the 2 types of recombinant hinnavinII were tested using a radial diffusion assay against selected 6 species of bacteria. As shown in Fig. 8, 25 μM concentration of the recombinant hinnavin II displayed obvious antibacterial activity against Gram-negative bacteria [*E. coli* K12, *E. coli* BL21 (DE3), *E. cloacae*] and Gram-positive bacteria (*B. megaterium*, *S. aureus*) except *B. subtilis*. It was found that conversion of C-terminal glycine residue to asparagine for adding the C-terminal amide systematically improved the activity of *A. rapae* hinnavinII against these bacteria. Similar results have been reported in cecropins, sarcotoxin IA, and some members in dermaseptin family (Nakajima and Qu, 1987; Andersons and Engstrom, 1991; Mor *et al*., 1994). This could be explained in enhanced activity as a result of stabilization of the structure through amidation, since many aspects of antimicrobial peptide activity are dependent on α-helical, amphipathic structure.

Conclusions

In summary, this work presents the expression and purification method that can be applied for the production of cabbage butterfly (*A. rapae*) antimicrobial peptide hinnavinII in *E. coli* cells. Above results showed that pET32-HIN expression system for the production of Trx-HIN could reach, with high expression level up to 15~20% of the total cellular proteins and more than 70% of the target proteins were in soluble form. Also, the fusion peptide with Trx overcame the antimicrobial peptide's inherent toxicity and difficulty of production due to its sensitivity to protease, and facilitated the purification of target peptide. Purified from the digested fusion protein, the recombinant HIN with asparagine residue containing an amide group at the C-terminus partially mimiced the amidated lysine residue. C-terminal amidated analogue of the recombinant HIN has been confirmed to display significantly enhanced activity of this peptide against certain microorganisms, which implies its possible application as an antimicrobial agent.

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