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Expression of Recombinant Hybrid Peptide Hinnavin II/α-Melanocyte-Stimulating Hormone in *Escherichia coli*: Purification and Characterization

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The increasing problem of antibiotic resistance among pathogenic bacteria requires novel strategies for the construction of multiple, joined genes of antimicrobial agents. The strategy used in this study involved synthesis of a cDNA-encoding hinnavin II/α-melanocyte-stimulating hormone (hin/MSH) hybrid peptide, which was cloned into the pET32a (+) vector to allow expression of the hybrid peptide as a fusion protein in *Escherichia coli* BL21 (DE3). The resulting expression of fusion protein Trx-hin/MSH could reach up to 20% of the total cell proteins. More than 50% of the target protein was in a soluble form. The target fusion protein from the soluble fraction, Trx-hin/MSH, was easily purified by Ni^{2+}-chelating chromatography. Then, enterokinase cleavage effectively cleaved the Trx-hin/MSH to release the recombinant hin/MSH (rhin/MSH) hybrid peptide. After removing the contaminants, we purified the recombinant hybrid peptide to homogeneity by reversed-phase FPLC and obtained 210 mg of pure, active rhin/MSH from 800 ml of culture medium. Antimicrobial activity assay demonstrated that rhin/MSH had a broader spectrum of activity than did the parental hinnavin II or MSH against fungi and Gram-positive and Gram-negative bacteria. These results suggest an efficient method for producing high-level expression of various kinds of antimicrobial peptides that are toxic to the host, a reliable and simple method for producing different hybrid peptides for biological studies.

Keywords: antimicrobial peptide, hinnavin, α-melanocyte stimulating hormone (MSH), hybrid peptide, fusion expression

Antimicrobial peptides (AMPs) have recently generated considerable interest as templates for the design of new antibiotics because of their ability to overcome the resistance of pathogenic bacteria, and they also play an important role in the innate immune response (Hancock, 1997; Zasloff, 2002; Jenssen *et al*., 2006). In order to develop more potent peptides, researchers have altered the original sequences via several strategies, including substitution at one or more positions, truncation, and deletion (Fink *et al*., 1989). Such strategies permit the determination of those residues or domains that are important in modulating activity and can result in analogs with more potent activity than the parental peptides (Tian *et al*., 2009). One especially effective method is the hybridization of two peptides with different properties to obtain novel AMPs (Boman *et al*., 1989; Saugar *et al*., 2006). Several hybrid segments of cecropin A combined with segments of melittin (Andreu *et al*., 1992; Giacometti *et al*., 2003; Giacometti *et al*., 2004; Ferre *et al*., 2006) and magainin (Sin *et al*., 2000; Lee *et al*., 2002; Kwak *et al*., 2003) have demonstrated broader antimicrobial spectra than the parental peptide cecropin A and lower hemolytic activity than melittin. Its broad spectrum and high antimicrobial activity suggest this hybrid peptide may be a promising candidate for therapeutants.

Recently, Yoe *et al.* (2006) purified a small cationic AMP, named hinnavin II, which belongs to cecropin family, from a homogenate of the cabbage butterfly, *Pieris rapae.* Hinnavin II is a cationic linear peptide with 38 amino acid residues, possessing more activity against Gram-negative than against Gram-positive bacteria. Significantly, hinnavin II with purified lysozyme has shown a powerful synergistic effect on the inhibition of bacterial growth.

α-Melanocyte stimulating hormone (MSH) is an endogenous linear peptide derived from pro-opiomelanocortin. MSH has potent anti-inflammatory, antipyretic, and pigmentary effects (Catania and Lipton, 1993; Lipton and Catania, 1997). In particular, MSH and its C-terminal tripeptide, Lys-Pro-Val, possess potent antimicrobial activity against two representative pathogens: *Staphylococcus aureus* and *Candida albicans* (Cutuli *et al*., 2000).

A biosynthetic method for producing a large quantity of AMPs has attracted much attention for its potential pharmaceutical applications. Bacterial expression of heterologous proteins is an easy and inexpensive way to produce large amounts of recombinant proteins for structural investigations. It has several advantages over chemical AMP synthesis, such as low cost, high specific activities of the products, and the possibility of performing manipulations and alterations at the genetic level (Zhou *et al*., 2007), while AMP isolation from natural sources is inefficient and time-consuming (Pazgier and Lubkowski, 2006; Ingham and Moore, 2007). However, in *E. coli*, recombinant AMP expression is not always successful, because of the AMPs' activity within the host and/or proteolysis

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of the recombinant peptides during synthesis (Skosyrev *et al*., 2003; Bao *et al*., 2006; Xu *et al*., 2007). To overcome this obstacle, research has developed several biological expression systems that fuse the antibacterial peptide with a partner protein possessing anionic properties (Rao *et al*., 2004; Kim *et al*., 2008). The commercial pET-32 series is designed for the cloning and expression of peptide sequences fused with thioredoxin (Trx) protein (LaVallie *et al*., 1993; Symersky *et al*., 2000). The carrier protein Trx can alleviate the recombinant protein's toxic effects on host cells and prevent target peptide proteolytic degradation. Moreover, Trx can also accelerate the high-level expression of soluble fusion proteins (Tenno *et al*., 2004; Hidari *et al*., 2005). Therefore, based on this knowledge, we inserted the cDNA encoding hybrid peptide hin/MSH, which incorporates 38 amino acid residues of hinnavin II (GenBank accession no. AY684243), and 13 residues of MSH substituted with C-terminal tripeptide Lys-Pro-Val, downstream of the Trx gene of the pET32a (+) vector. As expected, this expressed a high content of the soluble fusion protein Trx-hin/MSH in *E. coli* BL21 (DE3) with IPTG induction, and we recovered a large quantity of recombinant hin/MSH hybrid peptide and purified it, after enzymatic cleavage of the fusion protein by enterokinase. We then measured the minimal growth inhibition concentration (MIC) of the purified recombinant hin/MSH (rhin/MSH) hybrid peptide.

Materials and Methods

Materials

We used *E. coli* strains DH5α and BL21 (DE3) (Novagen, USA) as respective hosts for the cloning and expression of the fusion protein. With regard to purchased materials, the plasmid pET-32a (+), an expression vector for producing the fusion protein with the Trx, and the enterokinase came from Novagen; PCR reagents, restriction enzymes, and T4 DNA ligase came from Promega (USA); plasmid extraction kits and PCR production purification kits came from Bioneer (USA); and the HiTrap FF crude column and RESOUC RPC column came from Amersham Biosciences (Sweden). For fusion protein detection, we used mouse anti-$(His)_5$ antibody from GE Healthcare (UK) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG from Bio-Rad (USA). Other chemical reagents were of analytical grade.

Construction of recombinant plasmid pET32a-hin/MSH

We utilized the cloning vector pGEM-hinnavin II (Yoe *et al*., 2006), which contained cDNA encoding the hinnavin II, as a template for constructing the expression vector via PCR. To construct the full-length cDNA encoding the hin/MSH hybrid peptide, we added a nucleotide sequence coding for MSH (GenBank accession no. NM000939) at the 3'-terminus of hinnavin II cDNA. The forward primer (5'-GGGGTACC*GACGACGACGACAAG*AAATGGAAGAT TTTCAAGAAAATT-3') introduced a *Kpn*I site (underlined) and an enterokinase cleavage site (italicized). The designed reverse primer was (5'-CGGAATTCTTACACCGGCTTGCCCCAGCGGAAGTGC TCCATGGAGTAGGAACCCTTATA-3'), which included an *Eco*RI site (underlined) and stop codon. Therefore, the introduced MSH gene was downstream of the hinnavin II gene. The PCR technique was as follows: preheat at 95°C for 7 min, 30 cycles of 95°C for 20 sec, 53°C for 40 sec, and 72°C for 40 sec, and finally an elongation at 72°C for 10 min. We used the appropriate enzymes to digest the amplified PCR product and ligated it to the pET32a (+) at the corresponding restriction sites. Restriction endonuclease digestion and DNA sequencing confirmed the resulting plasmid.

Expression and purification of fusion protein Trx-hin/MSH

After we transferred the recombinant plasmid pET32a-hin/MSH into competent *E. coli* strain BL21 (DE3), we inoculated a single colony into an LB medium containing 100 g/ml ampicillin, cultured this overnight at 37°C, then diluted the cultures 1:100, grew them until OD_{600} reached 0.6, and finally, by addition of IPTG, induced them to a final concentration of 0.5 mM. We carried out the expression of Trx-hin/MSH for 5 h at 30°C under vigorous shaking, harvested the cells by centrifuging at 8,000×g for 10 min at 4°C, and stored them at -80°C. Next, we resuspended 2 g of frozen cells (wet weight from 800 ml culture) in 40 ml of lysis buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM EDTA; pH 8.0) and lysed them by sonication. We centrifuged the whole cell lysate at 1,2000×g for 10 min, to separate the soluble and insoluble forms. After decanting the soluble portion, we solubilized the insoluble form in 8 M urea containing 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl, by stirring for 16 h at 4°C. Finally, we analyzed the soluble and insoluble portions via the 15% SDS-PAGE protocol (Laemmli, 1970) and Western blotting (Towbin *et al*., 1979).

The fusion protein Trx-hin/MSH of the soluble form was loaded onto the HiTrap FF crude column, which was previously charged with $NiSO_4$ and equilibrated with binding buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole; pH 8.0). After extensive washing with a buffer identical but for containing 60 mM imidazole, the fusion protein was eluted with six column volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole; pH 8.0). After we collected the eluted fractions and analyzed them by means of the SDS-PAGE protocol, we pooled the purified fusion protein, dialyzed it against a cleavage buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, and 0.1% Tween 20; pH 8.0), with three changes within 24 h, and then lyophilized it.

Release and purification of the rhin/MSH

To release rhin/MSH from the fusion protein, we resuspended the lyophilized Trx-hin/MSH in cleavage buffer, to yield a final fusion protein concentration of 0.5 mg/ml, and added enterokinase (0.1 U/μl) to the protein pool, giving 0.1 U enzyme per 0.1 mg fusion protein. After 16 h incubation at 25°C, we loaded the reaction mixture to the HiTrap FF crude column again, to remove the Trx and undigested fusion proteins, dissolved the flow-through in 0.1% TFA, and loaded it on a reversed-phase FPLC on a 1 ml RESOURCE RPC column equilibrated with 0.1% TFA. We performed the elution with a linear gradient of 20-40% acetonitrile in 0.1% TFA, collected each peak monitored at 214 nm, tested the fractions for antimicrobial activity after lyophilization, and analyzed them via Tricine-SDS-PAGE gel (Schägger and von Jagow, 1987). Finally, we determined the protein and peptide concentrations via the Bradford assay (1976), using bovine serum albumin as the standard.

Mass spectrometry

For the analysis of the rhin/MSH's molecular weight and homogeneity, we employed mass spectrometry, carried out on an Applied Biosystems Voyager MALDI-TOF mass spectrometer at the Korea Basic Science Institute (KBSI).

Antibacterial activity

We tested the purified rhin/MSH antimicrobial activity against a number of microorganisms, including fungi and Gram-positive and

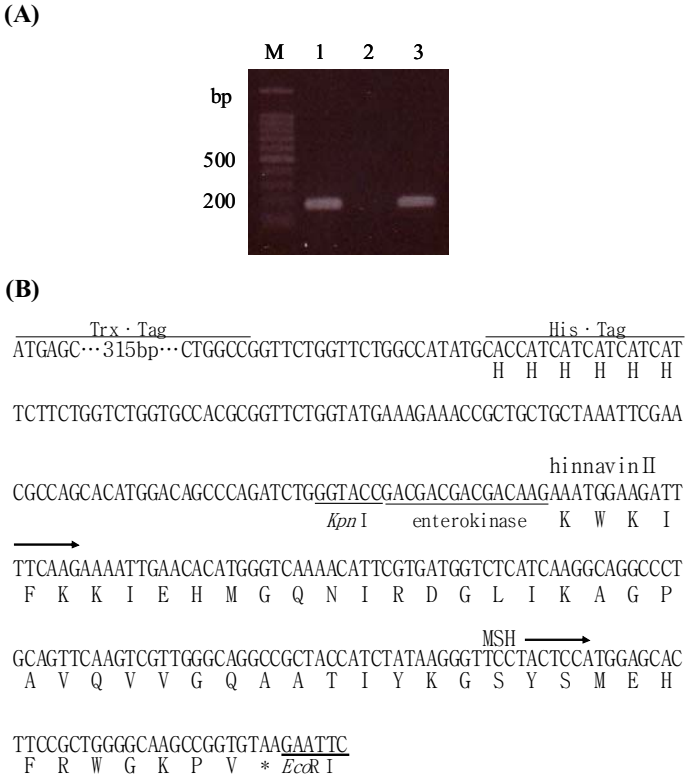

Fig. 1. (A) PCR screening of recombinant plasmid pET32a-hin/MSH. Lanes: 1 and 2, PCR products of pGEM-hinnavin II and a negative control; 3, PCR products of pET32a-hin/MSH; and M, DNA size markers. (B) Partial pET32a (+) and complete hin/MSH nucleotide sequences and predicted amino acid sequence. Amino acid sequence is indicated by the one-letter code written below the second nucleotide of each codon. The *Kpn*I site and *Eco*RI site are underlined. The EK site is double underlined. The stop codon is marked by an asterisk.

Gram-negative bacteria, as follows: *Bacillus megaterium* KCTC 1098, *S. aureus* KCTC 1927, *E. coli* K12 KCTC 1467, *Enterobacter cloacae* KCTC 2361, and *C. albicans* KCTC 7729, all from the Korean Collection for Type Cultures (KCTC), as well as *Bacillus subtilis* ATCC 6633 and *Aspergillus niger* ATCC 9642. We serially diluted the rhin/MSH 10-fold with 0.01% acetic acid and 0.2% BSA, distributed 10 μl aliquots from each dilution to a 96-well polypropylene microtiter plate, and inoculated each well with either a 100 μl suspension of mid-log phase bacteria (2×10^5 CFU/ml) in LB Broth or 80 μl of fungal spores in potato dextrose broth supplemented with tetracycline and cefotaxime. After 3 h incubation, at 37°C (bacteria) or 30°C (fungi), we added fresh media to the mixtures and continued incubation at the same temperatures for an additional 16 h. To determine growth inhibition, we measured the absorbance at 620 nm, using a microplate reader, and then determined each minimal growth inhibition concentration (MIC), expressed as the lowest concentration of the peptide at which no growth was observed, by means of a liquid growth inhibition assay. Each experiment was performed twice.

Results and Discussion

Construction of pET32a-hin/MSH

Many AMPs play an important role in the innate defense systems of the higher organisms. They have generated considerable interest as potential templates for the design and synthesis of new antibiotics (Sato and Feix, 2008). A cecropin family antibacterial peptide, hinnavin II has significant antibacterial activity against Gram-negative and Gram-positive bacteria, but it does not have antifungal activity in previously study (Yoe *et al*., 2006). Therefore, to improve broad-spectrum antimicrobial activity of hinnavin II, our study successfully amplified the cDNA fragment encoding the hin/MSH hybrid peptide by means of PCR, using designed primers from pGEM-hinnavin II as templates. As shown in Fig. 1A, 187 bp of the PCR product coded for hinnavin II, combining MSH at its C-terminus and enterokinase cleavage site at its N-terminus, which facilitated hin/MSH's release from the fusion protein by means of enterokinase. One pair of endonuclease restriction sites, *Kpn*I and *Eco*RI, flanked the PCR product up- and downstream, respectively (Fig. 1B). To construct an effective hin/MSH expression system, we selected a Trx-$(His)_6$-tag under the control of T7 promoter as the fusion partner and, therefore, constructed a recombinant pET32a-hin/MSH vector for expressing the Trx-hin/MSH fusion protein by modifying the pET32a (+) as shown in Fig. 2. The existence of a $(His)_6$-tag between the Trx and hin/MSH coding sequence benefits the facile purification of fusion protein Trx-hin/MSH by means of metal affinity chromatography. Also, we created an enterokinase cleavage site immediately upstream of hin/MSH, to facilitate enzymatic cleavage of the fusion protein for hin/MSH peptide release.

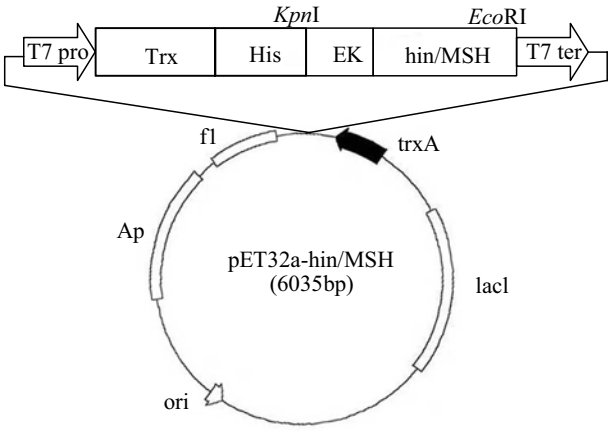

Fig. 2. Construction of the pET32a-hin/MSH expression vector. The *Kpn*I-*Eco*RI fragment (187 bp) containing hin/MSH cDNA was synthesized, annealed, and then ligated into a commercial vector, pET-32a (+). hin/MSH was expressed as a fusion protein with the Trx. To facilitate peptide cleavage from the fusion protein, an EK cleavage site was created between the Trx His-tag and hin/MSH.

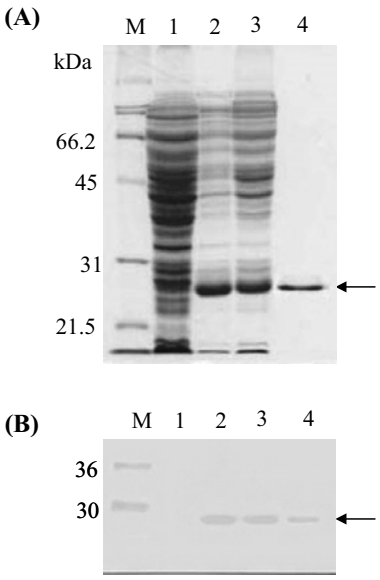

Fig. 3. (A) SDS-PAGE and (B) Western blotting analyses of the Trx-hin/MSH fusion protein. Lanes: 1, total cell lysate before induction; 2 and 3, soluble and insoluble protein extracts of cell lysate, respectively, after 5 h induction with IPTG; 4, Trx-hin/MSH fusion protein purified by Ni^{2+}-chelating column; and M, protein size markers.

Expression and purification of Trx-hin/MSH

The constructed expression vector, pET32a-hin/MSH, was transformed in *E. coli* BL21 (DE3). We analyzed several inducing factors affecting the fusion protein's expression, including bacterial cell density (OD_{600} between 0.1-1.0), concentrations of IPTG (0.1-1 mM), induction time (2-10 h) and temperature (20-37°C), obtaining optimal expression at a cell density of OD_{600}=0.6, with 0.5 mM IPTG, after 5 h incubation at 30°C. As shown in Fig. 3A, the target fusion proteins occurred in both soluble and insoluble forms (lanes 2 and 3), with an apparent molecular weight of 22.86 kDa. Also, we found the resulting fusion protein expression could reach 15-20% of the total proteins; more than 50% of the target protein was in a soluble form. Western blot analysis further confirmed this to be the target fusion protein Trx-hin/MSH (Fig. 3B). Consequently, we purified the soluble form of the fusion protein. Using a HisTrap FF column, we eluted most of the fusion protein in 200 mM imidazole and easily purified it to homogeneity. Analysis of the fusion protein on SDS-PAGE and via Western blotting revealed a protein band at about 22.86 kDa (Figs. 3A and B, lane 4). One-step affinity chromatography purified about 14 mg of fusion protein from 800 ml of culture medium.

Cleavage and purification of rhin/MSH

To generate rhin/MSH, we treated the desalted fusion protein with enterokinase at 22°C and pH 7.4, achieving complete digestion after incubating 0.5 mg fusion protein per unit of enterokinase for 12 h. After removing the Trx-$(His)_6$-tag and undigested fusion protein via a Ni^{2+}-chelating column, we purified rhin/MSH to homogeneity using a RESOURCE RPC column (Fig. 4). These procedures were highly efficient at producing large amounts of pure hin/MSH, producing 2.10 mg of pure hin/MSH from 800 ml of culture medium. As shown in Fig. 5, the fraction eluted at around 34% acetonitrile on the Tricine-SDS-PAGE revealed a molecular weight of about 5.8 kDa, identical to the anticipated theoretical molecular mass of hin/MSH (5,800 Da). MALDI-TOF MS analysis found the mass was 5,800.82 Da (Fig. 6), which agreed well with the calculated value. Table 1 summarizes the yields and purities of the fusion protein and rhin/MHS at certain key steps of the purification. The purity of the purified recombinant hin/MSH was nearly 95%.

Antimicrobial activity of the rhin/MSH

Recently, the rapid emergence of antibiotic-resistant bacterial and fungal strains have brought about considerable interest in the use of hybridization methods to obtain novel AMPs for use as therapeutic agents. The hybrid (chimeric) concept of AMPs was first tested on a peptide that contained residues 1-11 from cecropin A and 12-37 from cecropin D (Fink *et al*., 1989). Several hybrid peptides have shown improved biological activity as compared to their parental peptides (Boman *et al*., 1989; Andreu *et al*., 1992; Ferre *et al*., 2006); this may occur because the hybrid peptide can possess a better

Table 1. Purification of rhin/MSH hybrid from *E. coli*

Purification step	Total protein (mg)[a]	Protein of interest (mg)[b]	Purity (%)	Yield (%)[c]
Crude extracts[d]	190	30.4	16	100
Ni^{2+}-chelating column	27	14	51.8	46.1
RESOURCE RPC	2.2	2.1	95	6.9

[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).

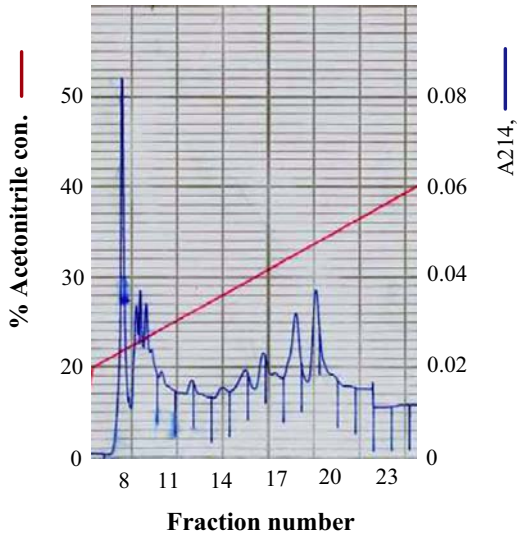

Fig. 4. Purification profile of rhin/MSH by RESOURCE RPC column of the eluted fraction from Ni^{2+}-chromatography.

combination of various parameters than the parental peptides. Using liquid growth inhibition assays of serial dilutions of rhin/MSH, our study tested the antimicrobial activity of the purified rhin/MSH against several bacterial and fungal strains (Table 2). The rhin/MSH was highly effective against the target microorganisms and showed a low MIC against all Gram-negative and Gram-positive bacteria tested. More interestingly, the hybrid rhin/MSH showed moderate activity against the fungi, which were not sensitive to hinnavin II. It seemed hybridization of two different peptides could not only rescue, but perhaps improve upon, antimicrobial activity.

Conclusions

For AMPs to be useful for pharmaceutical applications, they must possess strong antimicrobial activity against bacterial and fungal cells. In this study, we constructed a novel hybrid, hin/MSH, and expressed it as a Trx fusion protein in *E. coli* cells. Functionally expressed, fused with Trx, in soluble form in

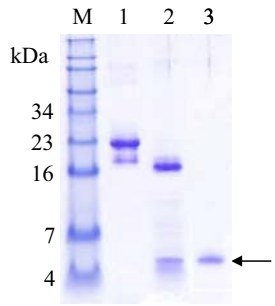

Fig. 5. Tricine SDS-PAGE analysis of rhin/MSH. Lanes: 1, Trx-hin/MSH fusion protein purified by Ni^{2+}-chelating column; 2, Trx-hin/MSH fusion protein cleaved by EK; 3, rhin/MSH purified by RESOURCE RPC column; and M, low protein size markers.

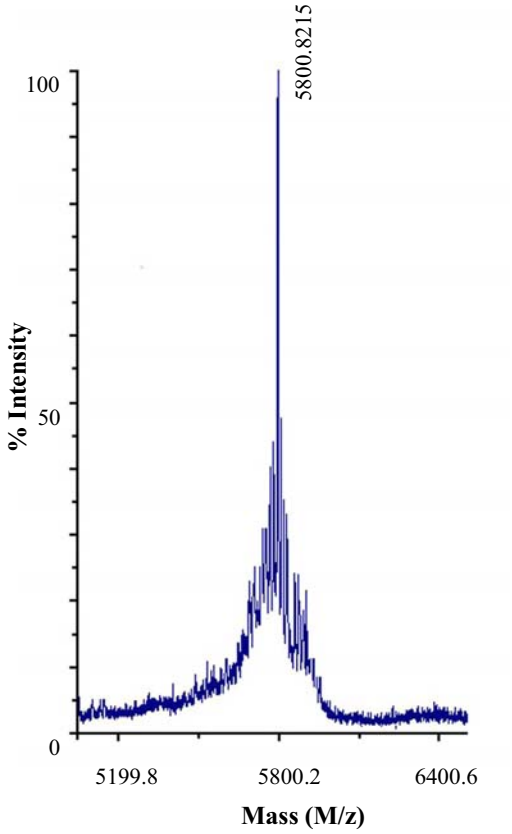

Fig. 6. Mass spectrum of purified rhin/MSH hybrid recorded on an applied biosystems voyager MALDI-TOF mass spectrometer.

E. coli, with the expression level up to 38 mg/L and to more than 15% of total cellular protein, hin/MSH appears to be a hybrid with broader antimicrobial spectra than the parentals, hinnavin II and MSH. This hin/MSH, which exhibits potent antimicrobial activity, may have potential as a model for the study of AMPs' action mechanisms-i.e., the antibiotic relationship of the peptides-as well as potentially being an excellent novel therapeutic agent.

Table 2. Antimicrobial activities of rhin/MSH

Microorganism	MIC (μM)
Gram-positive bacteria	
Bacillus megaterium KCTC 1098	2.4
Bacillus subtilis ATCC 6633	2.7
Staphylococcus aureus KCTC 1927	3.6
Gram-negative bacteria	
Escherichia coli BL21 (DE3)	2.1
Escherichia coli K12 KCTC 1467	2.3
Enterobacter cloacae KCTC 2361	3.4
Fungi	
Candida albicans KCTC 7729	19
Aspergillus niger ATCC 8642	20

Acknowledgements

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