DOI:10.1021/jf902574t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Expression of Acc-Royalisin Gene from Royal Jelly of Chinese Honeybee in *Escherichia coli* and Its Antibacterial Activity

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Royalisin is an antibacterial peptide found in Royal Jelly. Two gene fragments of Chinese honeybee (*Apis cerana cerana*) head, 280 bp cDNA encoding pre-pro-Acc-royalisin (PPAR) of 95 amino acid residues, and 165 bp cDNA encoding mature Acc-royalisin (MAR) of 51 amino acid residues were cloned into the pGEX-4T-2 vector. They were then transformed individually into *Escherichia coli* for expression. Two expressed fusion proteins, glutathione *S*-transferase (GST)-PPAR of 36 kDa and GST-MAR of 32 kDa were obtained, which were cross reacted with GST antibody accounting for up to 16.3% and 15.4% of bacterial protein, respectively. In addition, 41% of GST-PPAR and nearly 100% of GST-MAR were soluble proteins. Both lysates of the two purified fusion proteins displayed antibacterial activities, similar to that of nisin against Gram-positive bacteria strains, *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus*. MAR peptide released from the thrombin-cleaved GST-MAR fusion protein has a stronger antibacterial activity than that of GST-MAR fusion protein.

KEYWORDS: *Apis cerana cerana*; Royal Jelly; Acc-royalisin; prokaryotic expression; antibacterial activity

INTRODUCTION

Royal jelly (RJ), a principal food of the honeybee queen and larvae of worker within three days and drone, is secreted from the hypopharyngeal and mandibular glands of worker honeybees mainly between 5 and 15 days old of their imago life. Except for importance in regulating caste determination, development and reproduction, RJ has a wide range of biological activities, including acceleration of the growth rate of chick embryos, vasodilative, hypotensive, antitumor, antiinflammatory, disinfectant and antifatigue activities, and increase of the average life span of mice (*1*). RJ of the Western honeybee, *Apis mellifera*, consists of proteins (12−15%), which constitute 50% of the dry mass, carbohydrates (10−16%), lipids (3−6%), vitamins, free amino acids and a large number of bioactive substances such as 10-hydroxyl-2-decenoic acid (10-HDA), antibacterial peptides and stimulating factor for the development of genital organs in male mice (*2*). Therefore, RJ has been widely used as dietary supplement, medicinal food and cosmetic (*3*).

The antimicrobial activities of RJ against actinomycetes, molds and fungi have been known for many years (*2*). Recent studies revealed the inhibitory activities of RJ against both Gram-positive and Gram-negative bacteria, such as *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus* (*4*) and *Streptomyces griseus*, *Escherichia coli* (*5*). In previous studies 10-HDA was identified as the antibiotic component in RJ against many species of bacteria and fungi (*6*, *7*). Royalisin and four Jelleines in RJ of the Western honeybee were the active constituents which inhibit Gram-positive bacteria and fungi (*2*, *8*, *9*). Although with a narrower antibacterial spectrum than native RJ, royalisin strongly inhibits the growth of the Gram-positive bacteria including *Clostridium, Corynebacterium*, *Leuconostoc*, *Staphylococcus*, and *Streptococcus* at the effective concentration of 1 μM, the antibacterial potency of which is comparable to that of native RJ at 10 μg/mL. It also has inhibitory activity against other Gram-positive bacteria, i.e. *B. subtilis* and *Sarcina lutea* as well as American foulbrood caused by a Gram-positive bacterial pathogen, *Paenibacillus larvae larvae*. Moreover, the peptide fraction has been shown also to have antifungal activity such as model fungus *Botrytis cinerea* (*9*, *10*).

Numerous inducible antibacterial peptides have been characterized from insects, plants, amphibians and mammals, and they can be classified broadly into four major groups: cecropins, defensins or sapecins, attacin-like proteins and proline-rich peptides (*11*). Defensins, cationic antibacterial peptides, belong to the most common group of antimicrobial proteins in insects. Defensins with distinct structures have been identified as three subfamilies: classical defensins, β-defensins and insect defensins. Classical defensins and β-defensins have been found in mammalian neutrophils. Protein sequences of insect defensins share only limited sequence similarity with mammalian defensins, but this is the basis of their common structure comprising an amino-terminal loop, α-helix and two antipararallel β-strands stabilized by disulfide bridges. They act against Gram-positive bacteria, but Gram-negative bacteria are generally resistant to them (*11*). The mature peptide of Am-royalisin is composed of 51 amino acid residues, 5.52 kDa, belonging to defensins (*12*, *13*). In this paper, we investigated the biochemical properties of Acc-royalisin,

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 Published on Web 02/01/2010

a homologous peptide of Am-royalisin from the RJ of Chinese honeybee, *Apis cerana cerana*. In addition, we examined the antibacterial activity of the purified soluble expressed fusion proteins of both pre-pro peptide and mature peptide of Acc-royalisin against three Gram-positive bacteria strains, *S. aureus*, *B. subtili* and *M. luteus*.

MATERIALS AND METHODS

Bacteria Strains and Chemicals. The bacterial strains, *E. coli* TG1 and BL21 (DE3), *S. aureus* CMCC(B) 26003, *B. subtilis* CMCC(B) 63501 and *M. luteus* CMCC(B) 28001, the expression vector pGEX-4T-2 and the worker head cDNA library of Chinese honeybee sequenced by Waston Institute of Genome Science, Zhejiang University, were maintained in our laboratory (*14*). Restriction endonucleases, X-gal, isopropyl β-D-1-thiogalactopyranoside (IPTG), ethidium bromide (EB) and DL-2000 marker were purchased from TakaRa (Dalian, China). *Taq* polymerase, PCR purification kit and ampicillin were purchased from Sagon (Shanghai, China). pGEM-T easy vector and NBT/BCIP were purchased by Promega (Shanghai, China). Restriction endonucleases and T4 DNA Ligase were purchased from Fermentas International Corporation (Burlington, Canada). Plasmid DNA Extraction Kit was from OMEGA Bio-Tek Corporation (Doraville, GA). Antiglutathione *S*-transferases (GST) polyclonal antibody (purified from goat serum) was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG linked horseradish peroxidase (HRP) and diaminobenzidine (DAB) were from Boster Biological Technology, Ltd. (Wuhan, China). PVDF was from Millipore Corporation (Billerica, MA). SinoBio protein marker and thrombin protease were from Shanghai Sino Biotech Co., Ltd. (Shanghai, China). High-Affinity GST Resin Kit was from GenScript Corporation (Piscataway, NJ). Nisin (1000 IU/mg), one of the bacteriocins and an anti-Gram-positive bacteria peptide produced by bacteria, *Lactococcus lactis* subsp. Lactis, was from Lanzhou Weiri Biological Engineering Corporation (Lanzhou, China). TakaRa protein marker was from TakaRa Biotechnology Co., Ltd. (Dalian, China), and BioEev-Tech protein marker was from BioEev-Tech Scientific & Technical Co., Ltd. (Beijing, China).

DNA Sequencing and Data Analysis. Expression sequence tags (ESTs) were assembled into contig using CAP3 and PHRAP by sequence comparison with GenBank Nt Database, GenBank Nr and SWISSPROT database by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All ESTs in royalisin contigs were analyzed with DNAstar software. These ESTs containing the entire whole royalisin gene sequence were chosen for analysis. The collected clones were further sequenced by Shanghai Sangon Biotechnology Corporation further. The homologous sequences were searched with BLAST against data in the GenBank, and analyzed with CLUSTAL 1.8 and GeneDoc. The putative amino-terminal signal of putative amino acid sequence of royalisin was analyzed with Signal P (http://www.expasy.org/tools), and its protein structure was analyzed with ScanProsite (http://www.expasy.org/tools/scanprosite) and Soma programs.

Construction of Prokaryotic Expression Vectors and Expression of Acc-Royalisin in *E. coli*. The primers for both pre-pro-Acc-royalisin (PPAR) and mature Acc-royalisin (MAR) were derived from the Acc-royalisin cDNA (GenBank accession: EF660337): Accr-f1 (5′-AGGATCCATGAAGATCTATTT TATTG-3′) and Accr-f2 (5′-AGGATCCATGGTAACTTGTGACCTT-3′) which contained a *Bam*HI restriction site, respectively, and one reverse primer, Acc-r1 (5′-GCGGCCGCTTAACCGAAACGTTTGTC-3′), which contained a *Not*I restriction site. Amplification of the PPAR gene and MAR gene from the Acc-royalisin cDNA clone was performed using primers of Accr-f1 and Acc-r1, and primers of Accr-f2 and Acc-r1, respectively. Amplification of the polymerase chain reaction (PCR) was performed in a programmable thermal controller (BOER, China) with one cycle of 94 °C for 4 min followed by 30 cycles of denaturation (30 s at 94 °C), annealing (35 s at 56 °C) and extension (60 s at 72 °C) and a final step of 72 °C for 10 min. PCR products were visualized on a 2.5% gel with EB staining. The PCR products were subcloned into the pGEM-T easy vector to form the recombinant vector pGEM-T-PPAR and pGEM-T-MAR. The fragments of PPAR and MAR were excised and removed from the pGEM-PPAR and pGEM-T-MAR using *Bam*HI and *Not*I, and inserted into the GST fusion expression vector pGEX-4T-2, respectively. BL21 cells were transformed with the recombinant expression vector pGEX/PPAR and pGEX/MAR. Protein expression was induced by the addition of 1.0 mM IPTG with further growth at 37 °C for 3 h. Cells were harvested by centrifugation (5 min, 4000*g*, 4 °C), and cell samples were examined by Coomassie brilliant blue R250-stained sodium dodecyl sulfate polyacrylamide gel (12%, v/v) electrophoresis (SDS−PAGE). In order to determine the solubility, the harvest cells were suspended in 1× phosphate buffered saline (PBS), and then were lysed on ice. Ultracentrifugation of the cells and cell culture supernatant samples of the expressed proteins of GST-PPAR and GST-MARA was examined by SDS−PAGE gels described as above, respectively. The content of the expressed protein was measured by through scanning the profile of SDS−PAGE gels with the Gel Doc EQ imaging system (Bio-Rad Laboratories, U.S.A.).

Western Blot Assay. The recombinant proteins of GST-PPAR and GST-MARA were detected in SDS−PAGE as described by Sambrook et al (*24*). The electrophoresed proteins were transferred to a PVDF membrane for 15 min at 0.2 A in a Bio-Rad transblot apparatus. The membranes were incubated with the anti-GST polyclonal antibody (1:6000). After rinsing the membrane to remove unbound primary antibody, the membranes were exposed to the secondary antibody, goat anti-rabbit IgG/HRP and then reacted with DAB substrate for colorimetric detection according the manufacturer's protocol.

Purification of the Recombinant Protein Expressed by *E. coli*. Recombinant GST-PPAR, GST-MAR and GST, expressed in BL21 cells, were harvested after lysis by brief ultrasonic pulses on ice, then centrifugation at 12000*g* for 10 min at 4 °C. The supernatants (lysate proteins) were purified by using High-Affinity GST Resin in a disposable column according to the technical manual of the High-Affinity GST Resin Kit. After purification, the proteins were placed in the sealed dialysis bag and dipped in double distilled water to remove salts over 24−48 h, with at least 6 changes of water, and then lyophilized and weighed. The fusion protein of GST-MAR was cleaved by thrombin protease at the concentration 10 μL/mL in 1× PBS at 37 °C for 24 h. The MAR fraction was isolated with the High-Affinity GST Resin Kit as described.

Antibacterial Diffusion Assay. Antibacterial diffusion test on 1.5% (w/v) agar plates (inhibition zone assay) was performed according to Bilicova et al. (*9*). Briefly, 9 mL of Nutrition Agar (Hangzhou Microbiological Agents Co., Ltd., China) was spread and mixed with 1 mL of bacteria culture solutions of three bacterial strains, *S. aureus* or *M. luteus* (1×10^6 cells/mL), or spore suspension of *B. subtilis* (1×10^6 spores/mL), and 10 μL of protein samples (GST-PPAR, GST-MAR, MAR and GST in 10 mM Tris-HCl buffer, pH 8.0) at a protein concentration ranging from 2.0 mg/mL to 3.0 mg/mL and applied into 8 mm diameter paper filters on the surface of the agar medium plates. Nisin at a concentration ranging from 0.5 to 2.0 mg/mL dissolved in Tris-HCl was used as a positive control. Tris-HCl was used as the blank control. Plates were incubated at 37 °C for 16 to 20 h. Plates with inhibition zone assays were photographed against a black background to visualize the inhibition zone around the filter. The diameters of the inhibition zone around each filter paper were measured.

Statistical Analysis. The data analyses were performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL). One-way ANOVA with Duncan's post hoc tests was used to determine differences between each pair of treatment groups. The values are reported as mean ± SD; p values were two tailed, and a p-value < 0.05 was considered as significant.

RESULTS

Sequence Analysis of Acc-Royalisin cDNA. Sixty-nine ESTs containing PPAR were obtained from 8568 effective ESTs from the cDNA library of Chinese honeybee worker heads. PPAR ESTs, with homologous peptides for pre-pro-Am-royalisin, accounted for 0.81% of all the ESTs. Five ESTs containing the entire whole PPAR gene sequence were found. All putative amino acid sequences of PPAR were identical to those sequenced in this study. One of Acc-royalisin cDNA clones was further sequenced. The result showed that the clone contained the whole gene of 288 bp encoding 95 amino acids, with a predicted molecular weight of 10.45 kDa (**Figure 1**). Sequence analysis using

CLUSTAL X 1.8 and GeneDoc revealed that the PPAR (EF660337) shared 90−92% amino acid homologies with previously reported pre-pro-Am-royalisin (Nm0010116, AY496432 and AY333923) in the amino acid sequence. Analysis using Signal P showed that the precursor contained a putative signal peptide which was composed of 19 residues in the N-end, and the cleavage site of the putative pre-pro region was between Arg_{43} and Thr_{45}. The MAR containing an open-reading frame (ORF) encoding 51 amino acids shared 96% homologies with the mature Am-royalisin in amino acid sequence, with a predicted molecular weight of 5.52 kDa (**Figure 2**). Analysis with ScanProsite showed that the mature peptide likely contains six cysteine residues, had three disulfide bridges (**Figure 2**) and belonged to insect defensins which can inhibit Gram-positive bacteria.The consensus three-dimensional structure, predicted by Soma software, suggested that MAR consisted of α-helix (28.85%), antiparallel *β*-sheet (11.54%), amino-terminal loop (25.00%) and random coil (34.62%). PPAR was deposited in GenBank in 2007 as the first royalisin gene identified from Chinese honeybee (EF660337).

Two fragments, 280 bp cDNA encoding PPAR and 165 bp cDNA encoding MAR, were amplified by PCR with one of previously sequenced cDNA clone, rndcb_001106.y1.scf, as a template, respectively (**Figure 3**). The PCR products of the two fragments were purified and ligated individually into the easy vector, respectively. The plasmids of pGEM-T with the insertions of the two genes were confirmed by PCR, digested with *Bam*HI and *Not*I, and then sequenced.

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CGGCACGAGGCTTCTCAGTCTATTTTCGAATAGTTTGAACATTTATTGAAGTTTAACACG  60
TATTTAGCCAATATGAAGATCTATTTTATTGTCGCCTTTCTCTTCATGGCTATGGTTGCC 120
            M  K  I  Y  F  I  V  A  F  L  F  M  A  M  V  A
ATCATGGCTGCACCTGTTGAGGATGAATTCGAGCCACTTGAGCATCCTGAGAACGAAGAA 180
 I  M  A  A  P  V  E  D  E  F  E  P  L  E  H  P  E  N  E  E
CGTACCGATAGACATAGAAGAGTAACTTGTGACCTTCTCTCATTCAAAGGACAAGTCAAT 240
 R  T  D  R  H  R  R  V  T  C  D  L  L  S  F  K  G  Q  V  N
GACAGTGCTTGCGCTGCTAACTGTCTTAGTTTGGGTAAAGCTGGAGGTCATTGCAAGAAC 300
 D  S  A  C  A  A  N  C  L  S  L  G  K  A  G  G  H  C  K  N
GGAGTTTGTATTTGTCGAAAAACCAGTTTCAAAGATCTCTGGGACAAACGTTTCGGTTAA 360
 G  V  C  I  C  R  K  T  S  F  K  D  L  W  D  K  R  F  G  *
GTGCCATTATGAAGTTTCAACGATGAAATTTTTTAACAAAAAATATGAAAACGCAAAATG 420
AAAAATAAAAAATTATATCATCATTGAAAATTCTATGAATTTACTTATTTAATAAATAAT 480
ATAAATAAATTATCTTTTGGTTTAATTTTTTTAAAATATTATTTTTTGAATAAATAAATT 540
TTTTAAAAAAAT
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Figure 1. Nucleotide sequence and deduced amino acid sequence of pre-pro-Acc-royalisin. The start codon and stop codon are indicated in bold. The pre region (signal peptide) is underlined, and the pro region is boxed. Processing sites (signal peptidase), mature peptide cleavage site, and C-terminal amidation site are marked by vertical arrows.

Prokaryotic Expression. The genes of PPAR and MAR cleaved from the pGEM/PPAR and pGEM/MAR using *Bam*HI and *Not*I were inserted into vector pGEX-4T-2. The recombinant expression vectors with the inserted PPAR and MAR were identified by PCR and digestion with *Bam*HI and *Not*I. The pGEX/PPAR and pGEX/MAR were then transformed into *E. coli* BL21 for expression, respectively. The SDS−PAGE analysis showed that the expressed product of pGEX/PPAR contained a band of fusion protein of about 36 kDa (**Figure 4A**) which was identical to the predicted molecular mass of the recombinant protein composed of GST (26 kDa) and PPAR (10.45 kDa). The expressed product of pGEX/MAR produced a band of fusion protein of about 32 kDa (**Figure 5A**) which was identical to the predicted molecular mass of the recombinant protein composed of GST and MAR (5.52 kDa). The SDS−PAGE gel profiles showed that the expressed fusion protein GST-PPAR and GST-MAR accumulated up to about 16.3% and 14.7% of total protein of bacterial cells, respectively. Meanwhile, the PGEX-4T-2 vector was also transformed into BL21 cells and the GST protein about 26 kDa was expressed successfully (**Figure 5B**).

Analysis of SDS−PAGE gel profiles on the cells and cell culture supernatants from ultrasonic lysed bacteria expressing

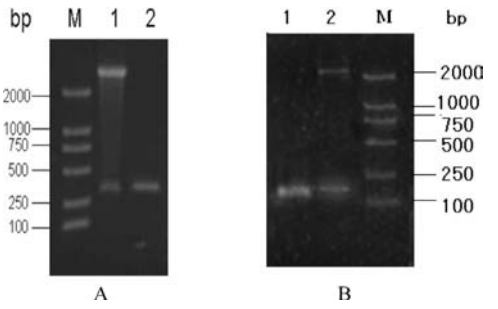

Figure 3. Identification of recombinant plasmids of pGEM/PPAR and pGEM/MAR. (**A**) Identification of pGEM/PPAR. Lane M, DNA marker. Lane 1, pGEM/PPAR digested by *Bam*HI and *Not*I. Lane 2, PPAR PCR product. (**B**) Identification of pGEM/MAR: Lane 1, MAR PCR product. Lane 2, pGEM/MAR digested by *Bam*HI and *Not*I. Lane M, DNA marker.

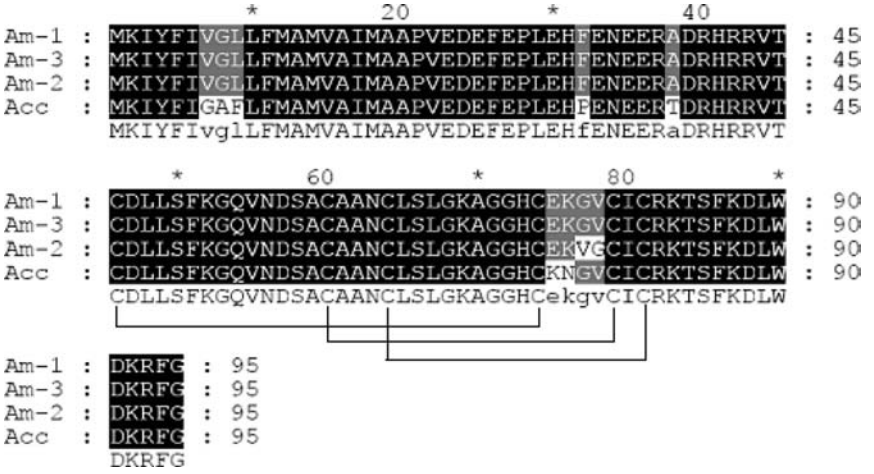

Figure 2. Amino acid sequence alignment of pre-pro-Acc-royalisin and pre-pro-Am-royalisin. The accession numbers in GenBank of the four sequences are EF660337 (Acc), Nm0010116 (Am-1), AY496432 (Am-2) and AY333923 (Am-3), respectively. Homologous amino acid residues are shaded. Asterisks represent omitted number 10, 30, 50, 70, and 90. Three disulfide bridges formed by six cystein residues are lined.

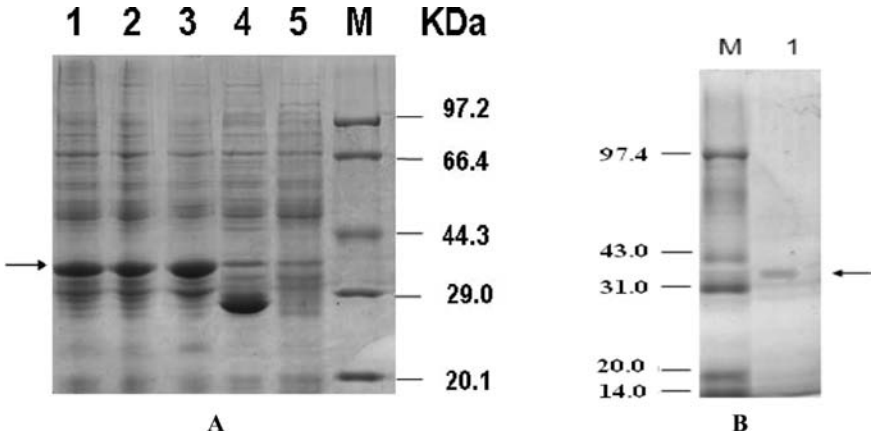

Figure 4. The SDS−PAGE pattern of PPAR fusion protein expressed in *E. coli* BL 21 and purified GST-PPAR protein. (**A**) The SDS−PAGE pattern of the expressed PPAR protein. Lane 1, lysate portion of GST-PPAR fusion protein. Lane 2, insoluble portion of GST-PPAR fusion protein. Lane 3, total protein of bacterial cells with the expressed GST-PPAR. Lane 4, total protein of bacterial cells transformed with pGEX-4T-2. Lane 5, total bacterial protein of BL21 cells. Lane M, protein marker. (**B**) The SDS−PAGE pattern of purified PPAR protein. Lane M, protein marker. Lane 1, purified GST-PPAR fusion protein.

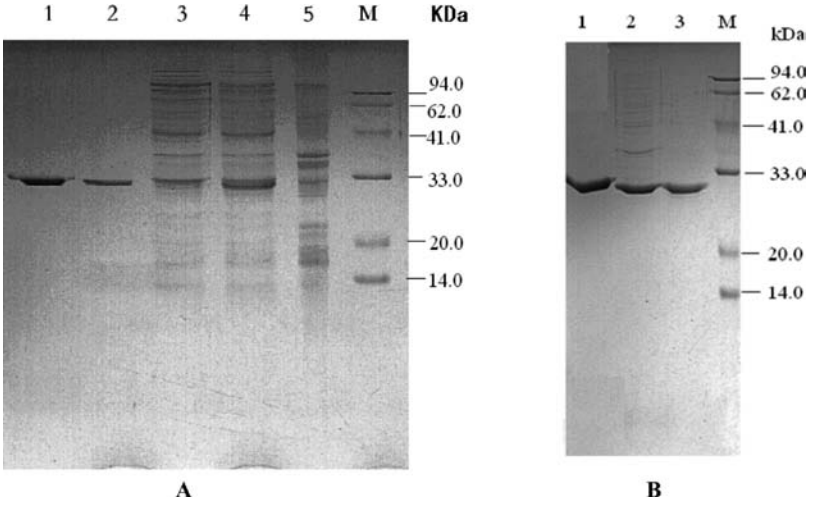

Figure 5. The SDS−PAGE pattern of expressed fusion proteins of GST-MAR and purified GST. (**A**) The SDS−PAGE pattern of expressed GST-MAR fusion protein and purified GST-MAR. Lanes 1 and 2, purified fusion protein of GST-MAR. Lanes 3 and 4, supernatant of bacterial cell lysate with the expressed GST-MAR. Lane 5, precipitate of bacterial cell lysate without expressed GST-MAR. Lane M, protein marker. (**B**) The SDS−PAGE pattern of purified GST protein. Lanes 1−3, purified GST protein. Lane M, protein marker.

the pGEX/PPAR fusion showed that the fusion protein is about 7.63% of total soluble bacterial protein, and about 8.80% of total protein in the inclusion body. Approximately 46.1% of the expressed fusion protein was soluble (**Figure 4A**). We also show that the GST-MAR fusion protein appeared in the supernatant only, indicating that essentially all of the GST-MAR fusion was soluble (**Figure 5A**). The expressed fusion proteins of both GST-PPAR and GST-MAR were recognized by the GST-antibody, respectively (**Figure 6**), which confirmed that the two expressed products were the expected fusion proteins of GST-PPAR and GST-MAR.

Purification of Expressed Fusion Proteins. Recombinant proteins of both PPAR and MAR were expressed in *E. coli* as the fusion proteins containing GST for affinity purification. The purity of the two soluble proteins was enriched up to 90% in a single step using high-affinity GST resin. The purified fusion protein bands of PPAR at 36 kDa (**Figure 4B**) and MAR at 32 kDa (**Figure 5A**) in molecular mass could be observed clearly on SDS−PAGE profiles. About 13.4 mg of the lyophilized fusion soluble protein GST-PPAR was obtained from 500 mL of the harvest recombinant expression bacterial cells. **Figure 7A** shows that the GST-MAR fusion has been cleaved by thrombin protease. The purified GST band could be observed easily on SDS−PAGE profiles. The purified bands of GST (26 kDa) and MAR (5.52 kDa) isolated from the thrombin-cleaved GST-MAR fusion protein could be observed clearly on SDS−PAGE profiles (**Figure 7B**). The lysate proteins of GST-PPAR, GST-MAR, MAR and GST were lyophilized for antibacterial tests.

Analysis of Antibacterial Activity of Expressed Lysate Proteins. The three lyophilized soluble proteins, GST-PPAR, GST-MAR and GST, diluted in Tris-HCl, were used to assay antibacterial properties. It was found that both GST-PPAR and GST-MAR inhibited growth of some Gram-positive bacterial strains such as *S. aureus*, *B. subtilis* and *M. luteus*. **Figure 8** presents the diffusion tests for the antibacterial effect of the fusion proteins, GST-PPAR (**Figure 8**, position 1 for plates **A**, **B**, **C**), and GST-MAR (**Figure 8**, position 2 for plates **A**, **B**, **C**), at concentration 2 mg/mL against the three bacterial strains mentioned as above. Nisin, a commercial bacteriocin used in treatment of food preservation, was used as a positive control of growth inhibition effect at concentration of 2 mg/mL (**Figure 8**, position 5 for plates **A**, **B**, **C**). No inhibition effect was observed for the negative control,

GST (**Figure 8**, position 3 for plates **A**, **B**, **C**) and blank control of Tris-HCl (**Figure 8**, position 4 for plates **A**, **B**, **C**) against the same Gram-positive bacterial strains.

The diameters of the inhibition zone around filters treated with various tested samples are shown in **Table 1**. The inhibition effects of GST-PPAR and GST-MAR against the three bacterial strains were less than nisin. There were differences of inhibition effects between GST-PPAR and GST-MAR for different bacterial strains. GST-PPAR has stronger effect against *B. subtillis* and *S. aureus*, and GST-MAR is stronger against *M. luteus*.

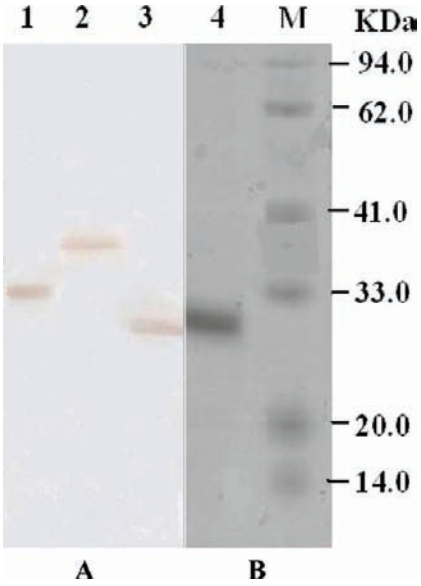

Figure 6. Western blot analysis of the expressed fusion proteins of GST-PPAR and GST-MAR. (**A**) Western blot analysis of the expressed fusion proteins. Lane 1, fusion protein of GST-MAR probed with anti-GST antibody. Lane 2, GST-PPAR protein probed with anti-GST antibody. Lane 3, fusion protein of GST probed with anti-GST antibody. (**B**) The SDS−PAGE pattern of purified GST protein. Lane 1, purified GST protein. Lane M, protein marker.

Figure 9 presents the diffusion tests for the antibacterial effect of the fusion proteins, GST-MAR (**Figure 9**, position 2 for plates **A**, **B**, **C**), at a concentration of 3.0 mg/mL, MAR isolated from the thrombin cleaved GST-MAR (**Figure 9**, position 3 for plates **A**, **B**, **C**) at 2.0 mg/mL and nisin (**Figure 9**, position 1 for plates **A**, **B**, **C**) at 0.5 mg/mL against the same three bacterial strains. No inhibition effect was observed for the Tris-HCl blank control (**Figure 9**, position 4 for plates **A**, **B**, **C**).

The diameters of the inhibition zone around filters treated with the four tested samples are presented in **Table 2**. The results indicate that the inhibition effect of MAR at 2.0 mg/mL was equivalent to GST-MAR fusion protein at 3.0 mg/mL and nisin at 0.5 mg/mL.

DISCUSSION

Since royalisin was first identified by Fujiwara et al. from RJ of Western honeybee (*2*), several similar other Am-royalisin sequences from the Western honeybee in amino acid and nucleotide sequence have been reported (*12*, *13*). This is the first report on nucleotide sequence of Acc-royalisin (GenBank accession: EF660337) in RJ of the Chinese honeybee. Comparative analysis showed that the royalisin precursors from Western honeybee and Chinese honeybee shared 90−92% of identity in amino acid sequences. Recently, the antibacterial properties of these peptides have been used to explain differences in resistance to adverse environmental factors, including pathogens of honeybee species (*15*).

In this study, PPAR and MAR were expressed in *E. coli* using the pGEX expression system. Thin layer scanning on the SDS−PAGE profiles of GST-PPAR and GST-MAR showed that the two expressed fusion proteins accounted for 16.3% and 14.5% of total proteins in bacterial cells. The GST fusion expression system is an integrated system for expression, purification, and detection of fusion proteins produced in *E. coli*. Fusion proteins were purified from bacterial lysates. The recombinant proteins were confirmed by Western blot. The purified soluble recombinant proteins of GST-PPAR, GST-MAR and MAR showed inhibitory effects against Gram-positive bacterial strains. By thrombin cleavage and removing GST, the antibacterial

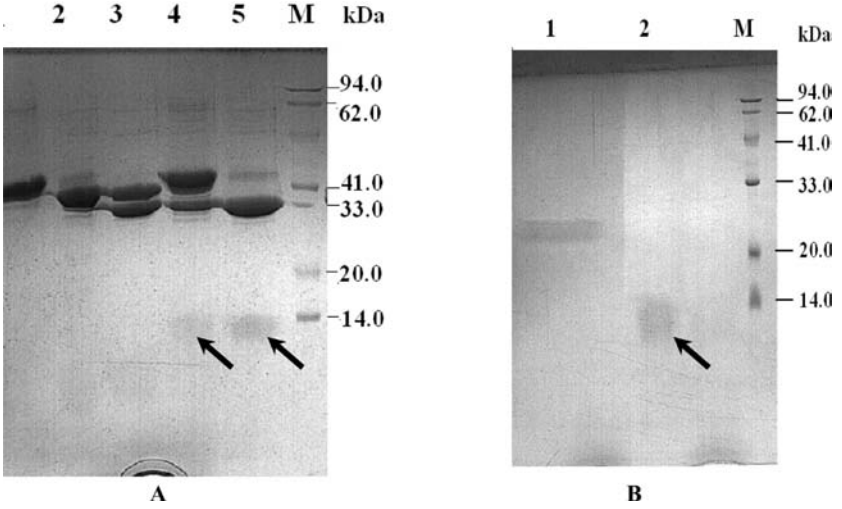

Figure 7. The SDS−PAGE pattern of thrombin cleavage of purified GST-MAR fusion protein and the affinity purification of thrombin-cleaved products. (**A**) The SDS−PAGE pattern of thrombin cleavage of purified GST-MAR fusion protein. Lane 1, purified GST-MAR fusion protein before thrombin cleavage thrombin-cleaved treatment. Lanes 2 and 3, during thrombin cleavage of purified GST-MAR protein. Lanes 4 and 5, the products of thrombin cleavage (the MAR are indicated by the black arrows). (**B**) The SDS−PAGE pattern of the affinity purification of thrombin-cleaved GST-MAR proteins. Lane 1, purified GST fraction from the thrombin-cleaved GST-MAR protein. Lane 2, the purified MAR fraction from the thrombin-cleaved GST-MAR protein (the MAR are indicated by the black arrow). Lane M, protein marker.

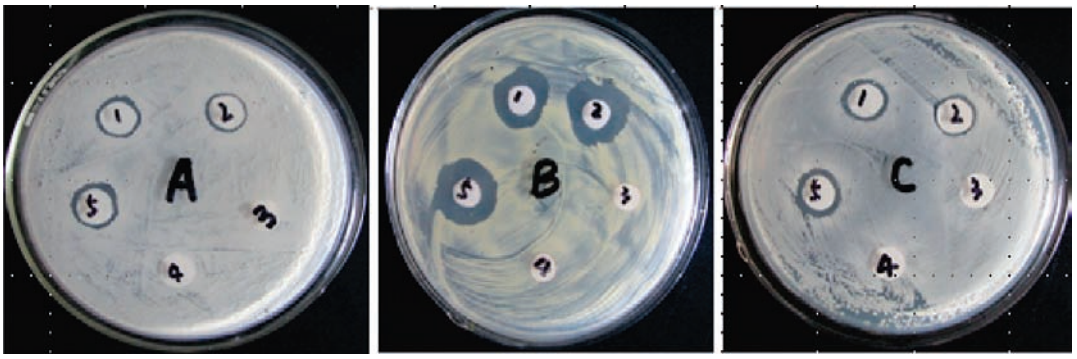

Figure 8. Antibacterial diffusion test on the agar plates for the purified fusion proteins of GST-PPAR and GST-MAR. (**A**) *B. subitillis*; (**B**) *M. luteus*; (**C**) *S. aureus*. Positions 1, 2, 3, 4, and 5 at plates **A**, **B**, **C** represented GST-PPAR, GST-MAR, GST, Tris-HCl and nisin, respectively. Tested protein concentrations of GST-PPAR, GST-MAR, GST and nisin are 2 mg/mL.

Table 1. Comparison of Antimicrobial Effects of GST-PPAR and GST-MAR on Three Gram-Positive Bacterial Strains ($n = 3$)[a]

	inhibition zone diameters (mm)		
treatment (2 mg/mL)	*S. aureus*	*B. subtilis*	*M. luteus*
GST-PPAR	11.53 ± 0.74 a	10.53 ± 0.32 ab	15.07 ± 0.97 b
GST-MAR	10.50 ± 0.20 b	9.83 ± 0.55 b	16.70 ± 0.52 a
nisin	12.30 ± 0.20 a	11.50 ± 0.79 a	17.16 ± 0.35 a

[a] GST-PPAR and GST-MAR represent GST-pre-pro-Acc-royalisin and GST-mature Acc-royalisin, respectively. After treatment, inhibition zone diameters were measured. Data represented as means ± SD of three independent experiments. Values within the columns with different letters were significant at p-value < 0.05 based on Duncan's multiple range test.

activity of MAR was raised, and MAR has stronger inhibition activity than that of GST-MAR fusion protein against Gram-positive bacteria. This was the first report of the royalisin gene from RJ expressed with molecular biological methodology, and the demonstration of antibacterial activity of recombinant royalisin protein. As the yield of lysate GST-MAR fusion protein was higher than that of GST-PPAR in *E. coli*, expression of the former in *E. coli* will be suitable for practical applications.

The beekeeping industry employing the Chinese honeybee, one subspecies of Asiatic honeybee, *A. cerana*, has a long, over 3000-year history in China (*16*). The industry has become an important source of income for Chinese farmers living on mountains at high altitude, such as in the Himalayan region. The total number of Chinese honeybee colonies kept by Chinese farmers is about 1.2 million or about 15% of the Western honeybee. The native species had been the only one breeding honeybee in China before the Western honeybee was introduced at the beginning of the 20th century. It plays an important role in ecology for the formation of the unique botanical layer through pollination. Although the Chinese and Western honeybees share many similarities, the former has unique biological characteristics and behavior. Importantly, it can tolerate the mite *Varroa destructor* and the microsporidan, *Nosema ceranae*, both of which are destructive to the Western honeybee. Therefore, the genomic resource of the Chinese honeybee will be valuable for breeding research concerning global apiculture (*1*, *15*, *17*). Four antimicrobial peptide families, abaecin, defensin, apidaecin and hymenoptaecin, are found in honeybees. Comparing four antimicrobial peptide gene families from the Chinese honeybee against *E. coli* with those from the Western honeybee reveals that there are more hymenoptaecin peptides in the Chinese honeybee (13 versus 1). Thus, Chinese honeybee may be under greater selective pressure from pathogens and parasites resulting in greater positive and diverse selection on antimicrobial peptides than the Western honeybee (*15*). Analysis of acidic extracts of honeybee heads, thoraxes, and RJs using a bacterial growth inhibition assay showed that royalisin was the peptide responsible for activity against the pathogens American foulbrood *Paenibacillus larvae larvae* and other tested Gram-positive bacteria. The analysis of RJs collected from individual colonies at two apiaries revealed differences in the content of the antibacterial peptide, suggesting that the differences might be associated with genetic variability between colonies (*10*). Two defensin genes, defensin-1 and defensin-2, have been identified from the thoracic salivary glands infected by bacteria. Defensin-1 is found in head tissue, but defensin-2 is not. Defensin-1 from head and thorax belongs to royalisin (*13*). Therefore, royalisin is valuable both for the prevention of honeybee diseases and RJ preservation. However, recombinant expression and the characterization of the corresponding products of the Am-royalisin and Acc-royalisin have not been investigated.

Because food safety has become an increasingly important international concern, the application of antimicrobial peptides from microorganisms, animals and plants that target food pathogens without toxic, adverse effects has received great attention. The increasing consumption of food formulated with chemical preservatives has been a critical issue in public health, demanding more "natural" and "minimally processed" food. As a result, there has been a great interest in naturally produced antimicrobial agents, especially antimicrobial peptides. Due to the low abundance of antimicrobial peptides in natural organism's cells or tissues, direct isolation is not feasible for industry production. On the other hand, chemical synthesis of the peptides is also unacceptable because of the complicated techniques applied and high cost involved.

Royalisin is an insect defensin and a food-borne antibacterial peptide from RJ, which has biological characters similar to nisin, which has been widely used as a food preservative in the food industry against Gram-positive bacteria *S. aureus, Listeria monocytogenes* and *Clostridium botulinum* associated with food-borne illnesses, as well as an alternate antibiotic for human or veterinary therapy to reduce resistance (*18*−*22*). However, the underlying molecular mechanism is unclear. The cell wall consists primarily of multiple layers of peptidoglycan with teichoic acid polymers dispersed throughout. The acidic character of the peptidoglycan cell naturally binds the highly positively charged antibacterial peptide. The mechanism of defensin A, a representative of insect defensins from the midge *Chironomus plumosus*, has been illustrated. When bound to the peptidoglycan layer of Gram-positive strains, defensin A disrupts the permeability barrier of the cytoplasmic membrane, resulting in the loss of cytoplasmic potassium, a partial depolarization of the inner

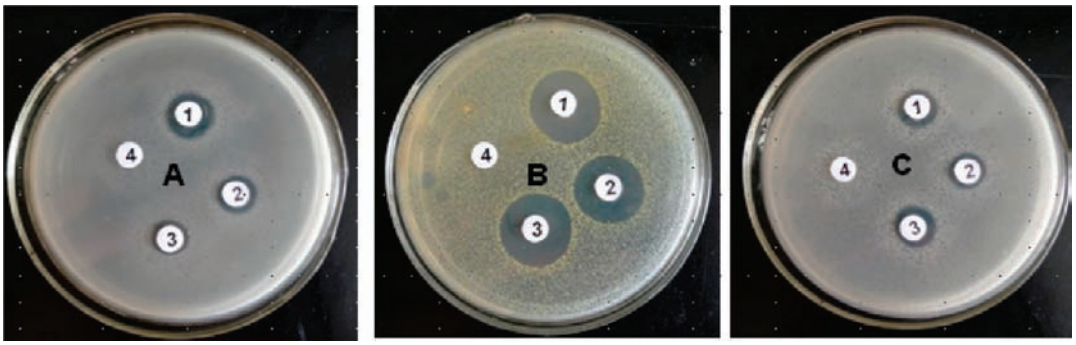

Figure 9. Antibacterial diffusion test on agar plates using purified MAR and GST-MAR. Fusion protein: (**A**) *B. subitillis*; (**B**) *M. luteus*; (**C**) *S. aureus*. Positions 1, 2, 3, and 4 of each plate represent nisin, GST-MAR, MAR and Tris-HCl, respectively. Tested protein concentrations of nisin, GST-MAR, MAR and GST are 0.5 mg/mL, 3.0 mg/mL and 2.0 mg/mL, respectively.

Table 2. Comparison of Antimicrobial Effects of MAR and GST-MAR Fusion Protein on Three Gram-Positive Bacterial Strains ($n = 3$)[a]

		inhibition zone diameters (mm)		
treatment	concn (mg/mL)	*S. aureus*	*B. subtilis*	*M. luteus*
GST-MAR	3.0	10.87 ± 0.67 b	11.37 ± 0.21 a	17.13 ± 0.20 b
MAR	2.0	10.83 ± 0.45 b	10.50 ± 0.27 b	17.10 ± 0.20 b
nisin	0.5	11.73 ± 0.60 a	11.86 ± 0.25 a	17.23 ± 0.31 a

[a] GST-MAR and MAR represented GST-mature Acc-royalisin fusion protein and purified mature Acc-royalisin released from thrombin-cleaved GST-MAR protein, respectively. After treatment, inhibition zone diameters were measured. Data are represented as means ± SD of three independent experiments. Values within the columns with different letters were significant at p-value <0.05 based on Duncan's multiple range test.

membrane, a decrease of cytoplasmic adenosine triphate (ATP) and inhibition of respiration (*23*).

As proteomic approaches have been used widely to predict the structures and functions of proteins and peptides, the biochemical composition and functions of RJ will soon be described in detail (*25*). Our results provide fundamental knowledge for further studies on the genetic engineering of RJ royalisin and its application in food and pharmaceutical industries as an antibacterial agent.

ABBREVIATIONS USED

RJ, Royal Jelly; 10-HDA, 10-hydroxyl-2-decenoic acid ; EST, expressed sequence tag; PPAR; pre-pro-Acc-royalisin, MAR; mature Acc-royalisin; EB, ethidium bromide; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PBS, phosphate buffered saline; 10 mM Tris-HCl (pH 8.0), Tris-HCl; PCR, polymerase chain reaction.

ACKNOWLEDGMENT

We are grateful to Dr. Laurence D. Parnell, Chao-Qiang Lai, Songbai Liu and Grace Harriman for editing the manuscript, and Dr. Weiliang Chen and Hui Zhang in Zhejiang University for their technical assistance.

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Received for review July 24, 2009. Revised manuscript received January 15, 2010. Accepted January 15, 2010. This project was supported by the grants from The Ministry of Science and Technology of China (No.2007AA10Z324).