Original Article

Sequence analysis of functional *Apisimin-2* cDNA from royal jelly of Chinese honeybee and its expression in *Escherichia coli*

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Apisimin is one of the functional peptides from royal jelly. The aim of this study was to analyze and *in vitro* express a new gene encoding Acc-apisimin-2 from Chinese honeybee (*Apis cerana cerana*) in *Escherichia coli*. Ninety-six clones containing apisimin expressed sequence tag (EST) were identified from 8568 effective ESTs of the cDNA library of Chinese honeybee worker heads. The coding region of the matured peptide from one clone containing Acc-apisimin-2 gene was sub-cloned into the prokaryotic expression vector pGEX-4T-2. The recombinant vector then was transformed into *E. coli* BL21 (DE3) for expression. The expression product was analyzed with SDS-PAGE and Western blot. The total length of the Acc-apisimin-2 cDNA was 379 bp, containing an open-reading frame (ORF) of 237 nucleotides encoding a 78 amino acid residue precursor. The Acc-apisimin-2 gene shared 100% homologies with Am-apisimin from *A. mellifera*, but 93% and 91% homologies with Aci-apisimin from *A. cerana indica* and the previously reported Acc-apisimin-1 sequence (AY278991) on a nucleotide level, respectively. The GST-Acc-apisimin-2 fusion protein expressed in the recombinant vector was about 31 kDa in size and accumulated up to about 22.1% of the total bacterial proteins. About 50% of the recombinant protein was soluble. The fusion protein purified through affinity chromatography was cross reactive with GST antibody, which confirmed the successful expression of GST-Acc-apisimin-2.

Key Words: ***Apis cerana cerana,*** **royal jelly, Acc-apisimin-2, sequence analysis, prokaryotic expression**

Introduction

Royal jelly (RJ), a principal food of the honeybee queen and young female larvae, is secreted from the hypopharyngeal and mandibular glands of 5-15 days old honeybee nurse. It has been reported that RJ has several nutritional functions and pharmacological activities, including vasodilative and hypotensive activities, increasing the growth rate of chick embryos, antitumo activitiy, anti-inflammatory activity, antihypertensive activity, antihypercholesterolemic activities, and ability to increase the average life span of mice[1]. Chemical composition analysis has shown that RJ of European honeybee, *Apis mellifera* consists mainly of proteins (12-15%) which constitute about 50% of its dry mass, carbohydrates (10-16%), lipids (3-6%), vitamins and free amino acids, together with a large number of bioactive substance such as 10-hydroxyl-2-decenoic acid, antibacterial protein, stimulating factor for the development of genital organs in male mice, etc.[2] Therefore, RJ has been widely used in commercial products, health foods and cosmetics in many countries.

Major RJ proteins (MRJPs) belong to a larger family and are produced in the hypopharyngeal gland, of which eight members with 49-87 kDa have been identified and represent 82-90% of the total proteins in RJ of the European honeybee.[3-5] The MRJP1, MRJP2, MRJP3 and MRJP5 represent about 82% of total protein content of RJ. They play a role as a source of essential amino acids and nitrogen for the nutrition of honeybee young female larvae and queen.[5] MRJP3 has the potent immunoregulatory effects *in vitro and vivo* and potent antiallergic activity.[6] MRJP1 which exhibited a molecular mass of 57-kDa as a monomer is the most abundant protein representing about 48% of RJ water-soluble proteins, can significantly stimulate rat hepatocyte DNA synthesis and promote live regeneration. The protein has certain structural properties causing its self oligomerization in RJ and formation of stabile complex with apisimin. Thus MRJP1 has been considered as a potential ingredient of functional foods and its cDNA was transformed into tobacco plant for expression.[6-9]

The full sequence of Am-apisimin cDNA was isolated and characterized from a cDNA library established from heads of European honeybee nurse. Am-apisimin is a small peptide in RJ with a molecular mass of 5.54 kDa, which may play a physiological role in honeybee colonies because a relatively high expression level of its small mRNA was observed in the heads of nurse and forager during the wholelife span of honeybee. [9]

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It seems that pure natural apisimin separated from RJ seems to bind strongly to MRJP1 and forms stable complex as monomer and as a variety of oligomers. The different forms of apisimin resemble oligomers of the antimicrobial peptides, magainin and dermaseptin isolated from frog skin.[9] It has been found that the major RJ proteins, MRJP1 could stimulate mouse macrophages to release TNF-R, which demonstrates that the protein could be used for its biological valuation.[10] Therefore, apisimin maybe an important physiologically active synergic ingredient. However, the gene has not been expressed *in vivo* through gene engineering.

In China, an alternative honeybee species, named Chinese honeybee, *A. cerana cerana*, a subspecies of Asian honeybee, *A. cerana* is indigenously and widely used for commercial beekeeping, primarily owing to its unique biological and behavioral characters, e.g. it has strong resistance to the bee mite, *Varroa destructor* which is the most significant pest damaging European honeybee[11], tolerance to the high and low temperatures, and ingenious olfactory ability to easily find low shrub and herbs which are usually distributed dispersedly and flowering scattered.[12-13] Queen-rearing experiments supported the differences in RJ from European honeybee and another Asian honeybee subspecies, Indian honeybee, *A. cerana indica* because the latter queens could not be successfully reared with *A. mellifera* RJ and vice versa.[14-15] The Aci-apisimin gene was obtained from the cDNA library of hypopharyngeal glands of India honeybee nurse bees. The sequence of the Aci-apisimin showed a 92.7% and 94.9% similarity with the nucleotide and amino acid sequences of Am-apisimin, respectively, which showed certain nutritional differences between two RJ.[16] One previously reported Acc-apisimin-1 sequence of Chinese honeybee was deposited in GenBank (accession number AY278991) in 2003 by Su, one of our colleagues.[17] However, we found a new Acc-apisimin sequence which is different from the two previously reported apisimin sequences of Asian honeybee.

The aim of the present work was to isolate and *in vitro* express the gene encoding Acc-apisimin-2 from Chinese honeybee in *Escherichia coli.* In addition, the gene sequence and its molecular structure of the deduced amino acid sequence were analyzed.

Material and methods

Honeybees

The colonies of Chinese bee were maintained in the field at Bee Research Facility, Zhejiang University. The colony was derived from a naturally mated queen which was developed from a wild field colony in Chunan County. Approximately 2000 adult workers were collected when they were 1, 3, 4, 5, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days old. To obtain bees of known age, 300 to 500 one-day-old bees every day were marked with various spot of paint on the thorax and then returned to their natal colony. Collections were made late in the afternoon. Bees were collected directly into liquid nitrogen to minimize the possible effects of collection on gene expression. Heads were dissected out, immediately placed in liquid nitrogen and stored at -80℃ until needed as describe by Whitfield et al (2002).[18]

Head cDNA Library construction

Total RNA (ca 1000 μg) was extracted from 130 worker heads collected 10 ones from every sample as above, respectively, with TRIzol (Invitrogen), and phenol etc., as described by Sambrook *et al.*[19] Poly (A+) RNA was purified by biotinylated oligo (dT) primer (Promega), followed by reverse transcription and first strand synthesis, the termini of cDNA was Blunted and added by *Eco*R I adapter (5'-*Eco*R I-GGCACGAGG-3'), after *Eco*R I ends phosphorylating and digested by *Xho* I (5'-*Xho* I-CTAGAG-3') all of length exceeding 500 bp cDNA inserts were cloned into and pBluescript® II XR plasmid vector and electroporate into DH10B *E. coli* strain after recollecting through fraction grading.

DNA sequencing and data analysis

Plasmid DNA was extracted from cDNA clones selected randomly, and sequencing was done in 96-well using MegaBACE 1000 sequencers from 5'-end in Waston Institute of Genome Science, Zhejiang University. Expression sequence tags (ESTs) were assembled using CAP3 and PHRAP by sequence comparison with GenBank Nt Database, GenBank Nr Database, SWISSPROT Database by Blast.[17] The clones in the cDNA Library containing apisimin gene were searched through the EST database of Chinese honeybee worker head by using the reported Am-apisimin (AY055108) in GenBank.[9] The collected clones were sequenced by Shanghai Sangon Biotechnology Corporation further. The homologous sequences of the result were blasted against data in the GenBank, and analyzed with CLUSTAL1.8 and GeneDoc. The putative amino acid sequence was analyzed with Signal P[20] and Motif-Scan programs.[17]

Construction of prokaryotic expression vectors and expression of Acc-apisimin in E. coli

Amplification of the Acc-apicimin-2 gene from the cDNA clone of Chinese honeybee worker head was performed using forward primer (5'-GGATCCATGAAAACATCA ATCAGTGTC-3') and reversed primer (5'-CTCGAGTTAAGCGAAAACATTAGCG-3') which containing the restriction sites of *Bam*H Ⅰ and *Xho* Ⅰ. Amplification was performed in a programmable thermal controller (Thermo-Hybaid, Hybaid Limited, UK) with regular PCR procedure[18]. PCR products were visualized on a 2.5% ethidium bromide stained gel. The PCR products were subcloned into the pMD-18T easy vector t form the recombinant vector pMD-Acc-apisimin-2T. Acc-apisimin-2T fragments were excised and removed from the pMD-Acc-apisimin-2T using *Bam*H I and *Xho* I and inserted into the GST fusion expression vector pGEX-4T-2. BL21 cells were transformed with the recombinant expression vector pGEX/Acc-apisimin-2. Protein expression was induced by the addition of 0.5 mM IPTG with further growth at 27℃ for 10 h. Cells were harvested by centrifugation (10 min, 4000 g) and broken up using ultrasonic, then were centrifugated again, the cells and cell culture supernatant samples were examined by Coomassie brilliant blue R250-stained SDS–PAGE gels to determine the soluble properties of the recombinant protein. The content of the expressed protein was measured through

scanning the profile of SDS-PAGE gel by using Gel Doc EQ imaging system (Bio-Rad Laboratories.U.S.A)

Purification of GST-Acc-apisimin-2 expressed by E. coli and its cleavage

After the recombinant protein was purified by using the Glutathione Sepharose 4B, the GST- fusion protein was cleaved by thrombin protease in 1×PBS at 37℃ for 10 h according to the method of the MicroSpin GST purification module.

SDS-PAGE and Western blot analysis

Purified GST-Acc-apisimin-2 was analyzed in SDS-PAGE. The electrophoresed proteins were transferred to a PVDF membrane for 15 min at 0.2 A in a Bio-Rad trans-blot apparatus. The membrane was incubated with rabbit anti-GST antibodies, and the antibody–antigen complex was detected with secondary antibodies, goat anti-rabbit IgG (H+L), conjugated with alkaline phosphatase, according to manufacturer's instruction. The PVDF membrane was photographed after it was dried.

Results

EST analysis on cDNA library of the worker heads

Analysis results for the obtained sequences from the cDNA library of the honeybee worker heads showed there were 8568 effective ESTs, of which 96 ESTs contained the apisimin fragment and accounted for 1.12% in the whole ESTs. It revealed that the mRNA transcriptional level of apisimin in Chinese honeybee head was very high.

Sequence analysis of Acc-apisimin cDNA

Three clones containing apisimin EST were searched with the known 5'-end Am-apisimin-2 fragment of about 50 bp, and were further sequenced. Sequencing results showed that three clones all contained the complement gene of 379 bp in full length, which contains an open-reading frame (ORF) encoding 78 amino acids, with a predicted molecular weight of 8.6 kDa (Fig 1). Sequence analysis results using CLUSTAL X 1.8 and GeneDoc revealed that the Acc-apisimin-2 gene encoding a mature peptide shared 100% homologies with Am-apisimin, but 91% and 93% homologies with Aci-apisimin and the previously deposited Acc-apisimin-1 sequence (AY278991) in nucleotide level, respectively. Valine and Serine, which accounts for 21.8% and 15.4% of the amino acid sequence of Acc-apisimin-2, respectively, are rich in the matured peptide. Analysis using Signal P showed that the precursor contains a putative signal peptide which is composed of 24 residues in the N-end, and the putative signal peptidase cleavage site was between Ala_{24} and Lys_{25}. The predicted molecular weight of the matured peptide is 5.9 kDa. One potential protein kinase C phosphorylation site was found at Ser_{29} through analysis by using Motif Scan programs.

Amplification of Acc-apisimin-2 cDNA and construction of pMD/Acc-apisimin-2

About 200 bp cDNA fragment encoding matured peptide of Acc-apisimin-2 was amplified by PCR with one of previously sequenced cDNA clone, rndcb_012678.y1.scf, as a template (Fig 1). The PCR product was purified and ligated into the pMD-18T easy vector. The pMD-18T with the insertion of Acc-apisimin gene was identified by

Figure 1. Nucleotide sequence and deduced amino acid sequence of Acc-apisimin-2. The signal peptide sequence is underlined. The putative protein kinase C phosphorylation site was boxed. The start codon, stop codon and polyadenylation signal sequences are indicated by bold letters, respectively.

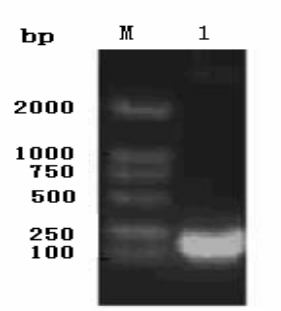

Figure 2. Acc-apisimin-2 fragment amplified from the selected clone

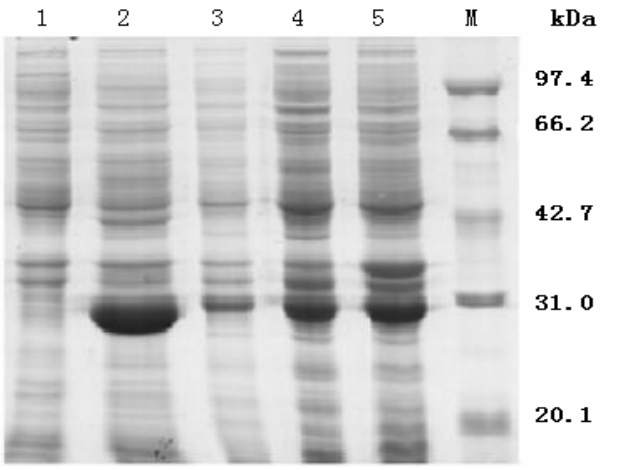

Figure 3. The SDS-PAGE pattern of the expression product. Lane 1: BL21 cell proteins with no plasmid transformed; 2: proteins from BL21 transformed with pGEX-4T-2 plasmid; 3: bacterial proteins from BL21 cells transformed with pGEX/Acc-apisimin-2; 4: expressed soluble GST-Acc-apisimin-2 fusion protein; 5: expressed insoluble GST-Acc-apisimin-2 fusion protein; M: protein marker

PCR and digestion with *Bam*HⅠand *Xho*Ⅰ, respectively, then further sequenced.

Prokaryotic expression

The Acc-apisimin-2 gene cleaved from the pMD/Acc-apisimin-2 using *Bam*HⅠand *Xho*Ⅰ was inserted into vector pGEX-4T-2. The recombinant expression vector with the insertion of Acc-apisimin-2 was identified by

PCR and digestion with *Bam*HⅠand *Xho*Ⅰ. The pGEX/Acc-apisimin-2 was then transformed into *E. coli* BL21 (DE3) for expression. The SDS-PAGE analysis results showed that the expression products contained a band of fusion protein of about 31 kDa (Fig 3) which was identical to the predicted molecular weight of the recombinant protein composed of GST (25 kDa) and Acc-apisimin-2 (5.9 kDa), and half of the expressed fusion protein was soluble (Fig 3). The scanning result of SDS-PAGE gel profile showed that the expressed fusion protein accumulated up to about 22.1% of total protein of bacterial cells.

Western blot analysis with GST-antibodies as the first antibody showed that the expressed fusion protein was recognized by the GST-antibody (Fig 4), which confirmed that this product of expression was the expected GST-Acc-apisimin-2 fusion protein.

Purification of the recombinant protein and its cleavage

Recombinant Acc-apisimin-2 was expressed in *E. coli* as a fusion protein containing GST for affinity purification. The purified GST-Acc-apisimn-2 fusion protein achieved up to 90% purity in a single step from the soluble portion of the total proteins using affinity chromatography of Glutathione Sepharose 4B. The results are shown in Figure 4 after GST-Acc-apisimin-2 fusion protein has been cleaved by thrombin protease at 37℃ for more than 10 h. The purified GST band could be seen clearly on SDS–PAGE profiles. But Acc-apisimin-2 band could not be shown because its molecular weight is only 5.9 kDa which is difficult to be detected with this method.

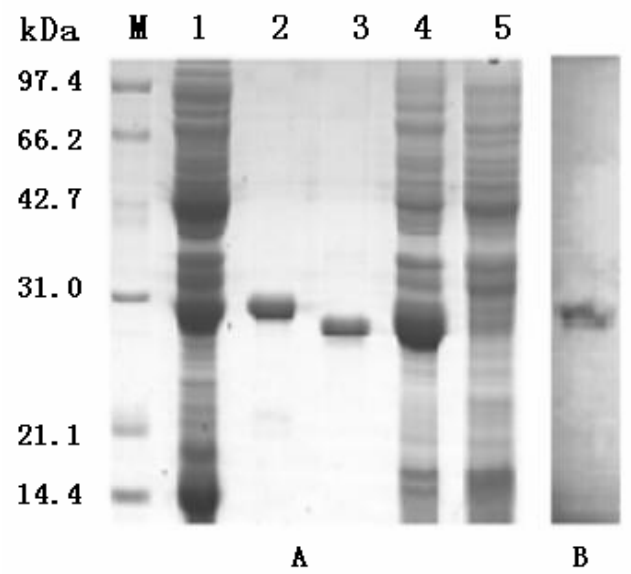

Figure 4. The SDS-PAGE pattern of expression product, purified protein and Western blot analysis. A. Lane M: protein marker; 1: expressed soluble GST-Acc-apisimin-2 fusion protein; 2: the purified GST-Acc-apisimin-2; 3: the purified thrombin-cleaved GST-Acc- apisimin-2; 4: proteins from BL21 transformed with pGEX-4T-2 plasmid ; 5: bacterial proteins from BL21 cells ; B. Western blot analysis using anti-GST antibody

Discussion

Statistics of ESTs revealed that apisimin gene was richly expressed in the Chinese honeybee head. The analysis result was based on our ongoing project, analysis and functional annotation of a ESTs collection from the brain of the Chinese honeybee. Another project conducted by Robinson's team on European honeybee in America was completed and obtained 15311 high-quality ESTs. They have annotated the ESTs on their homology to the well-characterized *Drosophilia* gene set. This approach led to the identification of honeybee orthologues[18]. These ESTs has been employed on DNA microarrays to study the messenger RNA changes associated with worker behavior of nurse and labor.[21] As an important work, MRJPs and peptides, enzymes in RJ should be analyzed and expressed in *E. coli* and insect cells, because these components have high nutritional value and biological activities, and potential economic value for the beekeeping industry which has a very important status in China. In recent 10 years, China has about 300 million colonies of honeybee every year, and has produced the most output of honey and RJ and has been the largest beekeeping country in the world.[12-13] It has shown that the EST data of Chinese honeybee head will offer valuable information for the research on RJ and behavior of honeybee.

The sequence analysis showed that the Acc-apisimin-2 gene in this paper is consistent with Am-apisimin, but different from Aci-apisimin and the previously reported Acc-apisimin-1 in nucleotide and amino acid level, so it was considered to be a new apisimin gene. This result revealed an important information that the apisimin gene shows polymorphism which has been found in MRJPs families, e.g., MJP3, MRJP2 and MRJP5 of Chinese honeybee[22], and will be further studied.

The GST fusion expression system is an integrated system for the expression, purification, and detection of fusion proteins produced in *E. coli*. Fusion proteins can be easily purified from bacterial lysates. Cleavage of the desired protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning sites on the pGEX plasmid. Previous studies in our lab have shown it is a good system for expression and purification of the bee venom genes, e.g., melittin from venom gland of Chinese honeybee.[23] Our results also proved that the Acc-apisimin-2 gene could be highly expressed in *E. coli* using the system with the optimized expression condition of melittin gene.

With the research development of the structure and function of protein and peptide recently, the basic studies on RJ composition by proteomic approach have shown acceleration in the field as well. Our present results may offer an important basis for further study of the genetic engineering of RJ apisimin and its functions, especially on the MRJP1 oligomerization complex with apisimin. The possible physiological properties of recombinant Acc-apicimin-2 will be further characterized.

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