

Expression of the melittin gene of *Apis cerana cerana* in *Escherichia coli*[☆]

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Abstract

A cDNA encoding melittin in *Apis cerana cerana* was obtained by PCR from the recombinant plasmid and cloned into the GST fusion expression vector pGEX-4T-2 for expression of the protein. The expressed protein of about 29 kDa was detected by Western blot and triple antibody sandwich ELISA, indicating that the recombinant protein is the fusion protein of GST-AccM. The expression conditions of GST-AccM fusion protein for *Escherichia coli* BL21 transformant were optimized. Thin layer scanning on the SDS-PAGE profiles of GST-AccM showed that the expressed protein accumulated up to about 15.2% of total protein of bacterial cells under the optimized expression condition. Purified and recovered recombinant melittin of *A. cerana cerana* showed bioactivity in activating rabbit platelets to aggregate.

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Keywords: *Apis cerana cerana*; Melittin; Protein expression

Melittin is a major protein component of venom from the European honeybee, *Apis mellifera*, comprising 50% of its dry weight [2]. It has important biological activities and been used as a model peptide for studying the general features of the structures of membrane proteins and their interactions with membrane lipids [1,5]. The direct cytolytic/necrotic action of melittin has been reported in erythrocytes and mast cells, mouse fibroblast cells, skeletal muscle cells, rat thymocytes, and a human lymphoblastoid cell line [6]. Currently, purification from the bee venom and chemical synthesis of melittin are the only approaches to obtaining melittin. The purification approach is often complicated by the presence of phospholipase A₂ in the samples, whereas chemical synthesis of melittin is very expensive, which limits any extensive applications and scientific research of melittin [11]. Vlask et al. [9] reported

that prepromelittin cDNA of *A. mellifera* was cloned and melittin is located in the COOH-terminal of the polypeptide chain (residues 44–70) [9]. However, expression information of melittin gene from *Apis cerana cerana* was not available up to date. Previous study has obtained a cDNA encoding prepromelittin by RT-PCR from the total RNA of venom gland of workers of the Chinese honeybee, *A. cerana cerana*. The PCR products were then ligated into pGEM-T easy vector named as pGEM-AccPPM [8]. Here, we reported an efficient expression of a melittin from the Chinese honeybee as a glutathione *S*-transferase (GST) fusion protein in *E. coli*.

Materials and methods

Materials

Honeybee (*A. cerana cerana*) workers were obtained from the beekeeping farm of Zhejiang University. The bacterial strain, *Escherichia coli* TG1, BL21, the GST fusion expression vector, and the recombinant plasmid pGEM-AccPPM were kept in the Molecular Laboratory of our Institute.

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Restriction endonucleases, X-gal, IPTG, and DL-2000 markers came from TakaRa (Dalian, China). *Taq* polymerase, NBT/BCIP, nitrocellulose, TEMED, and ampicillin were purchased from Sagon (Shanghai, China). Goat anti-rabbit IgG-HRP, alkaline phosphatase rabbit anti-goat IgG(H+L), and alkaline phosphatase goat anti-rabbit IgG(H+L) were purchased from Sino-American Biotechnology (Shanghai, China). Standard natural melittin of *A. mellifera*, ovalbumin (OVA), and thrombin protease came from Sigma (Shanghai, China). pGEM-T easy vector was manufactured by Promega (Shanghai, China). PCR purification kit was from the Qiagen (Shanghai, China). TXB₂ RIA kit was purchased from Furui (Beijing, China), goat anti-GST antibody, the expression vector pGEX-4T-2, GST 96-well detection module, and MicroSpin GST purification module were from the Pharmacia (Shanghai, China).

Methods

Cloning of the melittin cDNA

Amplification of melittin cDNA from the recombinant plasmid pGEM-AccPPM was performed using the forward primer FP1-AccM (5'-CGTGGATCCGGAA TTGGAGCAGTTCTC) and the reverse primer RP2-pGEM (5'-AGTCTCGAGCGCCTTTGAGTGAGCT GA). FP1-AccM was designed from *A. cerana cerana* melittin cDNA [8] whereas the reverse primer RP2-pGEM was designed at the site of pGEM-T easy vector 413 bp downstream from the 3'-region of melittin gene of recombinant pGEM-AccPPM. Amplification was performed in a programmable thermal controller (Thermo-Hyaid, Hyaid Limited, UK) with one cycle of 94 °C for 3 min followed by 30 cycles of denaturation (60 s at 94 °C), annealing (60 s at 54 °C), and extension (90 s at 94 °C) and a final step of 72 °C for 10 min. PCR products were visualized on a 1.2% ethidium bromide stained gel. After purification with the QIAquick purification kit, these PCR products were subcloned into the pGEM-T easy vector, and plasmids pGEM-AccMT were obtained.

Construction of vectors

AccMT fragments were excised from the pGEM-AccMT using *Bam*HI and *Xho*I, followed by purification from the agarose gel and insertion into the GST expression vector pGEX-4T-2. A 413-bp fragment of pGEM-T easy vector in the plasmid pGEM-AccMT was excised and removed using *Hind*III and *Xho*I. The ends of the remained pGEX-AccMT were repaired by Klenow fragment and ligated by T₄ DNA ligase to form the final recombinant expression vector pGEX-AccM.

Expression of AccM in *E. coli*

BL21 cells were transformed with recombinant pGEX-AccM DNA. Protein expression was induced by the addition of 0.5 mM IPTG with further growth at

25 °C for 3 h. Cells were harvested by centrifugation (10 min, 4000g), and cells and cell culture supernatant samples were examined by Coomassie brilliant blue R₂₅₀-stained SDS-PAGE gels.

Immunoblot analysis

Western blotting

Vertical polyacrylamide (12%) slab gel containing 0.1% SDS was run as described by Sambrook et al [7]. Proteins were electrotransferred to two nitrocellulose membranes for 1 h at 0.2 A in a Bio-Rad transblot apparatus. One membrane was incubated with rabbit anti-melittin (*A. mellifera*) antibodies, and the antibody-antigen complex was detected with secondary antibodies, goat anti-rabbit IgG(H+L), conjugated with alkaline phosphatase. The other membrane was incubated with goat anti-GST serums and the antibody-antigen complex was detected with the secondary antibodies, rabbit anti-goat IgG, conjugated with alkaline phosphatase, according to manufacturer's instruction.

Trip antibody sandwich ELISA

After sonication, the expressed protein was pipetted into each well of a GST 96-well detection plate, which has been coated with goat anti-GST antibody to capture GST fusion proteins by the supplier (Pharmacia) in advance. The captured fusion protein was then incubated with the secondary antibody, the rabbit anti-melittin antiserum. At last the antibody-antigen-antibody complex was detected with a third antibody, the goat anti-rabbit IgG-HRP, using the method based on manufacturer's instruction.

Optimization of the expression conditions

Induction in different bacterial concentrations (*OD*₆₀₀)

Several colonies of *E. coli* transformed with the pGEX-AccM recombinants were picked and each was transferred into a separate tube containing 6 ml of 2 × YT medium. Liquid cultures were incubated to an *A*₆₀₀ of 0.20, 0.40, 0.60, 0.80, 1.0, 1.20, 1.40, and 1.60, respectively, with vigorous agitation at 37 °C. By adding IPTG (final concentration = 0.5 mM), each culture volume was continuously incubated for an additional 3 h. Ten microliters of each resuspended cell culture was used for SDS-PAGE analysis.

Optimization of temperature, IPTG concentration, and induction duration

When liquid culture was incubated to show an absorbance of 1.0 at 600 nm with vigorous agitation at 37 °C, the culture was divided into different tubes and IPTG was added to each subculture with different final concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 5.0 mM). All the subcultures were continued to incubate for an additional

3 h at 25°C for the optimization of IPTG concentration. For optimization of expression temperature, the divided tubes were continued to incubate for an additional 3 h at 20, 25, 29, 33, and 37°C, respectively, with a final 0.1 mM IPTG concentration. For optimization of induction duration, the cultures were incubated for an additional 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, and 8.0 h, respectively, with a final concentration of IPTG at 0.1 mM. Ten microliters of each resuspended cell culture was used for SDS-PAGE analysis. Each treatment was repeated at least two times.

Purification of GST-AccM expressed by *E. coli* and its cleavage

After the recombinant protein was purified by using the Glutathione Sepharose 4B, the GST-AccM fusion protein was cleaved by thrombin protease in 1× PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.3) at 22°C for 16 h according to the method of the MicroSpin GST purification module. The resultant melittin protein was then purified directly from the liquid using the affinity matrix Glutathione Sepharose 4B.

Assay of the melittin activity

The bioactivity of the heterologously expressed melittin was evaluated based on the method of activation of melittin from *A. mellifera* venom to rabbit platelets as reported by Yu and Xiong [13]. The experiment was carried out with three groups: (1) Control, 0.9 ml rabbit platelets + 0.1 ml of 0.9% NaCl; (2) melittin, 0.9 ml rabbit platelets + purified and recovered recombinant melittin + 0.1 ml of 0.9% NaCl; and (3) standard, 0.9 ml rabbit platelets + melittin of *A. mellifera* + 0.1 ml of 0.9% NaCl. Then the amount of thromboxane B₂, which was produced when rabbit platelets aggregated under activation of melittin, were detected.

Results

Amplification of melittin cDNA and its fusion protein expression in *E. coli*

A 507-bp melittin cDNA fragment containing partial pGEM-T easy vector sequence was amplified by PCR from a previously sequenced prepromelittin recombinant DNA pGEM-AccPPM of *A. cerana cerana* (Fig. 1A). The recombinant pGEX-AccM is the expression vector pGEX-4T-2 with the insertion of melittin gene from *A. cerana cerana* identified by PCR and sequencing (Fig. 1B). Schematic representation of the expression vector pGEX-AccM is shown in Fig. 2. *E. coli* transformed with the pGEX-AccM recombinants was incubated for 3 h after induction of 0.5 mM IPTG at 25°C. The expressed protein band of about 29 kDa, which was consistent with the expected molecular weight

of the fusion protein, GST-AccM, appeared on the SDS-PAGE profile (Fig. 3).

Western blotting and triple antibody sandwich ELISA

Cell fractions and cell culture supernatants were analysed by Western blotting using a rabbit anti-melittin

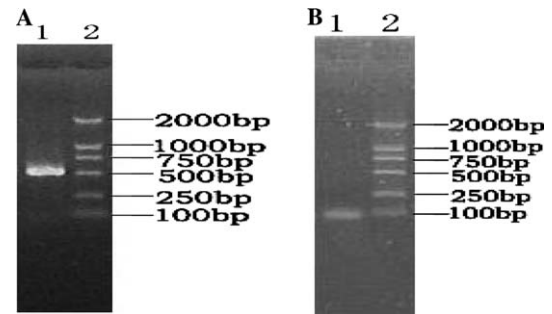


Fig. 1. PCR product of AccMT and AccM. (A) AccMT; (B) AccM. Lane 1, PCR product; lane 2, marker.

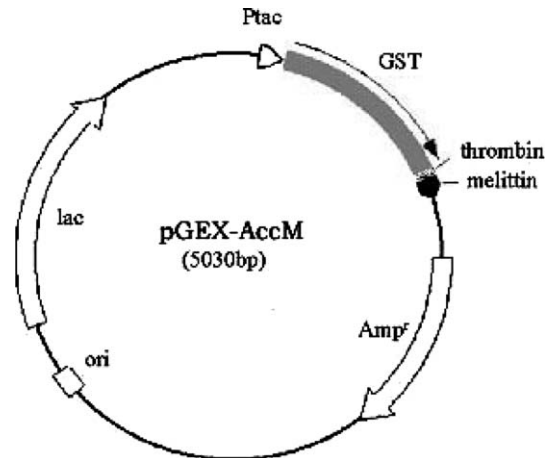


Fig. 2. Schematic representation of the expression vector pGEX-AccM.

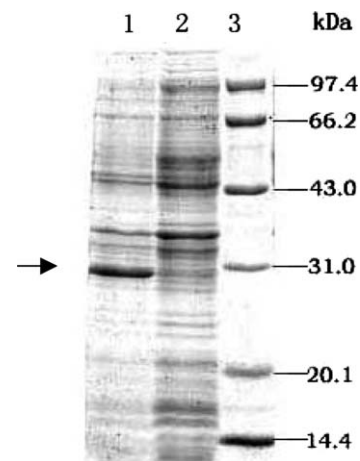


Fig. 3. SDS-PAGE profile of the expressed GST-AccM fusion protein in *E. coli*. Lane 1, BL21 cells transformed with recombinant pGEX-AccM; lane 2, BL21; and lane 3, marker.

antibody and goat anti-GST antibody, respectively. The protein was both recognized by these two antibodies (Fig. 4). It was proved that this product of expression was the expected fusion protein GST-AccM. After sonication, each sample was also identified by triple antibody sandwich ELISA (Table 1). The OD₄₉₀ of *E. coli* BL21 transformant of pGEX-AccM is two times more than that of the control *E. coli* BL21 using the anti-melittin binding OVA antibody and anti-GST antibody at $\leq 1:8000$ dilution. The result further confirmed that the expressed product was the GST-AccM fusion protein.

Induction in different bacterial concentrations (OD₆₀₀)

The high-expression was induced by IPTG addition to a final concentration of 0.5 mM at various stages of growth (OD₆₀₀: 0.20–1.61). The expression levels of the fusion protein at various phases of growth as detection by thin layer scanning on the SDS-PAGE profiles (Fig. 5) of GST-AccM are shown in Table 2. The result showed that as the OD₆₀₀ increased from 0.20 to 1.61, the expression level increased from 8 to 11% of the total bacterial proteins, the best OD₆₀₀ was about 1.00.

Induction in different concentrations of IPTG

The expression level of GST-AccM fusion protein from *E. coli* BL21 transformant in different concentrations of IPTG is shown in Table 3. The SDS-PAGE profile of the corresponding expression is shown in Fig. 6. Protein expression from a pGEX-AccM plasmid is under the control of the strong *tac* promoter. Even in the

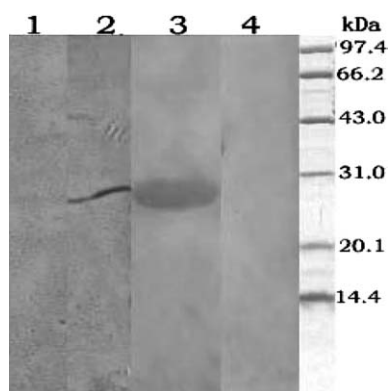


Fig. 4. Western blot analysis for GST-AccMelittin fusion protein. Lanes 1 and 4, BL21; lane 2, expressed product probed with the serum immunized with melittin binding OVA antigen; and lane 3 expressed product probed with anti-GST antibody.

Table 1

Data of expression products in *E. coli* by triple antibody sandwich ELISA

Dilution times of the anti-melittin antiserum	OD $\lambda = 490$ nm							
	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	1/128,000	1/256,000
PGEX-AccM	0.918	0.605	0.473	0.310	0.210	0.164	0.120	0.100
Check BL21	0.300	0.224	0.177	0.171	0.141	0.113	0.100	0.090

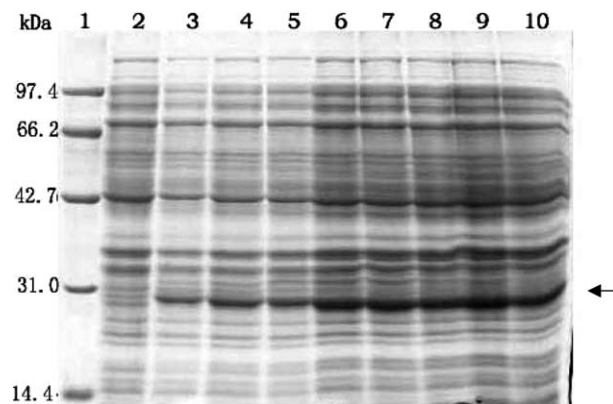


Fig. 5. SDS-PAGE analysis for expression of GST-AccM fusion protein from *E. coli* transformant in different concentrations of OD₆₀₀. Lane 1, marker; lane 2, BL21; lanes 3–10, bacterial proteins from BL21 transformed with pGEX-AccM induced with 0.5 mmol/L IPTG in different OD₆₀₀ from 0.20 to 1.61 (details for the lanes see Table 2).

absence of IPTG induction the pGEX-AccM plasmid was also strongly expressed. Thin layer scanning showed that the protein percentages decreased from 12 to 6% as the final concentrations of IPTG increased from 0.1 to 5.0 mM. And 0.1 mM IPTG final concentration was the best induction concentration in the experimental range.

Induction in different temperatures

The expression was induced by IPTG addition to a final concentration of 0.1 mM at various stages of growth temperature when the liquid cultures grew to an OD₆₀₀ of 1.0. The results of thin layer scanning are shown in Table 4. The corresponding SDS-PAGE profile is shown in Fig. 7. Among the five temperature

Table 2

Expression of GST-AccM fusion protein from *E. coli* BL21 transformant of pGEX-AccM in different bacterial concentrations (OD₆₀₀)

Lane	3	4	5	6	7	8	9	10
OD ₆₀₀	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.61
Expression level (%)	8	9	9	11	11	11	11	11

Table 3

Expression of GST-AccM fusion protein from *E. coli* BL21 transformant in different concentrations of IPTG

Lane	3	4	5	6	7	8	9	10
IPTG concentration (mM)	0	0.1	0.2	0.4	0.6	0.8	1.0	5.0
Expression level (%)	9	12	9	7	7	6	6	6

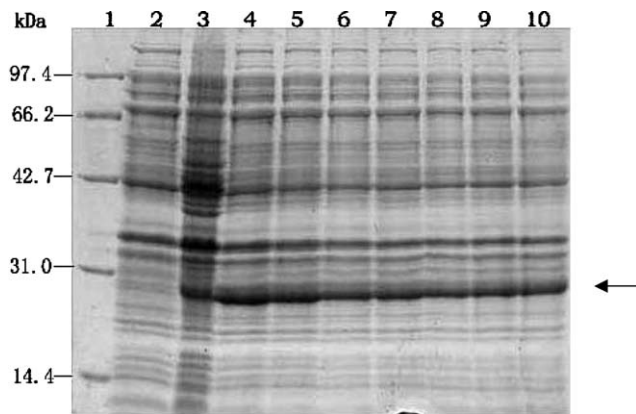


Fig. 6. SDS-PAGE analysis for expression of GST-AccM fusion protein from *E. coli* transformant in different concentrations of IPTG. Lane 1, marker; lane 2, BL21; and lanes 3–10, bacterial proteins from BL21 transformed with pGEX-AccM induced with IPTG from 0 to 5.0 mmol/L (details for the lanes see Table 3).

conditions ranged from 20 to 37 °C, the highest expression is at 20 °C.

Induction in different induction durations

Thin layer scanning of SDS-PAGE profile (Fig. 8; Table 5) showed the percentages of expressed product in the total bacterial protein were different in various induction durations. By adding IPTG to a final concen-

Table 4
Expression of GST-AccM fusion protein from *E. coli* BL21 transformant in different temperatures

Lane	3	4	5	6	7
Temperature (°C)	37	33	29	25	20
Expression level (%)	4	6	10	11	13

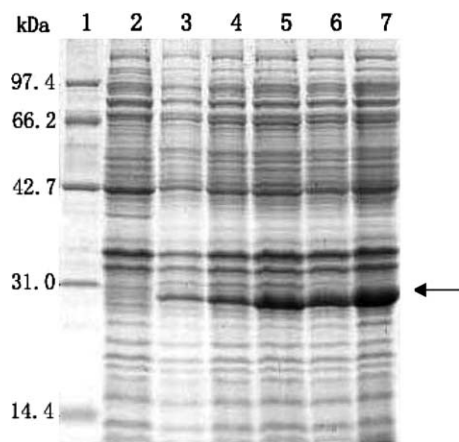


Fig. 7. SDS-PAGE analysis for expression of GST-AccM fusion protein from *E. coli* transformant in different temperatures. Lane 1, marker; lane 2, BL21; and lanes 3–7, bacterial proteins from BL21 transformed with pGEX-AccM induced with temperatures from 37 to 20 °C (details for the lanes see Table 4).

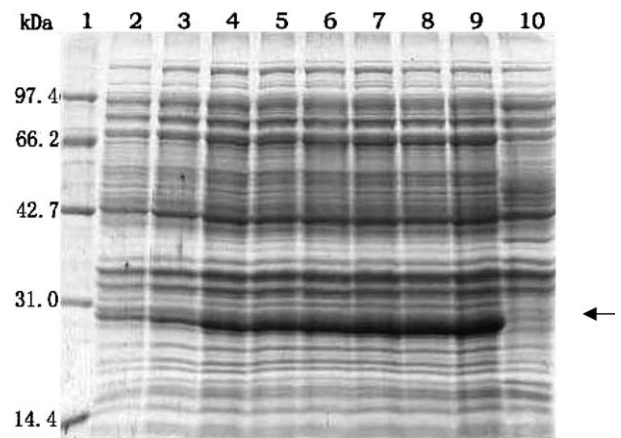


Fig. 8. SDS-PAGE analysis for the expression of GST-AccM fusion protein from *E. coli* transformant with different induction durations. Lane 1, marker; lanes 2–9, bacterial proteins from BL21 transformed with pGEX-AccM induced with durations from 0.5 to 8.0 h (details for the lanes see Table 5); and lane 10, BL21.

Table 5
Expression of GST-AccM fusion protein in *E. coli* BL21 transformant under different induction durations

Lane	2	3	4	5	6	7	8	9
Induction duration (h)	0.5	1.0	1.5	2.0	2.5	3.0	5.0	8.0
Expression level (%)	4	5	10	11	13	14	14	14

tration of 0.1 mM, the expression was the best for additional 3 h when the liquid culture grew to OD₆₀₀ of 1.0.

Expression under optimized conditions

Based on the above optimization experiments, it could be concluded that the best expression conditions for the fusion protein were when the culture was incubated to an A_{600} of 1.0, IPTG was added to a final concentration of 0.1 mM and then was continued to incubate for an additional 3 h at 20 °C. We used these optimized conditions to do an expression and the SDS-PAGE result is shown in Fig. 9. Thin layer scanning

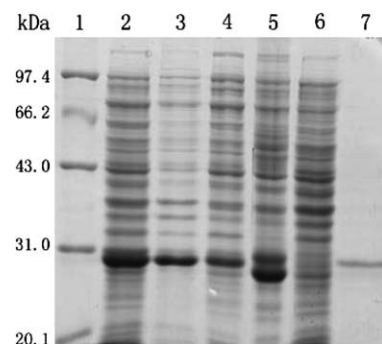


Fig. 9. SDS-PAGE analysis for expression of GST-AccM fusion protein from *E. coli* transformant in optimized conditions. Lane 1, marker; lane 2, total protein of bacterial cells with the expressed GST-AccM; lane 3, insoluble portion; lane 4, soluble portion; lane 5, BL21 with pGEX-4T-2; and lane 6, BL21.

showed that the expressed protein accumulated up to about 15.2%, highest up to 22.8%, of total protein of bacterial cells. About 40% of the expressed target protein was soluble.

Purification of the recombinant protein and its cleavage

Recombinant AccM was expressed in *E. coli* as a fusion protein containing GST for affinity purification. The purified GST-Acc fusion protein up to >90% of purity was achieved in a single step from the soluble portion of the total proteins using affinity chromatography of Glutathione Sepharose 4B. The results are shown in Fig. 10 after GST-AccM fusion protein has been cleaved by thrombin protease at 22 °C for more than 16 h.

Assay of bioactivity of the recovered recombinant melittin

After the cleavage of the recombinant fusion protein with thrombin protease, the GST was removed by affinity chromatography of Glutathione Sepharose 4B and the recombinant melittin was recovered. The recovered melittin was then used for the assay of bioactivity. Melittin bioactivity in activating aggregation of rabbit platelets was accompanied by the formation of thromboxane B₂. The results of formation of thromboxane B₂ of melittin activating rabbit platelets are shown in Table 6. The result

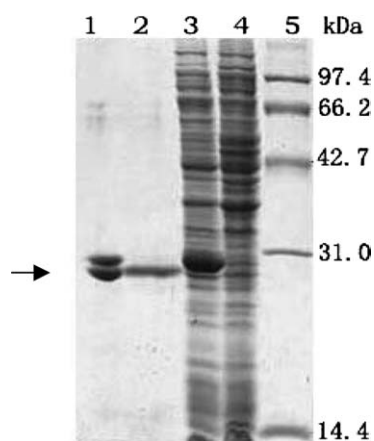


Fig. 10. SDS-PAGE analysis for proteolysis of purified GST-AccM protein. Lane 1, partial proteolysis of the purified GST-AccM protein; lane 2, complete proteolysis of the purified GST-AccM; lane 3, expression of pGEX-AccM from *E. coli* BL21 transformant; lane 4, BL21; and lane 5, marker.

Table 6
Formation of thromboxane B₂ of melittin activating rabbit platelets

	<i>A. cerana cerana</i> melittin (51 mg/L)	Standard melittin (51 mg/L)	Control
TXB ₂ (ng/L)	474 ± 2	431 ± 2	378 ± 2
TXB ₂ value with the control subtracted (ng/L)	96 ± 2	53 ± 2	

demonstrated that the recombinant melittin exhibited similar bioactivity as the natural melittin of *A. mellifera*.

Discussion

Melittin gene of the Chinese honeybee was highly expressed in *E. coli* using the pGEX expression system. The expression conditions of GST-AccM fusion protein for *E. coli* BL21 transformant were optimized, in different bacterial growth phases (OD₆₀₀), IPTG concentrations, temperature conditions, and induction durations. With full nutrition the optimized expression condition was obtained by the addition of IPTG of final concentration 0.1 mM at OD₆₀₀ of 1.0 and by allowing 3 h further cultivation at 20 °C. Thin layer scanning on the SDS-PAGE profiles of GST-AccM showed that the expressed melittin accounted for up to about 15.2% of total protein of bacterial cells under the optimized expression conditions. It is proved that the fusion protein was not wholly soluble. In fact, most of them (about 60%) are inclusion bodies. 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. Our result showed it is a good system for expression and purification of the melittin from *A. cerana cerana*.

Two cDNA fragments encoding prepromelittin were amplified by RT-PCR from the total RNA of venom gland of worker honeybees, *A. cerana cerana* and *A. mellifera*, respectively. Comparative analysis showed that the prepromelittins from two honeybee species shared more than 92% identities in amino acid sequences with each other, whereas their matured melittins, which are located in the COOH-terminal of the polypeptide chain (residues 44–70), shared 100% identities in amino acid sequences with each other [8]. So venom melittin of *A. mellifera* purchased from Sigma was considered as antigen. The expression product of melittin gene from *A. cerana cerana* was identified by its antiserum. The correct recombinant protein was confirmed by Western blotting and triple antibody sandwich ELISA.

Purified and recovered recombinant melittin of *A. cerana cerana* showed bioactivity in activating rabbit platelets to aggregate, accompanied by the formation of thromboxane B₂. 5-HT released during the aggregation would be helpful to aggregate the rabbit platelets. But the detailed mechanism of melittin activating rabbit platelets to aggregate is not yet clear.

Winder et al. [12] reported that expression constructs carrying melittin introduced into a human

bladder carcinoma-derived cell line and the resultant cell clones were analysed for tumorigenicity in nude mice to exhibit antitumour activity in tumour derived cell lines [12]. Wang et al. [10] reported that the cDNA encoding promelittin was obtained from the total RNA of the European honeybee (*A. mellifera*) poison gland by RT-PCR. Hydroxylamine cleavage site was arranged before the melittin sequence by site-directed mutagenesis. The expression vector containing the mutagenic promelittin sequence with partial sequence of β -galactosidase was constructed and the induced protein was expressed in *E. coli*. [10]. Wang et al. [11] also showed that the expression vector of promelittin fused with partial sequence of β -galactosidase was constructed by ligating the fragment inserted to pUC118 and it was expressed in the strain DH5 α of *E. coli*. Until now, however, purification of heterologously expressed melittin and recovery of recombinant melittin were not available [11]. Lazarev et al. [3] demonstrated inhibition of the infection caused by *Mycoplasma hominis* in cultured cells with controlled expression of the melittin gene incorporated in recombinant plasmid vectors. Lazarev et al. [4] also studied the effect of melittin, expressed within recombinant plasmid vectors, on the infection with urogenital pathogens *Chlamydia trachomatis* and *M. hominis* in HeLa cell culture and found the inhibition of *C. trachomatis* and *M. hominis* infection after the introduction of recombinant plasmid vectors expressing the melittin gene into the infected cell culture. Compared with the above works, the target fusion proteins were more highly expressed in *E. coli* in this study. It is very simple to purify the expression product and recover the recombinant melittin. As shown in this study, melittin can be purified up to >90% of purity in a single step while still preserves antigenicity and functionality of the protein.

Melittin is a cytolytic peptide extracted from bee venom [2]. Bees have highly specialized cells to keep themselves safe by melittin liberated from prepromelittin through several processing steps [9]. In this study, the melittin from *A. cerana cerana* is expressed at a high level in the GST fusion protein expression system. Our study may potentially lead to producing bee venom melittin through genetic engineering, which can be used for further study of its activities and applications on basic and clinical therapy and plant protection in agriculture.

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