Expression of Melittin in Fusion with GST in Escherichia coli and Its Purification as a Pure Peptide with Good Bacteriostatic Efficacy

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ABSTRACT: The expression and purification of melittin (MET) in microbials are difficult because of its antibacterial activities. In this work, MET was fused with a glutathione-S-transferase (GST) tag and expressed in Escherichia coli to overcome its lethality to host cells. The fusion protein GST-MET was highly expressed and then purified by glutathione sepharose high-performance affinity chromatography, digested with prescision protease, and further purified by Superdex Peptide 10/300 GL chromatography. Finally, 3.5 mg/L recombinant melittin (rMET) with a purity of >90% was obtained; its antibacterial activities against Gram-positive Bacillus pumilus and Staphylococcus pasteuri were similar to those of commercial MET. A circular dichroism spectroscopic assay showed that the rMET peptide secondary structure was similar to those of the commercial form. To our knowledge, this is the report of the preparation of active pure rMET without any tags. The successful expression and purification of rMET will enable large-scale, industrial biosynthesis of MET.

INTRODUCTION

Bioactive peptides are peptide compounds with low molecular weights and typically have significant biological activities. Melittin (MET) is an α-helical active peptide with an amphiphilic structure and has various physiological activities such as antibacterial, anti-inflammatory, antitumor, and antiviral activities. It is valuable in a wide range of applications in the pharmaceutical, healthcare, cosmetics, and other industries.

There are three methods for MET preparation: extraction from natural bee venom, chemical synthesis, and biological preparation. MET is derived from European bee (Apis mellifera) venom and constitutes only half of the bee venom. The extraction of MET is achieved at the expense of bees’ lives, which is not conducive to the continued development of bee colonies and beekeeping. Crude bee venom contains complex components with similar molecular weights, therefore MET isolation is challenging, cumbersome, and expensive. In particular, natural extraction results in significant contamination of MET with lipases, which can interfere with the membranolytic activity of the peptide. Chemical synthesis mainly uses amino acids as the raw materials and usually involves the synthesis of a polypeptide or protein by a solid-phase method. It has the disadvantages of causing serious pollution and giving a low target protein yield. Neither natural extraction nor chemical synthesis is an efficient method for large-scale preparation of MET.

There are many reports on the preparation of active peptides by biological methods, and such methods have considerable development prospects. However, the preparation of MET by biological methods still presents great challenges. MET is not easily expressed because of its high toxicity to cells and low molecular weight (only 26 amino acids, a molecular weight of about 2.9 kDa). Moreover, MET is susceptible to protease degradation in bacteria and therefore cannot be directly expressed in prokaryotic systems. More importantly, MET expression is not easy because of its high toxicity against a broad range of bacteria. Currently, MET is usually expressed in a fusion protein.

MET has been expressed by a fusion method. The fused MET sometimes has inclusion bodies or low-level expression. For example, the MET gene was directly linked to a soluble trimer of the tumor necrosis factor-related apoptosis-inducing ligand sTRAIL and expressed in Escherichia coli. This resulted in 90% of the inclusion bodies in the fusion protein sTRAIL-melittin.

Improvements in molecular biological techniques have enabled researchers to perform novel fusion expressions of MET. MET has been bound with other functional proteins to achieve the expression of a soluble form. Che et al. connected the active domain of cecropin A and MET to the hypersensitive (HR)-elicitor Hpa1 of rice leaf spot bacteria by rational design from scratch in E. coli. A novel binding protein, Hcm1, was purified. This acts as a potential insecticide by inducing disease resistance against viral, bacterial, and fungal pathogens. Shin et al. combined MET and gelonin genes to...
induce the expression of a MET-gelonin fusion protein in E. coli. They confirmed that the MET-gelonin toxin protein retained the equivalent intrinsic activity of MET and the inhibitory effect of unmodified gelonin on protein translation. Liu et al. linked MET and mutant human interleukin 2 (MhIL-2) on the vector pET-15b in E. coli and successfully expressed a new fusion protein MET-MhIL-2. The fusion protein showed the functional activities of IL-2 and MET and inhibited tumor growth in vivo. Su et al. fused MET with the amino-terminal fragment (ATF) of a urokinase-type plasminogen activator, which can target and recognize cancer cells. A pPICZαC-ATF-melittin eukaryotic expression vector was constructed and fusion proteins were expressed in Pichia pastoris, and an anticancer effect was detected in vitro after protein purification. Ishida et al. used calmodulin (CaM) as a fusion partner for MET and inserted a His tag at the N-terminus to induce protein expression in E. coli. The soluble fusion protein CaM-MET was obtained. After purification with a Ni column and subsequent digestion with TEV protease, 2.5 mg/mL of purified MET was collected by high-performance liquid chromatography (HPLC). However, they did not verify the antibacterial activity of the purified MET.

The glutathione-S-transferase (GST) tag is usually selected for fusion protein expression because it can help to protect against intracellular protease cleavage and stabilize recombinant proteins. Buhrman et al. achieved active peptide expression by constructing the plasmid pJB-HTS-MET to express GST and His-tagged MET in E. coli Rosetta cells. However, most of the MET was insoluble. Rayahin et al. produced MET in fusion with GST but did not cleave GST, which retained anti-inflammatory properties. Shi et al. further cleaved GST from MET using thrombin. The purified melittin with up to 90% of purity preserves the antigenicity and functionality of the protein. MET expression and purification from a fusion protein is still a challenge. To the best of our knowledge, there is no industrial biological method for preparing active MET. The preparation of soluble MET and improvement of its yield by biosynthetic methods would therefore have social and economic benefits.

In this work, we fused the MET peptide with a GST tag and achieved high heterologous expression of the fusion protein GST-MET in E. coli. More importantly, we purified the fusion protein GST-MET and obtained the active pure peptide MET with no tags. The antibacterial activities of recombinant MET (rMET) against E. coli, Bacillus pumilus, and Staphylococcus pasteuri were found to be almost the same. The successful expression, purification, and characterization of MET provides a novel approach to the biological preparation of MET and serves as a good example of active peptide biosynthesis. More importantly, the biosynthesis of MET will reduce the sacrifice of bee colonies that result from MET extraction from bee venom.

## RESULTS AND DISCUSSION

### Overexpression of Fusion Proteins GST-proMET and GST-MET

Promelittin (proMET) is a natural precursor of MET. It has a molecular weight of 7.6 kDa and contains a signal peptide, a leader peptide, and a mature peptide (2.9 kDa). In the biosynthesis by honeybees, the mature peptide is released after cleavage of the signal peptide and the leader peptide to form MET. Because MET is lethal to bacterial cells, it is usually expressed in a fusion protein by combining MET with another protein tag. The GST tag is widely used to promote the solubility and folding of recombinant proteins. In this work, we used the expression plasmid pGEX-6P-1 and GST fusion with the target peptide MET.

As described in the Materials and methods section, the two recombinant strains E. coli BL21/pGEX-proMET and E. coli BL21/pGEX-MET were obtained. Protein expression was optimized by the optimization of the temperature and isopropyl-β-D-thiogalactoside (IPTG) concentration. SDS-PAGE results (Figure 1A) showed that GST-proMET was expressed in the sediment, consistent with its theoretical molecular weight of 35 kDa. The protein GST-proMET was not observed in cell-free extracts of E. coli BL21/pGEX-proMET. These results suggest that GST-proMET is mainly expressed in the insoluble form. In contrast, GST-MET was expressed in the supernatant by induction with 0.1 mM IPTG at 25 °C; the band size was consistent with the theoretical molecular mass of 30 kDa (Figure 1B). A band at about 30 kDa was also observed in the sediment of E. coli BL21/pGEX-MET. These results show that the fusion protein GST-proMET could not be expressed, whereas GST-MET was partially expressed as a soluble protein with about 15% of the total soluble proteins (~20 mg/L culture). It was reported that the presence of signal peptides and leader peptides generally had positive effects on the expression or folding of the core peptide. However, their presence sometimes led to high expression levels that might cause the target protein to accumulate in the cell and be expressed as inclusion bodies.

In the expression of GST-proMET, the corresponding bands in the sediment were thick, which indicates that GST-proMET was strongly expressed, and therefore, inclusion bodies were formed because of overaccumulation.

### Purification of Fusion Protein GST-MET

We purified the fusion protein GST-MET using glutathione sepharose high-performance affinity chromatography. Most untagged E. coli proteins were removed by washing, and the fusion protein was eluted with 10 mM reduced glutathione. The GST protein was also purified for comparison. The purity of the protein was determined by SDS-PAGE. As shown in Figure 2A, lane 3, a band at 27 kDa represented purified GST, whereas GST-MET gave two bands at about 27 and 30 kDa (lanes 2 and 4). The lower band was consistent with the molecular mass of GST. This is possible because GST-MET expression simultaneously induced overexpression of GST. After purification and
enrichment by glutathione sepharose high-performance affinity chromatography, a clear band that was consistent with the molecular mass of GST was observed on the SDS-PAGE gel. It can be deduced that this portion of the GST will affect the binding rate of the target fusion protein GST-MET to glutathione sepharose high-performance medium during the purification process.

The purified fusion protein GST-MET was digested overnight with PPase in glutathione sepharose high-performance medium. The purified GST-MET was digested overnight at 4 °C with PPase of different concentrations. Figure 2B shows that when the concentration ratio of GST-MET to PPase was 10:1, 5:1, 2.5:1, or 1:1, the band at 30 kDa, which is consistent with the molecular weight of GST-MET, almost disappeared. The two bands at 30 and 27 kDa were replaced by one band at 27 kDa, which indicates that GST-MET was completely digested by PPase. However, the band for MET (at about 3 kDa) was not observed in SDS-PAGE. These results are attributed to the difference between the molecular sizes of GST and MET after the digestion of GST-MET by PPase. The relative molecular weight of MET was only 3 kDa, and that of the GST label was 27 kDa. Their different molecular weights might affect the SDS-PAGE analysis results.

Figure 2. (A) Tris-SDS-PAGE analysis of purification of GST-MET and GST with glutathione-affinity chromatography; M, Protein MW Marker (Low); Lanes 1 and 2, soluble proteins from E. coli BL21/pGEX-6p-1 and E. coli BL21/pGEX-MET; Lanes 3 and 4, the elution of GST and GST-MET by 10 mM reduced glutathione elutes GST; (B) The digestion of GST-MET with different concentrations of PPase. Lane 1, GST-MET before the digestion; Lanes 2–5, the digestion of GST-MET under the treatment of different concentration ratios of GST-MET and PPase: 10:1, 5:1, 2.5:1, and 1:1. (C) Tricine-SDS-PAGE analysis of MET purification with glutathione sepharose high-performance medium. M1, protein molecular weight marker (Low); M2, ultralow molecular weight protein marker; Lane 1, before digestion; Lane 2, the purified MET.

Figure 3. Purification of MET using Superdex Peptide 10/300 GL chromatography. (A) Chromatogram of Superdex peptide 10/300 GL; (B) Tricine-SDS-PAGE analysis of the peak sample. M, ultralow molecular weight protein Marker; Lane 1, the sample before loading; Lane 2, the sample of peak 1; Lane 3, the concentrated sample of peak 2; Lane 4, cMET; (C) SDS-PAGE analysis of rMET in peak 2 and cMET.

is consistent with the size of pure rMET. The rMET was purified to apparent homogeneity by SDS-PAGE. Calculations of the corresponding $A_{215}$ value and SDS-PAGE analysis value showed that about 15% of the rMET was in peak 1 and about 85% was in peak 2. Further experiments were performed on the rMET in peak 2. After several chromatography procedures, about 5.8% rMET (~3.5 mg/L culture) of purity greater than 90% was obtained. Mass spectrometric analysis showed the presence of cMET of molecular mass 2847.5 and 98% purity; the rMET in peak 2 had a molecular mass of 3447.2 Da and a purity of about 92% (Figure 4).
Determination of Antibacterial Activity of rMET. MET has antibacterial activity because it destroys bacterial membranes, and it is often used as a model antibacterial agent.\(^{40}\) The antibacterial activity of pure rMET was determined by Oxford cup experiments with *E. coli* JM109, *B. pumilus*, and *S. pasteuri* as test strains. As shown in Figure 5A–C, the three cultured bacteria were treated with MET (100 μL, 50 μg/mL). The inhibition zone of *B. pumilus* was significantly larger than those of *E. coli* and *S. pasteuri*. The inhibition zone of rMET against *E. coli* was the smallest among the three strains. These results show that the purified rMET had significant antibacterial effects against *E. coli*, *B. pumilus*, and *S. pasteuri*.

The antibacterial activities of rMET in inhibition of the growth of Gram-negative *E. coli* JM109 and Gram-positive *B. pumilus* and *S. pasteuri* were further tested in 96-well microplates containing LB broth. As shown in Figure 5, rMET and cMET significantly inhibited *B. pumilus* growth at a concentration of 5 μg/mL. *B. pumilus* was the most susceptible to rMET and showed growth inhibition rates of 97% by rMET and 100% by cMET (Figure 5D). For *S. pasteuri*, rMET gave 68.6% growth inhibition compared with 72.7% by cMET. Ampicillin (10 μg/mL) showed only 49.9% growth inhibition of *E. coli*, whereas 5 μg/mL rMET was sufficient to inhibit about 52.5% growth of *E. coli*; inhibition by cMET was about 63%. These results indicate that the antibacterial effects of rMET on Gram-positive strains were significantly higher than those on Gram-negative strains. The antibacterial activities of cMET and rMET were similar and the same antimicrobial activities were obtained against Gram-positive *B. pumilus* and *S. pasteuri*.

These evaluations enabled the determination of the minimum inhibitory concentrations (MICs) of rMET and cMET for the bacterial species studied. As shown by the data in Table 1, the sensitivity of *B. pumilus* to rMET is greater than those of *E. coli* and *S. pasteuri*. The MIC for *B. pumilus* is about sixfold lower than that for *E. coli* and 5.5-fold lower than that for *S. pasteuri*. The MIC of rMET is slightly higher than that of cMET against the three bacterial species. This suggests that the bacterial resistance for rMET is similar to that for cMET.

**Table 1. Minimum inhibitory concentration (MIC) of recombinant melittin (rMET) and commercial melittin (cMET) against *E. coli*, *B. pumilus*, and *S. pasteuri***

| bacterial species | rMET (μg/mL) | cMET (μg/mL) |
|------------------|-------------|--------------|
| *E. coli*        | 41.0–43.5   | 40.0–42.5    |
| *B. pumilus*     | 6.5–8.0     | 6.0–7.5      |
| *S. pasteuri*    | 35.5–37.0   | 33.0–35.0    |

**Analysis of the rMET Protein Structure.** The antibacterial activity of purified rMET did not obviously differ from that of cMET, and therefore, further structural analysis was required. After purification, there are five more amino acid residues encoded by the plasmid at the N-terminus of rMET, i.e., Gly–Pro from the protease cleavage site, Leu in the middle, and Gly–Ser encoded by the recognition sequence of BamH I. A comparison of the sequences of native MET (GIGAVLKVLTTGGLAPISWIKRKKRQQQ) and rMET (GPLGSMIGAVLKVLTTGGLAPISWIKRKKRQQQ) shows that six N-terminal amino acids and one C-terminal residue...
are different. The circular dichroism spectroscopic assay of the rMET and cMET spectra showed a similar trend in appearance, indicating that their secondary structures are similar. The residue ellipticities of rMET and cMET were $14.03 \times 10^{-4}$ and $14.21 \times 10^{-4}$ ° cm$^2$/dmol, respectively at 197 nm (Figure 6).

![Figure 6. Circular dichroism analysis of rMET and cMET.](Image)

**CONCLUSIONS**

In summary, we heterologously expressed the fused protein GST-MET in *E. coli* and purified it by several chromatography steps. Recombinant MET, i.e., rMET, of purity over 90% was obtained. The antibacterial activities of rMET against Gram-positive *B. pumilus* and *S. pasteurii* were almost the same as those of cMET, but the activity was slightly lower against Gram-negative *E. coli*. This study provides a novel approach to the heterologous expression of active peptides and provides a good example for future biological preparations of active peptides. Further toxicological experiments will be performed to study the effects of the five amino acid residues at the N-terminus of rMET. Other biosystems for MET expression such as *B. subtilis*, which do not produce toxins, will be constructed by DNA sequencing in Tianlin Biotechnology Co., Ltd. (Wuxi, China). Then, the fragment promet with the BamHI/Not I site was cloned into the corresponding site of expression vector pGEX-6p-1 to construct pGEX-proMET (Figure 7). The HRV protease cleavage site in the vector pGEX-6p-1 was between LEVLFQ and GP.

![Figure 7. Schematic diagram of pGEX-proMET and pGEX-MET plasmid construction.](Image)

The recombinant plasmid pGEX-MET was amplified using pGEX-proMET as a template and primer 3 and primer 4 as primers (Figure 1). After the amplified fragment was digested with Dpn I enzyme, it was transformed into *E. coli* JM109. The recombinant plasmid pGEX-MET was obtained after nucleotide sequencing. The constructed recombinant plasmids pGEX-proMET and pGEX-MET were transformed into *E. coli* BL21 (DE3) to obtain the recombinant strains *E. coli* BL21/pGEX-proMET and *E. coli* BL21/pGEX-MET.

**Protein Expression.** The recombinant *E. coli* BL21/pGEX-proMET and *E. coli* BL21/pGEX-MET were cultured in LB broth containing 100 μg/mL ampicillin with shaking at

| Table 2. Primers, plasmids, and strains used in this study* |
|----------------|-------------------|------------------|-------------------|
| **strains/plasmids/primers** | **characteristics** | **sources** | **locations** |
| **Strains** | | | |
| *E. coli* JM109 | cloning host, antibacterial activity test | Invitrogen | |
| *E. coli* BL21 (DE3) | expression host | Invitrogen | |
| *B. pumilus* | antibacterial activity test | lab stock | |
| *S. pasteurii* | antibacterial activity test | lab stock | |
| *E. coli* JM109/T-proMET | *E. coli* JM109 harboring T-proMET | this study | |
| *E. coli* BL21/pGEX-proMET | *E. coli* BL21 harboring pGEX-proMET | this study | |
| *E. coli* BL21/pGEX-MET | *E. coli* BL21 harboring pGEX-MET | this study | |
| **Plasmids** | | | |
| pMD19-T | cloning vector | Takara | |
| pGEX-6p-1 | expression vector | lab stock | |
| T-proMET | pMD19-T harboring promet gene | this study | |
| pGEX-proMET | pGEX-6p-1 harboring promet gene | this study | |
| pGEX-MET | pGEX-6p-1 harboring met gene | this study | |
| **Primers** | | | |
| primer 1 | CGCGGATCCATGAAATTCCTGGT-TAACGGTGCTCCTGG (BamHI) | | |
| primer 2 | TTGGCAGCGGCTAAACCCCTGCT-GGCGTTTTACGTTGATAC (Not I) | | |
| primer 3 | CAGGGGCCCCCTGGAGTCATCGGG-TATCGGTGCTGTTCTGAAAGTTCTG | | |
| primer 4 | AGAACAGCACGGATACCATCGGATGACTCC-AGGAGGGCTCGTTGAAGAAGCTTCC | | |

*The underlined bases are the restriction endonuclease recognition sequences.*
37 °C. When the OD$_{600}$ value of the culture was about 0.6, 0.1–0.5 mM isopropyl $\beta$-D-thiogalactoside (IPTG) was added to induce protein expression at 20–37 °C for 16 h. The cells were harvested by centrifugation at 8000g for 10 min and resuspended in buffer A1 (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ 1.8 mM KH$_2$PO$_4$ pH 7.3), and lysed by sonication at 4 °C. The supernatant was collected by centrifugation at 10 000g for 45 min. The samples were analyzed by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Soluble expressed proteins were used for subsequent purification.

**Purification of Fusion Protein GST-MET.** Glutathione Sepharose High-Performance medium (GE Healthcare, Piscataway, NJ) was equilibrated with buffer A1. The supernatants were filtered with a 0.22 μm microporous membrane, and then they were loaded onto the resin at a speed of 1 mL/min. The column was washed with 10 mL of buffer A1 to eliminate unbound proteins. The protein was eluted with buffer B1 (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The eluted fractions were collected for SDS-PAGE analysis.

**Purification and Pure MET.** After the hybrid protein in the supernatant was washed, the fusion protein GST-MET in Glutathione Sepharose High-Performance medium was treated with Prescission protease (PPhase) in buffer A2 (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5) at 4 °C overnight.

After digestion, to remove the GST-tagged protein and the PPhase from the target protein, the medium was segmented by centrifugation at 500g for 5 min. The supernatant was collected for size-exclusion chromatography.

Superdex Peptide 10/300 GL (GE Healthcare, Piscataway, NJ) was equilibrated with the A3 buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with an AKTA purifier system. The collected samples were centrifuged at 10 000g for 30 min to remove insoluble matter, and then the supernatant was loaded in Superdex Peptide 10/300 GL. The column was eluted with buffer A3 at room temperature. UV absorption was measured at UV 215 nm, and the elution peaks were collected for Tricine-SDS-PAGE with 18% polyacrylamide analysis.

**Antibacterial Activity Determination.** Bacterial cultures were grown in aerobic conditions to the mid-log phase as determined when the OD$_{600}$ was 0.5. Then, 1 mL of the bacterial solution was diluted in 200 mL of agar medium kept at 50 °C, and 5 mL was quickly added to the plate to be flattened. After the upper medium was solidified, the sterilized Oxford cup (8 × 6 × 10 mm$^3$) stands vertically near the plate mark. The purifi d rMET and chemically synthesized MET (cMET) of 50 μg/mL were added to the Oxford Cup, ampicillin was used at 200 μg/mL as a positive control, and ddH$_2$O was used as a negative control. The plate was incubated at 37 °C for 16–18 h. The inhibition zones were observed.

**E. coli, B. pumilus,** and **S. pasteuri** were selected to culture in LB liquid medium to the logarithmic growth phase. Approximately 2 × 10$^6$ colony forming units (CFU) per mL of each strain were incubated in a 96-well microplate (Thermo Fisher Scientific, China) with rMET or cMET. The final concentration of rMET or cMET was 5 μg/mL in each well. Untreated strains cultured in LB served as blank controls. Ampicillin (10 μg/mL) was used as a control for growth inhibition. Cultures were incubated at 37 °C for 2 h and monitored by counting live colonies (CFU) on LB agar medium by serial 10-fold dilutions.

For the determination of minimum inhibitory concentration (MIC), the microdilution technique was used. Melittin was dissolved in sterile water and filtered using a 0.22 μm syringe filter. Bacteria (200 μL) were added to the diluted samples (20 μL) in 96-well plates. The microplates were incubated at 37 °C for 24 h. The MIC value was determined visually by comparison of bacterial growth with positive control. The MIC was between the lowest concentration capable of promoting inhibition of bacterial growth and the lowest concentration that did not promote inhibition. The experiments were performed in triplicate.

**Mass Spectrum and Circular Dichroism.** Mass spectral measurement was performed using Waters MALDI SYNAPT G2-Si MS (Milford). Circular dichroism (CD) assays were performed using a Jasco J720 spectropolarimeter (JASCO, Inc., Easton, MD). Wavelength scan data were collected from 170 to 350 nm in a phosphate buffer (pH 7.5) for every 30 scans with the following settings: response, 1 s; sensitivity, 100 mdeg; and speed, 50 nm/min.

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### Author Contributions

L.Z. conducted the investigation; devised the methodology; did experiments; and wrote the original draft. R.Z. supervised the work and revised the manuscript. L.L. performed formal data analysis. Z.L., G.X., and K.X. helped to perform experiments and analyzed the protein structure. Y.X. was involved in revising the manuscript.

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■ ABBREVIATIONS AND NOMENCLATURE
cMET, commercial melittin; GST, glutathione-S-transferase; MET, melittin; PPhase, presscission protease; proMET, promelittin; rMET, recombinant melittin

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