Efficient Production of Mastoparan B, a Cationic Venom Peptide, via the Artificial Oil Body-Cyanogen Bromide Purification Platform

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Abstract-Mastoparan B (MPB), a cationic venom peptide found in Vespa basalis, belongs to an evolutionarily conserved component of the innate immune response against microbes. MPB possesses significant biological potency, especially in the anti-microbial activity, and poreforming ability. These properties make MPB extremely attractive for further studies and applications. However, this cationic MPB is difficult to obtain and production. In the present study, we successfully to develop a new and reliable recombinant oleosin-based fusion expression procedure in Escherichia coli and coupled with the artificial oil bodies (AOB)-cyanogen bromide purification platform to produce bioactive MPB. This procedure could yield ~2.0mg of MPB from 1L of cultured cell pellet. And, enhanced the yield by >2-fold of purified MPB compared to reported previously. Take together, this study provides a new insight into the over-production of active MPB, which will facilitate studies and applications of this peptide in the future.

Index Terms—artificial oil bodies, cyanogen bromide, Escherichia coli, mastoparan B, oleosin-fusion protein

I. INTRODUCTION

Mastoparan B (MPB) is a venom peptide isolated from Vespa basalis (black-bellied hornet), the dangerous vespine wasps found in Taiwan [1]. As reported, MPB (LKLKSIVSWAKKVL) is an amphiphilic, α-amidated, and cationic tetradecapeptide, with a molecular mass of 1.6 kDa [1], [2]. The precursor polypeptide of MPB and its processing enzyme, dipeptidyl peptidase IV (DPP-IV), have been successfully cloned in our laboratory [2]. MPB belongs to an evolutionarily conserved component of the innate immune response against microbes [3], [4]. Additionally, many biological activities of MPB have been reported, including the activation of also phospholipase A, C, and D, degranulation of mast cells, release of histamine, stimulation of G-proteins, hemolytic effect, antimicrobial activity, and pore-forming ability [5]-[9]. It is noteworthy that the amphiphilic structure of MPB is related to its hemolytic effect, antimicrobial activity, and pore-forming ability [7]-[14]. Among different venom peptide, MPB is the only peptide identified and characterized that induces short-term hypotension [8]-[12]. Taken together, these properties make MPB more attractive for further studies than other venom peptides. However, the peptide is difficult to obtain, either isolated from Vespa basalis or synthesized by chemical methods [15], and both processes are timeconsuming and expensive. It is worth to note that MPB has been expressed in Escherichia coli via Artificial Oil Bodies (AOB) system [16], the yield was equivalent to 0.9mg per liter of cell culture. Accordingly, in this study, we further attempted to modify and design a reliable recombinant oleosin-based fusion expression in Escherichia coli coupled with the AOB-cyanogen bromide (CNBr) platform to increase the yield of MP-B production.

II. MATERIALS AND METHODS

A. Construction of Plasmids pET30-His₆-rOle-MPB

Plasmid pET30-His₆-rOle was generated using pET29r-Ole $({}^{127}M \rightarrow L)$ as a template and subcloned into the KpnI and SalI restriction sites in the expression vector pET30a(+) (Novagen, San Diego, USA) to form an inframe open reading frame, a leader polypeptide at the Nterminus of rOle (which include the open reading frame of the pET30a(+), amino acid residue 1-38) and a linker polypeptide (PWLISDPNSSSVD) at the C-terminus of rOle. Finally, a double-stranded DNA (coding for methionine-MPB peptide) was synthesized (Canada). Following the desired methionine-MPB DNA sequences were ligated in frame to the SalI/XhoI-digested pET30-His₆-rOle. The resulting plasmid was designated pET30-His₆-rOle-MPB (Fig. 1). The recombinant plasmid was verified by PCR, restriction endonuclease analysis, and DNA sequencing.

B. Optimal Condition for His6-rOle-MPB Fusion Protein Overexpression

In this experiment, different IPTG concentration, culture temperature and induction times were tested to

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optimize His_6 -rOle-MPB expression. Briefly, a single clone from transformed *E. coli* C43(DE3) was added to 100 mL LB-kanamycin (inoculum 1%). Once the cultures reached OD_{600} =0.5, cells were induced with 1, 5 or 10mM IPTG for 2h. Additionally, different time periods (1, 2, and 3h) were tested in this study. These cultures were grown with 180rpm shaking. At the end of the growth period, inclusion bodies were harvested, and protein content was analyzed by SDS-PAGE and western blotting. Quantitative analysis of the expressed His₆-rOle-MPB fusion proteins (theoretical molecular weight of 22.6kDa) were determined by scanning and digitizing the immunoblotted membrane using an image-analyzing system and the ImageJ software (Alpha Innotech Corporation, San Leandro, CA).

C. Purification and Identification of MPB Peptide

First, His₆-rOle-MPB fusion protein was incubated with 1:1000 (w/w) thrombin using the thrombin cleavage capture kit (Novagen, San Diego, CA) in cleavage buffer (200mM Tris-HCl, pH8.4, 1.5M NaCl, 25mM CaCl₂) for 16h at 20 °C. Thrombin recognizes the consensus sequence Leu-Val-Pro-Arg-Gly-Ser and then cleaves the peptide bond between Arg and Gly, allowing removal of the upstream polypeptide (leader polypeptide), which is at the N-terminus of the His₆-rOle-MPB fusion protein. AOBs were constituted using 600µg of rOle-MPB proteins, 15mg of triacycerols (Sigma) and 150µg of phospholipid (Sigma) in 1 mL of 10mM sodium phosphate buffer (pH 7.5), mixed, sonicated, and collected and resuspended in the sodium phosphate buffer (pH 7.5), according to the protocol described by Peng et al. [16], [17]. Afterwards, 50 microliters of AOBs were dissolved in 30 µL of 88% formic acid, 15 µL of water, and 5 µL of 5 M CNBr (5 M CNBr stock in acetonitrile), to formulate a 100-µL reaction volume containing 250mM CNBr. The reaction was carried out with modification of the literature [18], [19]. The purified MPB was visualized on a 16.8% tricine SDS gel by silver staining and then was analyzed by the QSTAR Elite system (Applied Biosystems, Framingham, MA) and the peptide was identified using the Mascot software. To quantitatively analyze the protein, the Tris-tricine gel was scanned and digitized using an AlphaImager imageanalyzing system (Alpha Innotech, San Leandro, CA) and the computer program AlphaImagerTM2200 version 5.5. Two micrograms of purified MPB was used as a reference for calculation.

D. Antimicrobial Sensitivity Test

The antimicrobial sensitivity test was performed by the disc diffusion method [20] with some modification. The test *E. coli* strain J96 was cultured in LB broth. The sterile nutrient agar was poured into sterile 9-cm petri dish, allowed to solidify, and was inoculated with $500 \,\mu\text{L}$ of *E. coli* J96 (in exponential phase; $0.5 < \text{OD}_{600} < 0.6$). Meanwhile, sterile filter paper discs (Difco, Detroit, Mich) of 6mm in diameter were impregnated with $20 \,\mu\text{L}$ of the purified MPB ($10 \,\mu\text{g}/\text{disc}$) and then placed on the surface of the test plate (a LB-agar plate inoculated with *E. coli* J96) after the water had evaporated. Sterile PBS disc was

used as a mock control. Then, the test plate was incubated overnight (18h) at 37 °C. At the end of the incubation, the zone of inhibition was observed.

III. RESULTS AND DISCUSSION

A. Design of the Plasmid Pet30-His₆-Role-Mpb

For production and purification of MPB, the plasmid pET30-His₆-rOle-MPB was generated in this study. Fig. 1 depicts the process by which the recombinant plasmid pET30-His₆-rOle-MPB was constructed. Based on the design, the MPB peptide was interlinked with recombinant oleosin by a methionine residue, a CNBr cleavage site. CNBr hydrolyzes peptide bonds at the Cterminal end of methionine residues; therefore, excess methionine residues (at position 127;127Met) must be removed from the oleosin sequence. To that end, the ³⁷⁹A (adenine) nucleotide of the oleosin coding sequence was replaced with ³⁷⁹C (cytosine) in order to change ¹²⁷Met (ATG) to 127 Leu (CTG). This resulting plasmid was named pET29-rOle(${}^{127M \rightarrow L}$). Afterwards, pET29-rOle(${}^{127M \rightarrow L}$) was used as a template to generate an intermediate donor plasmid pET30-His₆-rOle-MPB (Fig. 1). Finally, an additional methionine and the MPB coding sequences were fused in frame to the linker nucleotides; this resulting plasmid was designed pET30-His₆-rOle-MPB (Fig. 1).

pET30-His₆-rOle-MP-B



Figure 1. Schematic representation of recombinant plasmid pET30His₆rOle-MP-B. The His₆-rOle-MPB protein are shown in the lower panel which contain an N-terminal leader polypeptide (38 amino acids), a linker polypeptide (13 amino acids), and the MPB peptide (14 amino acids).

In summary, pET30-His₆-rOle-MPB is designed with a His_{6} -tag to facilitate purification and retrieval of the His_{6} -rOle-MPB fusion protein by Ni-NTA affinity column. On the other side, the thrombin protease recognition sites allow the removal of the leader polypeptide which is at the N-terminus of the His₆-rOle-MPB fusion protein. Cleavage of His_{6} -rOle-MPB with thrombin results in rOle-MPB. Finally, the MPB peptide is obtained by CNBr digestion.

B. Optimized Expression of His₆-Role-Mpb in C43(De3)

We tested whether the presence or absence of IPTG affected the expression of His₆-rOle-MPB. Protein expression was not detected in uninduced C43(DE3) cells (data not shown). The results indicated the protein expression is tightly controlled by the IPTG-induced T7 promoter/lac operon. Furthermore, varying concentrations of IPTG and induction time were tested for optimal protein expression. Results showed that expression His₆-rOle-MPB, induced with 5mM IPTG was 1.2- and 2-fold higher than expression induced with 1mM

and 10mM IPTG, respectively, since the relative intensities of the detected band were 133, 160 and 90 when expression was induced with 1, 5 and 10mM IPTG, respectively (Fig. 2A, lower panel). To determine the optimal induction time, 5mM IPTG-induced cells were harvested at 1, 2 or 3h. His₆-rOle-MPB expression reached the highest level between 1 and 2h, when the relative intensities of the detected band were 146, 174 and 106, at 1, 2 and 3h, respectively, (Fig. 2B, lower panel). Together, these results suggest that His₆-rOle-MPB expression level is optimal when cells are cultured at 37 °C for 2h after induction with 5mM IPTG.

C. Mpb Peptide Release and Identification

The purified product was detected as a major band with a molecular mass of ~26.6 kDa, which is similar to the size of His₆-rOle-MPB, by SDS-PAGE and western blot analysis (Fig. 3A). The purified His₆-rOle-MPB was selectively cleaved by thrombin protease in order to remove the His₆-tag and the leader peptide from the N-terminus.



Figure 2. Optimal production of His₆-rOle-MPB in *E. coli* C43(DE3). (A) Effects of increasing IPTG concentration His₆-rOle-MPB expression levels. pET30His₆-rOle-MPB transformed *E. coli* C43(DE3) was grown to OD₆₀₀ 0.6 and then induced with 1mM, 5mM or 10mM

IPTG at 37 °C for 2h. (B) Effects of IPTG-induced times on His₆-rOle-MPB expression. pET30His₆-rOle-MPB transformed *E. coli* C43(DE3) was grown to OD₆₀₀ 0.6, and then induced with 5mM IPTG at 37 °C for 1, 2 or 3h.

A major band of approximately 18-kDa was detected on a 16.8% tricine gel by silver staining (Fig. 3B, Lane 1), which corresponded to the estimated molecular weight of rOle-MPB protein. Subsequently, rOle-MPB protein was constituted into AOB and incubated with CNBr for a cleavage reaction, which resulted in the release of the MPB peptide from rOle-MPB protein. As mentioned previously, a linker peptide was expressed at the C terminus of rOle to increase the exposure of MPB on the surface of AOB, this facilitates the CNBr treatment. The purification process employed here takes advantage of CNBr, which cleaves specifically at C-terminal side of methionine [14], [15]. The purification process employed here takes advantage of CNBr, which cleaves specifically at C-terminal side of methionine. This will facilitate the production of an identical MPB without additional amino acids. As shown in Fig. 3B, a single band was detected with close to the predicted molecular weight of MPB (Fig. 3B, Lane 2). The putative MPB, and over two-third of the peptide sequence was verified by the MALDI-MS analysis (Fig. 3C). Taken together, approximately 1.9mg of purified MPB peptide was obtained from 1L of cultured cell pellet after induction with 5mM IPTG for 2h (Fig. 3B and Fig. 3C). The results reported herein indicate that the expression yield obtained in this study is significantly higher than that reported previously [16], with a yield of approximately 0.9mg from 1L of cultured cell pellet. Meanwhile, this purification platform will provide a more convenient strategy to produce different variation of MPB, which is more suitable for medicinal applications such as hypotension and antibacterial effect.



LKLKSIVSWAKKVL

Figure 3. Purification and identification of MPB peptide. (A). SDS-PAGE analysis of purified His₆-rOle-MPB by nickel-chelated affinity chromatography. (B). Tricine-PAGE analysis (silver staining) of the thrombin and CNBr cleavage product. Lane 1, cleavage product after His₆-rOle-MPB incubation with thrombin. Arrow indicates the cleavage product, expected rOle-MPB protein ~18kDa. Lane 2, cleavage product after rOle-MPB incubation with CNBr via AOB purification platform. Arrow indicates the position of expected MPB peptide ~1.6kDa. (C). MALDI-MS was used to identify the purified MPB. Two peptide fragments of purified MPB verified by MALDI-MS analysis are underlined.



Figure 4. Antimicrobial activity of purified MPB. Effects of purified MPB against *E*. coli stain J96 by disc diffusion assay. PBS as the mock control (left); 10 µg of purified MPB was tested (right).

D. Antimicrobial Activity

The antimicrobial activity of purified MPB was tested against *E. coli* J96. As shown in Fig. 4, an inhibition zone was observed around the disc of purified MPB while no inhibition zone formed around the disc of mock control. These results indicated that the purified MPB was bioactive and very effective in anti-bacterial (*E. coli* J96) activity. The antimicrobial test showed that the purified MPB significantly inhibits the growth of *E. coli* (gramnegative bacteria) is consistent with the fact that MPB possesses antibacterial activity [14], [21].

IV. CONCLUSION

In this study, the recombinant oleosin based fusion strategy coupled with AOB-CNBr purification platform open a new avenue for the production of active MPB. Meanwhile, it will not only facilitate the studies and applications of the peptide in the future, but also this purification platform will provide a more convenient strategy to produce different variation of MPB, that is more suitable for medicinal applications such as hypotension and antibacterial effect.

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