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Expression and Purification of the venom peptide -Mastoparan B of *Vespa basalis* in *Escherichia coli*

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Abstract. Mastoparan B is a cationic, amphiphilic tetradecapeptide toxin isolated from the venom of the black-bellied hornet (*Vespa basalis*), the most dangerous species of wasps found in Taiwan. Mastoparan B was evaluate possess antibacterial activity and cardiovascular depress activity. However, mastoparan B is low abundance in the venom (3.4% of crude venom). In this study, mastoparan B was overexpressed in *Escherichia coli* BL21 as a recombinant protein fused the C-terminus of oleosin by a linker polypeptide, intein *S*. Artifical oil bodies (AOBs) were reconstituted with triacylglycerol, phospholipid to obtain the insoluble recombinant protein. Mastoparan B was subsequently released through self-splicing of intein induced by temperature alteration from artifical oli bodies, and the recombinant mastoparan B was collected it in the supernatant after centrifugation. Recombinant mastoparan B release from AOBs was exhibited bacteriostatic and bactericidal activity. These results have shown that mastoparan B was successfully expressed and purified via the efficient AOB expression/purification system.

Keywords: *Vespa basalis*, Mastoparan B, Artifical oil bodies (AOBs) expression/puruification system, Bacteriostatic and Bactericidal activity

1. Introduction

Mastoparan B, a cationic tetradecapeptide isolated from the venom of black-bellied hornet, *Vespa basalis*, the most dangerous species of vespine wasps found in Taiwan [1]. Mostoparan B exhibit several biological activities including mast cell degranulation and release of histamine [2, 3], hemolysis [2, 3], activation of phospholipase A2 [4, 5], 46 phospholipase C [5], G proteins [6], and antibacterial activity. Unlike other vespid mastoparan toxins, mastoparan B more hydrophilic amino acid residues and was capable of inducing short-term hypotension in rats [8, 9, 10]. As various biological actions of mastoparan B are probably played through different mechanisms, the hypotensive effect may be useful for developing this peptide into an anti-hypertension agent if its potency could be enhanced and its hemolytic activity abolished.

The most polypeptide expression and purify are via recombinant protein system. However, biosynthesis of small peptides in a heterologous host produced with low yield/or are substantially degraded due to sensitivity to cellular protease [11]. These difficulties result from the extracellular nature of antimicrobial peptides that have no feature favoring their intracellular accumulation. Artificial oil bodies (AOBs) systems were established as an expression/purification system provides a facilitation method of recombinant protein or peptide purification [12-14]. In this study, a matoparan B gene form *Vespa basalis* was cloned and expressed as an oleosin-fused protein in *E.coli* BL21 (DE3), affinity-purified by formation of AOBs, released from oleosin by intein-mediated peptide cleavage, and finally harvested by concentration of the supernatant. The anti-microbial activity of recombinant mastoparan B was analyzed by bactericidal (MIC) and bacteriostatic activity (MBC) of several bacterial species.

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2. Materials and Methods

2.1. Construction of Mastoparan B Expression Plasmid

PCR was performed to synthesize the DNA bearing the mastoparan B gene with its mature peptide (mature mastoparan B) from the venom gland cDNA library [15] using the oligonucleotide forward primer, MB-I1-f (5'ATGCTCTTCCAACTTGAAACTGAAGTCTATTGTATCAT 3'), containing a *Sap* I site (underlined) and a reverse primer, MB-I1-r (5'ATGAATTCTTATAGTACTTTCTTAGCCCATGATAC 3'), containing a *Eco*RI site (underlined). The desired PCR product was purified and digested with *Sap* I and *Eco*RI, and then ligated at 16^{o}C overnight with *Sap* I-*Eco*R I digested pJO1-OSP1 plasmid [12]. The resulting plasmid, pJO1-OSP1-Mastoparan B was use transformed *E. coli* competent cells on a LB agar plate containing ampicillin (100μg/ml)(Sigma Chemicsl CO., St. Louis, MO), and the accuracy of plasmid construction was also confirmed by direct sequencing.

2.2. Overexpression of Oleosin-intein *S*-mastoparan B Fusion Protein

The recombinant plasmid pJO1-OSP1-Mastoparan B was transformed to *E.coli* BL21(DE3). Over-expression of the recombinant fusion protein, oleosin-intein *S*-Mastoparan B was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM in a bacteriophage T7 RNA polymerase/promoter system. Two hours after induction, the *E. coli* cells were harvested, lysed by sonication in the 50 mM sodium phosphate buffer, pH8.5, fractionated into supernatant and pellet by centrifugation, and then subjected to SDS-PAGE analysis.

2.3. AOB Preparation and Protein Recovery

AOBs were prepared according to the reported method [16]. The pellet fraction of E. coli cell lysate were resuspended in 1 ml of wash buffer (50 mM sodium phosphate, 10 m M EDTA, 100 mM NaCl, 0.5 % triton-X-100, pH8.5), mixed well for washing the pellet, and fractioned into supernatant and pellet parts by centrifugation. Then, 15 mg of TAG (olive oil from Sigma) and 150 μg phospholipid (Sigma) were added into the pellet fraction of *E. coli* cell lysate containing 800 μg of oleosin fused recombinant polypeptides and mixture was subjected to sonication. Subsequently, the reconstituded AOBs were collected after centrifugation and resuspended in the sodium phosphate buffer (0.1 M, pH7.4). To retrieve the target protein, AOBs were placed at 4 ° C for 16 h. Finally, centrifugation was applied to segregate the oil body and aqueous phases, and the protein in each phase was analyzed by SDS-PAGE.

2.4. Antimicrobial Activity Assay

Microorganisms were grown in vitro in sterile 96-well microtiter plate (Iwaki Inc, Japan) in final volume of 300 μl. The MIC assay mixture contained 100 μl of each microorganism (final concentration 1-5 x 10^5 CFU/ml), 100 μl of culture medium (Tryptone Soys Broth, (TSB)) and 100 μl of the purified mastoparan B sample solution. Plates were incubated at the appropriate growth conditions. Bacterial growth was determined by optical density (OD) measurements (590mn) using a Bio-teck μQuant microplate spectroplate spectrophotometer (Bio-TEK, VT, USA). The MIC was determined as the lowest peptide concentration that prevented increase in OD. Each peptide concentration was test in triplicate in three independent experiments [17]. The MBC, corresponding to the concentration the kill 99.9% of the total bacterial, was determined by spotting 20 μl from each well of the MIC showing no visible growth on TSB-agar and incubating overnight. Each peptide concentration was test in triplicate in three independent experiments [18].

2.5. Time-kill Determination

The bactericidal activity of mastoparan B was measured in 6 of the 10 strains using the time-killing method. Three gram positive bacterial *Streptococcus alactolyticus*, *Staphylococcus aureus* and *Staphylococcus intermedius B,* with two gram negative bacterial*, Salmonella cholerasuis* and *Vibrio parahaemolyticus* were used, because both MIC and MBC activities were observed. Each strain of the bacterium contains 1 x 10^5 bacterial colony forming units with 2x MBC of mastoparan B, respectively. The reactions were terminated by plating on TSB agar plate, and the plates were incubated overnight at 37 ℃ [19].

3. Results

3.1. Expression of Recombinat Protein Oleosin-intein *S*-mastoparan B in *E. Coli.*

PCRs were set up to subclone the mature peptide DNA fragment of mastoparan B gene into AOB expression/purification system vector pJO1-OSP1. The recombinant oleosin-intein *S*-mastoparan B fusion protein was expressed in *E.coli* BL21 (DE3). After induction with IPTG at 37°C, the expressed protein products of induced and non-induced recombinant bacterial containing the masto-paran B gene were analyzed using SDS-PAGE. A band of about 34.4 kDa corresponding to the oelosin-intein *S*-mastoparan B fusion protein was observed in the induced recombinant bacteria (**Figure 1**). The expressed recombinant protein, oelosin-intein *S*-mastoparan B was predominately found in the insoluble of fraction of cell lysate after centrifugation (ppt-1). (**Figure 1**) These insoluble pellets resuspended with wash buffer and collected pellet (ppt-2) parts by centrifugation. Most bacterial proteins would be removed by this wash buffer. AOBs were reconstituted with the insoluble pellet (ppt-2) of cell lysate consisting mainly of oelosin-intein *S*-mastoparan B. The oleosin-intein *S*-mastoparan B as well as other insoluble bacterial proteins was almost entirely present in the oil body fraction with the supernatant (sup-2) and nearly no visible pellet (ppt-2) after centrifugation (**Figure 1**).

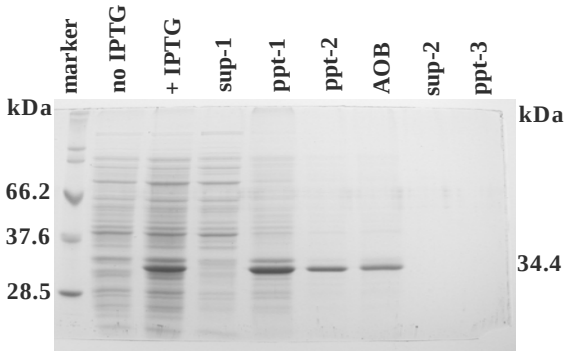

Fig. 1: Glycine SDS-PAGE analysis of oleosin-intein *S* matoparan B over-expressed in *E.coli*. With or without IPTG induction, total proteins of *E. coli* expressing mastoparan B were fractionated into supernatant (sup-1) and precipitate (ppt-1), the pellet fraction (ppt-2) was fractionated by washing buffer mixed after centrifugation. AOBs were constituted with the pellet fraction (ppt-2) of *E. coli* containing oleosin-intein *S* matoparan B. After constitution, three fractions, supernatant (sup-2), precipitate (ppt-3) and resolved in 10% SDS-PAGE.

3.2. Purification of Mastoparan B

Release of mastoparan B from AOBs was achieved via self-splicing of the intein *S* linker induced by shifting the temperature to 4°C. After centrifugation, mastoparan B was found in the supernatant (sup-3), whereas oleosin-intein *S* remained in digested AOBs (**Figures 2**). A band of about 1.61 kDa corresponding to the mastoparan B peptide was observed in the supernatant (sup-3), digested AOBs and venom of *Vespa basalis* (**Figures 2**). This assay was performed by Tricine SDS-PAGE. The identity of recombinant mastoparan B was further confirmed by N-terminal sequencing (Mission Biotech, Taipei, Taiwan). The yield of purified mastoparan B was about 0.9 mg per liter of cell culture.

3.3. Antimicrobial Activity of Recombinant Mastoparan B

Recombinant mastoparan B was tested against 4 strains of gram positive bacterial and 6 strains of gram negative bacterial for bactericidal and bacteriostatic activity. Bacteria were chosen which represent general animal pathogens. Results show that *Staphylococcus alactolyticus* was sensitive to mastoparan B , while *Citrobacter koseri, Klebsiella pneumoniae* and the hemolytic *Escherichia coli* were resistant to mastoparan B. Result show that mastoparan B acts better against Gram-positive than Gram-negative bacteria. In general, the bacteriostatic concentration (MIC) is lower than the bactericidal concentration (MBC) of mastoparan B (Table 1).

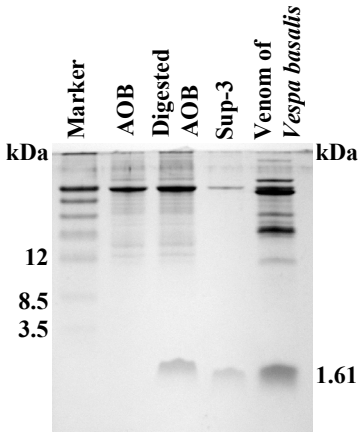

Fig. 2: Tricine SDS-PAGE analysis of Mastoparan B from AOBs. AOBs were constituted with the pellet fraction (ppt-2) of *E. coli* containing oleosin-intein *S*-matoparan B for self-splicing of intein linker by elevating the temperature from 4 to 25°C. A band of about 1.61 kDa corresponding to mastoparan B was observed in the supernatant (sup-3), digested AOBs and venom of *Vespa basalis*.

Table 1: Bacteriostatic and bactericidal activity of mastoparan B against 10 different animals and animal-specific pathogens

Bacteria	MIC (μg/ml)	MBC (μg/ml)
Gram Positive		
Staphylococcus intermedius B	28	32
Staphylococcus xylosus	32	25
Staphylococcus aureus	28	32
Streptococcus alactolyticus	16	20
Gram negative		
Vibro parahaemolyticus	24	24
Salmonella cholearasuls	12	16
Citrobacter koseri	**NI**[a]	**NB**[b]
Klebsiella pneumoniae	**NI**	**NB**
hemolytic Escherichia coli	**NI**	**NB**
Pseudomonas aeruginosa	16	20

[a]No inhibition activity
[b]No bactericidal activity

To determine the rate of bactericidal activity of the mastoparan B, a kinetic study of mastoparan B was performed on the bacteria that were inhibited in the MBC assay. The time courses to kill the bacterial culture suspension of Gram-positive bacteria, *Streptococcus alactolyticus, Staphylococcus aureus, Staphylococcus intermedius B, Staphy-lococcus xylosus* (Figure 3A) and Gram-negative bacterial for *Salmonella cholerasuis*, *Vibrio Parahaemolyticus* (Figure 3B) were compared to each evaluate the bactericidal activity of mastoparan B. At the 2x MBC of the mastoparan B for treating the Gram-positive and the gram negative bacterial, respectively, the bactericidal action was found to be time-dependent. As the incubation time of the bacterial incubated with mastoparan B was longer, the bactericidal activity of the mastoparan B seemed more powerful.

4. Disscussion

Most small peptides (<5 kDa) were expressed via fusion protein system like GST-fusion protein and purified by expensive ligand-couple columns [11]. An improved artificial oil body-based system, as exemplified by the production of nattokinase and hydantoinase [12,13] in *E. coli*, have been developed for bacterial expression and purification of functional recombinant proteins offers a lower cost for replacing the

expensive affinity chromatography. In this study, we expressed mastoparan B as an oleosin-intein *S*-mastoparan B in *E.coli* BL21 (DE3) and mixed triacylglycerol, phospholipid to construct AOBs. Released mastoparan B from AOBs and collected supernatant by centrifuged. Result show the purified recombinant mastoparan B display high antibacterial both gram-positive (16-32 μg/ml) and gram-negative bacterial (12-24μg/ml) (table 1) conformed to the chemistry synthesis of mastoparan B for gram-positive (3.13-25 μg/ml) and gram-negative bacterial (6.25-25 μg/ml) [20-21].

The considerably antibacterial activity of recombi-nant mastoparan B released from oleosin-intein *S*-mastoparan B suggest that mastoparan B immobilized on the AOB surface might fold into an optimal structure ready for separated form AOBs by the intein-mediated self-splicing. Finally artificial oil bodies (AOBs) expression/puruification system can useful in recombinant expression of a broad spectrum of short peptides.

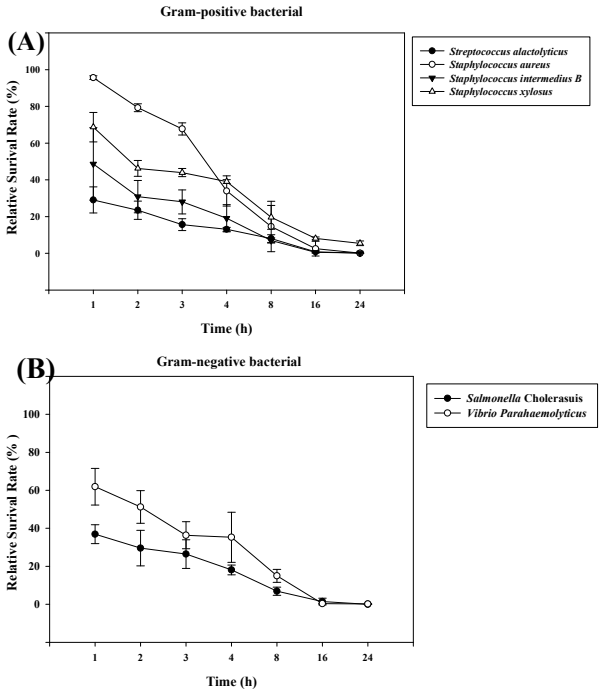

Fig. 3: Kinetic of mastoparan B bacterial versus gram positive and Gram-negative bacteria. At the 2x MBC of the mastoparan B for treating the bacterial was incubated at 37 ^{o}C for increasing timers (0-24h).

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