Cost-Effective Expression and Purification of Recombinant 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 membrane permeabilization activity, bacteriostatic and bactericidal activity. These results have shown that mastoparan B was successfully expressed and purified via the efficient AOB expression/purification system.**

***Index Terms*—Vespa basalis, mastoparan B, artifical oil bodies (AOBs) expression/puruification system, bacteriostatic and bactericidal activity.**

I. 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], 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 [7]-[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 system, oleosin, a unique structural protein of seed oil bodies, is used as the carries. Oleosins possess a lipophlilic segment embedded into triacylglycerol core, with two amphipathic arms protruding on the surface of oil bodies. Thus, the target protein can be expressed in *E. coli* as an insoluble recombinant protein fused to N-and/or C-terminus of oleosin by an intein *S/M* fragment. Stable artificial oil bodies are constituted with triacylglycerol, phospholipid and the insoluble oleosin fusion protein. Target protein is subsequently released through self-splicing of intein *S/M* induced by DDT or temperature alternation, with remaining olesoin-intein *S/M* residing in oil bodies. Finally, the target protein is harvested by concentrating the supernatant [12].

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 membrane permeabilization activity, bactericidal (MIC) and bacteriostatic activity (MBC) of several bacterial species.

II. Materials and Methods

A. 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'ATGCTCTTCCAACTTGAAACTGAAGTCTATTGTA TCAT 3'), containing a *Sap* I site (underlined) and a reverse primer, MB-I1-r (5'ATGAATTCTTATAGTACTTTCTTAGCCCATGATA C 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°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

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(100μg/ml)(Sigma Chemicsl CO., St. Louis, MO), and the accuracy of plasmid construction was also confirmed by direct sequencing.

B. 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.

C. 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 analyzes in SDS-PAGE and western blotting. Antibodies against oleosin were raised in chicken using sesame 15-kDa oleosin purified from sesame oil bodies [17].

D. 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 [18]. 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 [19].

E. 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 ℃ [20].

F. Membrane Permeabilization Assay

The membrane permeabilization activity of mastoparan B was determined by measurement in Staphylococcus aureus of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6-phosphate) as substrate [21]. *S. aureus* was grown at 30 ℃ for 16 h in LB medoum containing 1% galactose as an inducer for phosphor-β-galactosidase, a cytoplasmic enzyme of *S. aureus* [22]. The bacteria were harvested from 0.5 ml of the broth, washed three times with 1ml of 10 mM sodium phosphate buffer (pH 7.0), containing 130 mM NaCl and resuspended in the same buffer (absorbance at 650 nm was adjusted to 0.13). An aliquot of the cell suspension (48 μl) was added to incremental amounts of the purified recombinant mastoparan B dissolved in 6 μl of 10 mM sodium phosphate buffer (pH 7.0), containing 130 mM NaCl. The mixture were then incubated at 30 ℃ for 20 min. 6 μl of 15 mM ONPG-6-phosphate was added to each mixture. After incubating the mixtures at 30 ℃ for an additional 90 min, the reaction was stopped by the addition of 0.8 M NaOH (10 μl) and the production of o-nitrophenyl was assayed by measuring the absorbance at 405 nm.

III. Results

A. 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 (Fig.1A). The expressed recombinant protein, oelosin-intein *S*-mastoparan B was predominately found in the insoluble of fraction of cell lysate after centrifugation (ppt-1). (Fig.1A) 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 (Fig 1A). Moreover, the presence of the oleosin in this recombinant protein was confirmed in the western blot result (Fig 1B), in which the 34.4 kDa recombinant protein was recognized by the antibody against sesame oleosin.

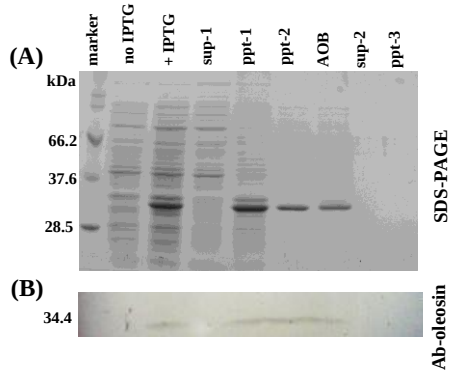

Fig. 1. SDS-PAGE and western blotting of oleosin-intein *S* matoparan B overexpressed in *E. coli*.

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 (A). Duplicated SDS-PAGE gels were transferred to nitrocellulose membrane, and then subjected to immunoassaying using chicken antibodies against sesame oleosin (B).

B. 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 ℃. After centrifugation, mastoparan B was found in the supernatant (sup-3), whereas oleosin-intein *S* remained in digested AOBs (Fig 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* (Fig. 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.

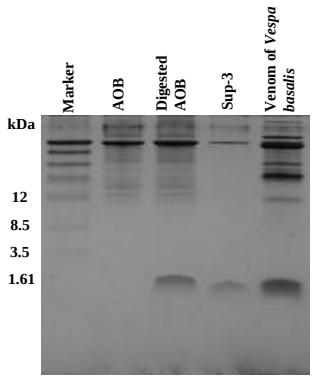

Fig. 2. Tricine SDS-PAGE analysis of mastoparan B from AOBs.

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*.

C. 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 I).

TABLE I: 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* (Fig. 3A) and Gram-negative bacterial for *Salmonella cholerasuis, Vibrio Parahaemolyticus* (Fig. 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.

D. Effect of Mastoparan B on the Permeability of Bacterial Cytoplasmic Membrane

Since mastoparan B show the bactericidal activity of *S. aureus* (Table I and Fig. 3), it was hypothesized thata target of mastoparan B was the bacterial membrane [23]. Hence, the effect of the mastoparan B on the permeability of *S. aureus*

cytoplasmic membrane was investigated. The change in permeability was assayed by measuring the level of the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside-6-phosphate, a chromogenic substrate for phosphor-b-galactosidase of *S. aureus*. As a result, the hydrolysis of ONPG-6-phosphate was increase by the concentration of the mastoparan B, suggest the mastoparan B increases the permeability of the cytoplasmic membrane of *S. aureus*.

The cells containing induced phosphor-b-galactosidase activity with galactose were incubated at 30℃, for 20 min with the indicated concentration of mastoparan b. After addition of onpg-6-phosphate, the level of hydrolysis of the substrate was assay by measuring the absorbance at 405 nm. The level of the hydrolysis after incubating the cells at 30℃ for 20 min with triton x-100 was defined as 100%.

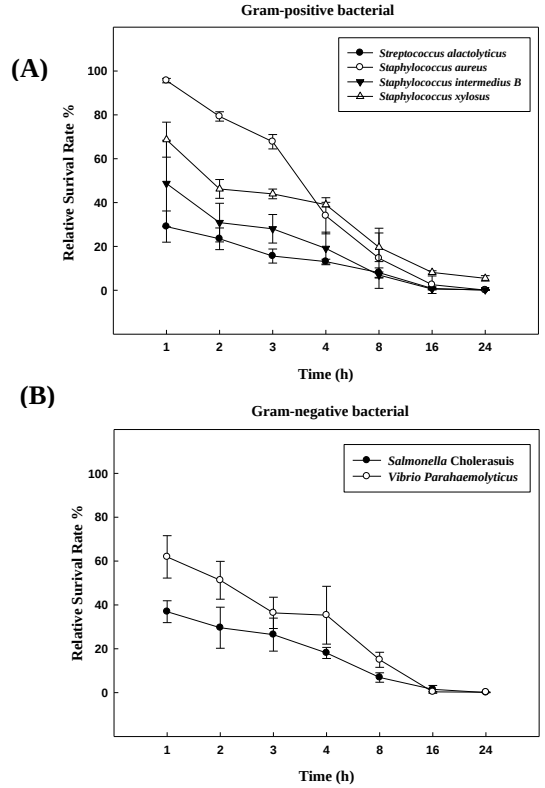

Fig. 3. Kinetic of mastoparan B bacterial versus gram positive and Gram-negative bacteria. At the 2x MBC of the mastoparan B for treating the bacterial was incubated at 37 ºC for increasing timers (0-24h).

TABLE II: EFFECT OF MASTOPARAN B ON THE PERMEABILITY OF *S.* AURESUS MEMBRANE.

Mastoparan B (μg/ml)	**Hydrplysis of ONPG-6-phosphate (%)**
0.1	3.5
0.2	10.2
0.4	17.5
0.8	25.7
1.6	28.4
3.2	51.7
6.4	72.6

IV. 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 I) 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) [24]-[25].

Membrane-lytic peptides have been studied extensively in order to understand general aspects related to peptide-lipid interactions, as well as the relation of these interactions to the biological function of these peptides. The largest and most studied group out of those described above includes melittin [26], cecropin [27], magainin [28], alamethicin [29], and δ-hemolysin[30], all of these polypeptides considerably in chain length, hydrophobicity and overall distribution of charge, but share a common α-helical structure when associated with phospholipid membranes [31]. Mastoparan B exhibited a CD spectrum rich in α-helix conformation in Tris-buffered saline containing 20% trifluoroethanol (Ho et al., 1996; Chuang et al., 1996). And the results indicated that a target of mastoparan B is the bacterial membrane (Table I). The α-helix structure of mastoparan B seems to be responsible for the increase the permeability of *S. auresus*. It is like melittin which exhibit an α-helix structure would bind and permeabilize bacterial membranes.

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.

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