Optimization for Expression of *Culex Quinquefasciatus* Antimicrobial Peptide Gambicin

Lalitra Udomrak* Phanthila Sirichaiyakul* Dr.Yoshihura Matsuura**

Dr.Toru Okamoto*** Dr.Dumrongkiet Arthan****

ABSTRACT

Conventional antibiotic resistance in pathogenic bacteria has been rising during the last few decades. Therefore, antimicrobial peptides (AMPs) are interesting option for developing compound as alternative antibiotics. Previously, *Culex quinquefasciatus* (CQ) gambicin, an AMPs mosquito has been successfully expressed in *Pichia pastoris* GS115 but no activity was observed. We hypothesized that recombinant CQ gambicin peptide without antimicrobial activity may be caused by the proteolytic degradation by protease which is unusually found during the production of recombinant proteins and improper protein expression system. To address these problems, we therefore aimed to express CQ gambicin in not only protease-deficient *Pichia pastoris* strain but also baculovirus expression system to obtain active CQ gambicin. The CQ gambicin without signal peptide was successfully expressed in protease-deficient *Pichia pastoris* SMD 1168 because SDS-PAGE revealed protein band with a molecular weight of about 7 kDa. However, no its antimicrobial activity was observed. For the baculovirus expression system, the CQ gambicin without signal peptide was successfully fused with honeybee melittin, maltose-binding protein, and tobacco etch virus protease (HBM-MBP-TEV) tag which is useful for facilitating the proper folding and solubility of the target protein. It showed a chemiluminescent band at 53 kDa corresponding to CQ gambicin fused with HBM-MBP-TEV. In addition, the CQ gambicin without signal peptide was successfully fused with Glutathione S-transferases (GST) tag which is useful for protecting protein from the intracellular protease. It showed a chemiluminescent band at 34 kDa for CQ gambicin fused with GST. Neither recombinant CQ gambicin fused with HBM-MBP-TEV nor GST showed no antimicrobial activity. To recover active CQ gambicin, further study are required for protein refolding approach. In addition, expression of recombinant CQ gambicin in mammalian cells may be an alternative way to obtain the active CQ gambicin.

Keywords: Gambicin, Antimicrobial peptides (AMPs), *Culex quinquefasciatus*

---

* *Student, Master of Science Program in Tropical Medicine, Faculty of Tropical Medicine, Mahidol University*

** *Professor, Department of Molecular Virology, Research Institute for Microbial Disease, Osaka University*

*** *Assistant Professor, Department of Molecular Virology, Research Institute for Microbial Disease, Osaka University*

**** *Assistant Professor, Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University*

Introduction

The frequency and spectrum of antibiotic resistant bacterial infections have been rising during the last few decades. Antibiotics resistance in bacteria is also an important cause of morbidity and mortality. The resistant ability of microorganisms was associated with over-used drugs, inappropriate treatment and prolonged therapy of infected patients. Therefore, it is an urgently need for the development of novel generations of effective antibiotics.

Antimicrobial peptides (AMPs), small cationic peptides produced by various organisms, are currently being considered as potentially alternative antibiotics. It has been widely recognized that many organisms use AMPs as a major part of their immediately effective, particularly innate immunity. Since AMPs typically have a broad spectrum of activity against pathogenic bacteria and fungi, enveloped viruses, parasites and even cancerous cells (Nguyen *et al*., 2011). Focusing on AMPs in mosquito, three well-known AMPs are cecropin, defensin and gambicin (Koczulla, Bals, 2003). Cecropin and defensin have been studied in depth, but not gambicin. A gambicin from *Anopheles gambiae* has been reported for antimicrobial activity against gram positive and gram negative bacteria, filamentous fungi and plasmodium ookinetes (Vizioli *et al*., 2001). However, there have not been studies about the potential of AMPs which are isolated and characterized from *Culex quinquefasciatus* (CQ). Since CQ is mosquito species of which lives in the dirty water contains plenty of pathogenic bacteria, therefore we hypothesized that CQ gambicin may possess higher potential antimicrobial activity comparing to other mosquito gambicins.

Based on Sirichaiyakul *et al*. (2014)'s study, the recombinant CQ gambicin peptide was successfully expressed by methylotrophic yeast *Pichia pastoris* GS115. However, the recombinant CQ gambicin showed no antimicrobial activity against *Escherichia coli* using agar-well diffusion method.

We hypothesized that recombinant CQ gambicin peptide without antimicrobial activity may be caused by (i) the proteolytic degradation by protease which is unusually found during the production of recombinant proteins (ii) improper protein expression system. To address these problems, we will perform experiments including; (i) expression of CQ gambicin in protease-deficient *Pichia pastoris* strain (ii) express CQ gambicin in baculovirus expression system.

Objective of the study

This study aimed to optimize the expression system for producing active antimicrobial peptide from *Culex quinquefasciatus* including yeast expression system using protease-deficient *Pichia pastoris* strain and baculovirus expression system.

Research Methodology

Expression CQ gambicin in protease-deficient *Pichia pastoris* SMD1168

Transformation of recombinant plasmid into *P. pastoris* SMD1168

The pPICZαB plasmid harboring CQ gambicin gene without signal peptide was successfully constructed by Sirichaiyakul *et al*. (2014). Expression of the recombinant protein in protease-deficient *P. pastoris* SMD1168 was performed according to the

manufacture protocol (Invitrogen). Briefly, the selected recombinant expression plasmid was linearized with SacI (TAKARA), and then transformed into a *P. pastoris* SMD1168 by electroporation. Transformants were first screened from YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) plates containing zeocin at 150 µg/ml. A negative control was *P. pastoris* transformed with an empty pPICZαB plasmid.

Expression of recombinant CQ gambicin in *P. pastoris* SMD1168

The *P. pastoris* SMD1168 transformant harboring CQ gambicin peptide was used for production of the recombinant protein. Each transformant was inoculated in BMGY broth (1% yeast extract; 2% peptone; 1 M potassium phosphate pH 6.0; 1.34% YNB; 4 x 10–5 % biotin; 1% glycerol) and cultured in a shaking incubator at 30°C and 250 rpm, until A_{600} reaching at 5. Cells were harvested and resuspended in BMMY medium (1% yeast extract; 2% peptone; 1 M potassium phosphate pH 6.0; 1.34% YNB; 4 x 10–5 % biotin; 0.5% methanol) to induce recombinant CQ gambicin expression. Subsequently, the cell solution was cultured in a shaking incubator at 250 rpm and 16°C. Methanol at 0.5% was added to the culture every 24 hr to maintain induction. After 5 days, the cell-free supernatants were collected and stored at 4°C until used.

Optimization the condition for recombinant CQ gambicin expression

Expression of the recombinant CQ gambicin in *P. pastoris* SMD1168 was performed as previous described. The temperature at 16°C and 30°C were used to optimize the expression condition. Additionally, methanol-induction was adjusted to a final concentration of 0.5%, 1%, and 2% to optimize the condition as well.

SDS-PAGE analysis

SDS-PAGE was performed with 4% stacking gel and 15% resolving gel according to Laemmli (1970) to determine both degree of purity and the subunit molecular weight.

Table 1 specific primer for PCR-amplified

Gene	Primers sequences (5'-3')
CQ gambicin fused with GST	F: GGTTCCGCGTGGATCCTGGGTGTATGTCTATGCGAA
	R: GGGAATTCGGGGATCCTCACCCAATGAAGCACTCGG
CQ gambicin fused with GST and 6xhistidine	F: GGTTCCGCGTGGATCCTGGGTGTATGTCTATGCGAA
	R: GGGAATTCGGGGATCCCTAATGGTGATGGTGATGAT
CQ gambicin fused with HBM-MBP-TEV	F: CTTCCAATCCGAATTCTGGGTGTATGTCTATGCGAA
	R: GCTCGTCGACGAATTCTCACCCAATGAAGCACTCGG
CQ gambicin fused with HBM-MBP-TEV and 6xhistidine	F: CTTCCAATCCGAATTCTGGGTGTATGTCTATGCGAA
	R: GCTCGTCGACGAATTCCTAATGGTGATGGTGATGAT

Expression CQ gambicin in baculovirus expression system

Construction of recombinant plasmids harboring CQ gambicin gene without signal peptide

The full-length CQ gambicin gene from Sirichaiyakul *et al*. (2014) was used as a template for PCR-amplified CQ gambicin using specific primers as shown in Table 1. The PCR products including CQ gambicin fused with GST, CQ gambicin fused with GST and 6xhistidine, CQ gambicin fused with HBM-MBP-TEV, and CQ gambicin fused with HBM-MBP-TEV and 6xhistidine were cloned into pFastBac1GST vector and pFastBac1HBM-MBP-TEV vector.

Subsequently, the plasmids were transformed into competent *E. coli* STBL2. After DNA sequencing, each positive clones was extracted for their recombinant plasmid by Mediprep of PureLink HiPure Plasmid DNA Purification Kit (Invitrogen).

Construction of recombinant bacmids harboring CQ gambicin gene

A recombinant bacmid harboring CQ gambicin without signal peptide were generated according to the modified instruction manual of Invitrogen Company. Briefly, the recombinant plasmids including pFastBac1GST harbouring CQ gambicin gene, pFastBac1GST harbouring CQ gambicin gene with 6xhistidine, pFastBac1HBM-MBP-TEV harbouring CQ gambicin gene, and pFastBac1HBM-MBP-TEV harbouring CQ gambicin gene with 6xhistidine were transformed into DH10Bac™. Then the recombinant plasmid clones were screened by the selection of the blue/white colony.

Production of recombinant CQ gambicin in Sf9 cells

The Sf9 cell lines were cultured in Sf900-II free serum medium (Gibco) and verified for their number and viability. The 1 x 10^6 cells were plated in each well of 6-well plate. Sf900-II free serum medium (Gibco) without penicillin/streptomycin 500 µl was added in each recombinant bacmid harboring gambicin. Then Sf9 cell lines were transfected with the recombinant bacmid with transfection reagent (X-tremeGENE HP DNA transfection reagent, Roche). The Sf9 cell lines were incubated at 27°C for viral infection for 4 days. The supernatant of culture medium was harvested and stored as the initial virus production (P1 viral stock) which was used to infect into Sf9 cells. After infecting for 4 days, the supernatant of infected culture medium were stored as the second passage of amplified virus (P2 viral stock). The cell pellet were lysed and detected for the CQ gambicin expression.

Western blot analysis for recombinant CQ gambicin expressed in baculovirus system

The SDS-PAGE analysis of cell lysates were performed. The protein bands on the SDS-PAGE gel was transferred to the nitrocellulose membranes using iBlot™ Gel Transfer Device (Invitrogen). For detecting GST fused with CQ gambicin, the membrane was incubated with skim milk containing a 1:1000 dilution of primary antibody; α-GST Goat (GE Healthcare Life Sciences) and skim milk containing 1:2500 dilution of secondary antibody; a α-Goat HRP (GE Healthcare Life Sciences). For detecting HBM-MBP-TEV fused with CQ gambicin the membrane was incubated with skim milk containing a 1:1000 dilution of α-FLAG Goat HRP (GE Healthcare Life

Sciences). Finally, the membrane was incubated with the SuperSignal West femto chemiluminescent substrate, (Thermo Scientific) and visualized under luminescent image analyzer (ImageQuant LAS4000mini, GE Healthcare Life Sciences).

Antimicrobial activity assay

To assay antimicrobial activity, MIC of recombinant CQ gambicin was determined by agar-well diffusion method. Cells in broth was adjusted to 0.5 McFarland standards by using 0.9% normal saline solution. These cultured medium was streaked onto Mueller-Hinton agar plate for 3 dimensions. The wells were then done by punching with a diameter of 5 mm. All tested solutions and ampicillin as a positive control were filled in agar wells. The agar plate was incubated at 37°C for 18 hr. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the recombinant CQ gambicin in agar plates illustrating visible inhibition zone.

Results

Expression CQ gambicin in protease-deficient *Pichia pastoris* SMD1168

To induce the expression, methanol at 0.5% was added into the cultured *P. pastoris* transformant harboring CQ gambicin every 24 hr. Cell-free supernatants (CFS) were collected at day 5 to determine the protein profiles by SDS-PAGE. *P. pastoris* transformants harboring CQ gambicin gene without signal peptide displayed a band of protein at molecular weight about 7 kDa (Figure 1, lane 3 and 4), whereas those of harboring a full-length CQ gambicin showed no band at 7 kDa (Figure 1, lane 5 and 6).

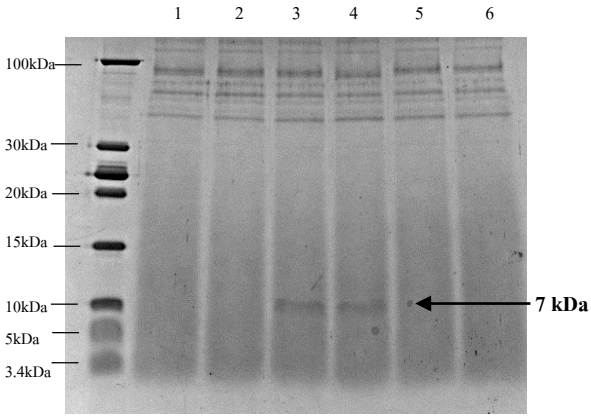

Figure 1 SDS-PAGE analysis of CFS obtained from clones of *P. pastoris* harboring empty pPICZaB vector (lane 1 and 2), CQ gambicin gene without signal peptide (lane 3 and 4) and full-length CQ gambicin gene (lane 5 and 6).

Expression of the recombinant CQ gambicin at 16°C showed a protein band on SDS-PAGE with a molecular weight of about 7 kDa but not 30°C (data not shown). For methanol-induction, the band at 7 kDa of 1% methanol showed more intense band than the other (Figure 2, lane 3). Therefore, the optimized condition for CQ gambicin expression was at 16°C and 1% of methanol.

Expression CQ gambicin in baculovirus expression system

DNA sequencing revealed that CQ gambicin genes without signal peptide (with/without 6xhistide) were successfully cloned into pFastBac1HBM-MBP-TEV and pFastBac1GST. The positive clones harboring recombinant bacmid were obtained as white colony.

After transfection of recombinant bacmid harboring CQ gambicin fused with HBM-MBP-TEV

and GST protein tag into Sf9 cells, in which the target protein was intracellularly expressed, the supernatant was collected as a viral stock P1. Then viral stock of bacmid harboring HBM-MBP-TEV-gambicin (with/without 6xhistide) and GST-gambicin (with/without 6xhistide) were infected into Sf9 cells. The infected cell lysates were detected for recombinant CQ gambicin fused with protein tag by western blot analysis as shown in Figure3.

The result revealed the chemiluminescent band at 53 kDa (Figure 3A, lane 1) and 54 kDa (Figure 3A, lane 2) corresponding to CQ gambicin fused with HBM-MBP-TEV and that for CQ gambicin fused with HBM-MBP-TEV and 6xhistide, respectively, which were larger than that for HBM-MBP-TEV fusion protein tag at 46 kDa. In addition, CQ gambicin fused with GST with- and without- 6xhistide showed the chemiluminescent band at 34 kDa and 35 kDa, respectively (Figure 3B, lane 1 and 2), which were larger than that for GST fusion protein tag at 27 kDa.

Antimicrobial activity testing for recombinant CQ gambicin

Cell-free supernatants (CFS) collected at day 5 from clones of *P. pastoris* harboring CQ gambicin gene without signal peptide were tested for their antimicrobial activity against *E. coli* by agar-well diffusion assay. The results showed no inhibition zones when compared with ampicillin as a positive control (Figure 4). After 10-fold concentrated clude recombinant CQ gambicin, the result still showed no antimicrobial activity were observed.

There was no any inhibition zone of the recombinant CQ gambicin fused with HBM-MBP-TEV (with/without 6xhistide) as well as recombinant CQ gambicin fused with GST (with/without 6xhistide) expressed by baculovirus expression system comparing to ampicillin as s positive control.

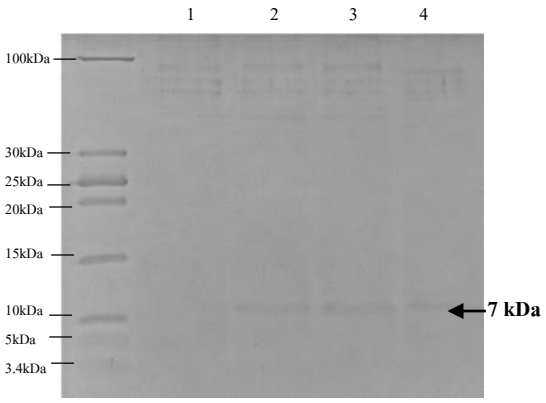

Figure 2 SDS-PAGE analysis of CSF obtained from clones of *P. pastoris* harboring CQ gambicin gene without signal peptide. Induction the recombinant CQ gambicin by adding methanol at 0.5%, 1% and 2% were shown in lane 2, 3 and 4, respectively.

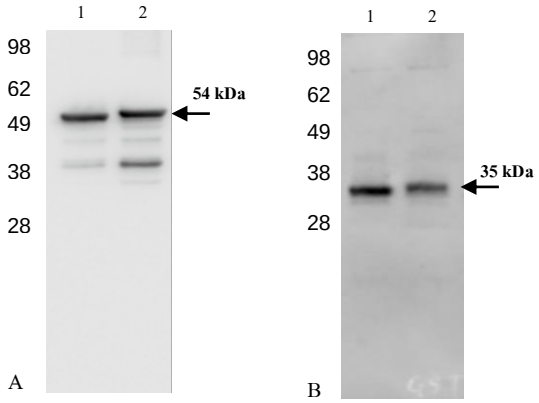

Figure 3 Western blot analysis of gambicin obtained from lysed sf9 cells. A CQ gambicin fused with HBM-MBP-TEV tag; lane 1 with 6xhistidine, lane 2 without 6xhistidine. B CQ gambicin fused with GST tag; lane 1 with 6xhistidine, lane 2 without 6xhistidine.

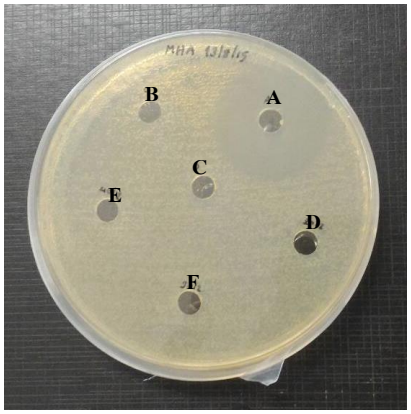

Figure 4 The result of agar-well diffusion assay displayed no inhibition zone. (A) ampicillin (positive control); (B) pPICZαB (negative control); (C) sterile water (negative control); (D), (E), (F) recombinant CQ gambicin

Discussion and Conclusion

The *P. pastoris* expression has become successfully for the production of several recombinant protein, also antimicrobial peptide (Jin *et al*., 2006; Rashid *et al*., 2009). This expression system offers several advantages over bacterial expression system including appropriate folding of molecules and disulfide bond formation, as well as execution of post-translational modifications which conserve protein function (Guo *et al*., 2012).

Recombinant *Culex quinquefasciatus* gambicin peptide was successfully expressed in *P. pastoris* GS115, but two bands of recombinant gambicin were detected on SDS-PAGE (Sirichaiyakul *et al*., 2014). We hypothesized that two bands of recombinant CQ gambicin may be caused by the proteolytic cleavage. To solve this problem, *P. pastoris* protease-deficient strain, namely *P. pastoris* SMD1168 were used, the result revealed that only a band of recombinant CQ gambicin was found on SDS-PAGE, indicating that no proteolytic cleavage of CQ gambicin, yet it still exhibited no antimicrobial activity against *E. coli*. Inactive gambicin might cause by the incorrect disulfide bond formation during translation process due to disulfide mispairing and irreversible aggregation (Wu *et al*., 2003; Szyk *et al*., 2006; Yamaguchi, Miyazaki, 2014).

For the baculovirus expression system, western blot analysis revealed that neither recombinant CQ gambicin fused with GST nor HBM-MBP-TEV successfully expressed in insect Sf9 cells exhibited no antimicrobial activity. Inactive CQ gambicin might be affected from interfering of the fusion protein to its proper folding of 3-dimensional structure. Although, GST-tag can help to protect protein from the intracellular protease and stabilize the recombinant protein, it may impede the correct protein folding. For this reason, further study are required for the cleavage GST fusion tag by a site-specific protease, thrombin (Terpe, 2003), which may recovery of antimicrobial activity. Similarly, the role of HBM, MBP fusion tags are involved in enhancing the solubility of many proteins (Raran-Kurussi *et al*., 2015) and facilitate to proper folding of disulfide-bonded proteins (Riggs, 2000; Lebendiker, Danieli, 2011). It may not correct the protein folding as their natural structure.

In conclusion, we can produce the recombinant CQ gambicin form both yeast and baculovirus expression system. However, it still exhibited no antimicrobial activity against *E. coli*. Further studies aim to perform refolding approach to recover its correct native structure. In addition, the expression of CQ gambicin in mammalian cells is alternative way to obtain the active CQ gambicin.

Acknowledgements

This project was financially supported by the Faculty of Tropical Medicine (Mahidol University). P.S. received the Global Education Scholarships by Mahidol University and partially supported by Department of Molecular Virology, Research Institute for Microbial Disease (Osaka University) to be trained at Osaka University. Additionally, the authors are grateful to Dr. Amornrat Aroonnual and Assist. Prof. Dr. Yuvadee Mahakunkijcharoen from Faculty of Tropical Medicine, Mahidol University.

References

Guo C, Huang Y, Zheng H, Tang L, He J, Xiang L, *et al*. Secretion and activity of antimicrobial peptide cecropin D expressed in *Pichia pastoris.* Experimental and therapeutic medicine 2012; 4: 1063-1068.

Jin F, Xu X, Zhang W, Gu D. Expression and characterization of a housefly cecropin gene in the methylotrophic yeast, *Pichia pastoris*. Protein Expression and Purification 2006; 49: 39-46.

Koczulla A., Bals R. Antimicrobial peptides: current status and therapeutic potential. Drugs 2003; 63: 389-406.

Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Lebendiker M, Danieli T. Purification of proteins fused to maltose-binding protein. Methods in Molecular Biology 2011; 681: 281-293.

Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. Trends in Biotechnology 2011; 29: 464-472.

Raran-Kurussi S, Keefe K, Waugh DS. Protein Expression and Purification 2015; 110: 159-164.

Rashid F, Ali I, Sajid I, Elamin S, Yuan Q. Recombinant expression of an antimicrobial peptide Hepcidin in *Pichia pastoris*. Pakistan Journal of Zoology 2009; (5)41: 349-356.

Riggs P. Expression and purification of recombinant proteins by fusion to maltose-binding protein. Molecular Biotechnology 2000; (1)15: 51-63.

Sirichaiyakul P, Suthangkornkul R, Thepouyporn A, Tamaki O, Matsuura Y, Takedaet N, *et al*. Expression and characterization of antimicrobial peptide gambicin from *Culex quinquefasciatus* in *Pichia pastoris.* Proceedings of the 2nd ASEAN Plus Three Graduate Research Congress (2ndAGRC); 2014 Feb 5-7; Bangkok. 2014; 607-614.

Szyk A, Wu Z, Tucker K, Yang D, Lu W, Lubkowski J. Crystal structures of human a-defensins HNP4, HD5, and HD6. Protein Science 2006; 15: 2749–2760.

Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Applied Microbiology and Biotechnology 2003; 60: 523-533.

Vizioli J, Richman AM, Uttenweiler-Joseph S, Blass C, Bulet P. The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. Insect Biochemistry and Molecular Biology 2001; 31: 241-248.

Wu Z, Powell R, Lu W. Productive Folding of Human Neutrophil r-Defensins in Vitro without the Pro-peptide. Journal of the American Chemical Society 2003; 125: 2402-2403.

Yamaguchi H, Miyazaki M. Refolding Techniques for Recovering Biologically Active Recombinant Proteins from Inclusion Bodies. Biomolecules 2014; 4: 235-251.