ลักษณะสมบัติของ Viral Responsive Protein 15 และบทบาทที่เป็นไปได้ในการนำไวรัสเข้า/ออกนิวเคลียสในกุ้งกุลาดำ *Penaeus monodon*

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CHARACTERIZATION OF VIRAL RESPONSIVE PROTEIN 15 AND ITS POSSIBLE ROLE IN NUCLEAR IMPORT/EXPORT OF VIRUS IN BLACK TIGER SHRIMP *Penaeus monodon*

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Miss Krisadaporn Jaturontakul

A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Biochemistry
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By	Miss Krisadaporn Jaturontakul
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Assistant Professor Kuakarun Krusong, Ph.D.
Thesis Co-Advisor	Professor Anchalee Tassanakajon, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Faculty of Science

(Professor Supot Hannongbua, Ph.D.)

576808664

THESIS COMMITTEE

....Chairman

(Professor Piamsook Pongsawasdi, Ph.D.)

....Thesis Advisor

(Assistant Professor Kuakarun Krusong, Ph.D.)

....Thesis Co-Advisor

(Professor Anchalee Tassanakajon, Ph.D.)

....Examiner

(Assistant Professor Rath Pichyangkura, Ph.D.)

....External Examiner

(Kallaya Sritunyalucksana-Dangtip, Ph.D.)

กฤษดาพร จาตุรนตกุล : ลักษณะสมบัติของ Viral Responsive Protein 15 และบทบาทที่เป็นไปได้ในการนำไวรัส เข้า/ออกนิวเคลียสในกุ้งกุลาดำ *Penaeus monodon*. (CHARACTERIZATION OF VIRAL RESPONSIVE PROTEIN 15 AND ITS POSSIBLE ROLE IN NUCLEAR IMPORT/EXPORT OF VIRUS IN BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.เกื้อการุณย์ ครูส่ง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.อัญชลี ทัศนาขจร, 134 หน้า.

Viral responsive protein 15 (*Pm*VRP15) ค้นพบจากการระบุยีนที่มีการแสดงออกเพิ่มสูงขึ้นในเซลล์เม็ดเลือดของกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาวในระยะต้น (24 ชม.) และระยะปลาย (48 และ 72 ชม.) ด้วยเทคนิค suppression subtractive hybridization (SSH) เมื่อทำนายโครงสร้างโปรตีนพบว่า *Pm*VRP15 ประกอบด้วยส่วนของ transmembrane อยู่ 1 ส่วน จากการเลี้ยงเซลล์เม็ดเลือดกุ้งกุลาดำ (primary hemocyte cultures) พบว่ายีน *Pm*VRP15 มีการแสดงออกสูงขึ้นประมาณ 1.1, 2.6, 3.6, 6.7 และ 4.1 เท่าหลังจากติดเชื้อไวรัสตัวแดงดวงขาว ที่เวลา 6, 12, 24, 48 และ 72 ชม. ตามลำดับ ผลการยับยั้งการแสดงออกของยีน *Pm*VRP15 ด้วยเทคนิค RNA interference พบว่า เมื่อฉีด dsRNA *Pm*VRP15 ส่งผลให้อัตราการเพิ่มจำนวนของไวรัสโรคตัวแดงดวงขาวลดลงถึง 4.2 เท่าเมื่อเปรียบเทียบกับกลุ่มควบคุม จึงกล่าวได้ว่ายีนดังกล่าวมีผลต่อการเพิ่มจำนวนของไวรัสตัวแดงดวงขาว เมื่อวิเคราะห์บริเวณที่มีการแสดงออกของโปรตีน *Pm*VRP15 ด้วยเทคนิค confocal laser scanning microscopy พบว่าโปรตีน *Pm*VRP15 มีการแสดงออกบริเวณนิวเคลียร์เมมเบรน และเมื่อสกัดแยกเซลล์เม็ดเลือดกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว พบโปรตีน *Pm*VRP15 อยู่ในส่วนใสของนิวเคลียสและส่วนที่จับกับโครมาติน ดังนั้น *Pm*VRP15 อาจเกี่ยวข้องหรือทำหน้าที่เป็นส่วนหนึ่งของเมมเบรนโปรตีนในนิวเคลียส เป็นไปได้ว่า *Pm*VRP15 เกี่ยวข้องกับการเข้าสู่นิวเคลียสหรือออกจากนิวเคลียสของเชื้อไวรัสตัวแดงดวงขาว จึงทดสอบโดยการหาจำนวนไวรัสตัวแดงดวงขาวในส่วนนิวเคลียสและไซโทพลาสซึมในสภาวะที่ยับยั้งการแสดงออกของยีน *Pm*VRP15 ในกุ้งติดเชื้อไวรัสตัวแดงดวงขาวเปรียบเทียบกับกลุ่มควบคุม ผลการทดลองพบว่าการยับยั้งการแสดงออกของยีน *Pm*VRP15 ส่งผลให้จำนวนไวรัสตัวแดงดวงขาวในส่วนนิวเคลียสต่อไซโทพลาสซึมลดลงกว่ากลุ่มควบคุมถึง 9.3 เท่า จึงกล่าวได้ว่า *Pm*VRP15 อาจมีผลต่อการเข้าสู่นิวเคลียสของเชื้อไวรัสตัวแดงดวงขาว เนื่องจากการตกผลึกโปรตีนเป็นอีกวัตถุประสงค์หนึ่งของงานวิจัยนี้ จึงทำการผลิตโปรตีนรีคอมบิแนนท์ *Pm*VRP15 ทั้งในระบบยีสต์ *Saccharomyces cerevisiae* และแบคทีเรีย *Escherichia coli* พบว่าโปรตีนรีคอมบิแนนท์ *Pm*VRP15 มีการแสดงออกเพียงเล็กน้อยในระบบยีสต์ *S. cerevisiae* สายพันธุ์ FGY217 แต่มีการแสดงออกสูงในแบคทีเรีย *E. coli* สายพันธุ์ C43 (DE3) ที่เวลา 1-3 ชม.หลังจากเหนี่ยวนำการแสดงออกของโปรตีนด้วย IPTG เข้มข้น 1 มิลลิโมลาร์ จากนั้นทำโปรตีนให้บริสุทธิ์ด้วยนิกเกิลคอลัมน์และคอลัมน์แบบแลกเปลี่ยนไอออน DEAE ตามลำดับ เมื่อวิเคราะห์น้ำหนักโมเลกุลของโปรตีนด้วย MALDI-TOF พบว่า โปรตีน r*Pm*VRP15 มีขนาด 15.899 กิโลดาลตัน และจากการวิเคราะห์โครงสร้างทุติยภูมิด้วยเทคนิค Circular Dichroism spectroscopy พบว่าโปรตีน r*Pm*VRP15 ประกอบด้วยส่วนของแอลฟาเฮลิกซ์ 48.45 % และเบต้าชีท 13.57 % นอกจากนี้ยังหาสภาวะที่เหมาะสมในการตกผลึกโปรตีน r*Pm*VRP15 เพื่อใช้ในการวิเคราะห์โครงสร้างสามมิติด้วยเทคนิค X-ray crystallography โดยสภาวะที่เหมาะสมที่ทำให้เกิดผลึกคือ Index^{TM} D12 ซึ่งพบจุดที่เกิดจากการเลี้ยวเบนของโปรตีน อย่างไรก็ดีจะทำการปรับปรุงสภาวะเพื่อให้ได้ผลึกที่เหมาะสำหรับการวิเคราะห์โครงสร้างต่อไป

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KRISADAPORN JATURONTAKUL: CHARACTERIZATION OF VIRAL RESPONSIVE PROTEIN 15 AND ITS POSSIBLE ROLE IN NUCLEAR IMPORT/EXPORT OF VIRUS IN BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: ASST. PROF. KUAKARUN KRUSONG, Ph.D., CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., 134 pp.

A viral responsive protein 15 (*Pm*VRP15) has been identified from suppression subtractive hybridization library at the early (24 h) and late phase (48/72h). *Pm*VRP15 was highly up-regulated in the hemocyte of white spot syndrome virus-infected *Penaeus monodon*. A protein domain prediction indicated that *Pm*VRP15 consisted of a transmembrane helix. In primary hemocyte cultures, *Pm*VRP15 mRNA expression was up-regulated about 1.1, 2.6, 3.6, 6.7 and 4.1 fold at 6, 12, 24, 48 and 72 h post-WSSV infection, respectively. After *Pm*VRP15 gene silencing by RNA interference (RNAi), VP28 transcript was reduced by 4.2 fold at 24 h post WSSV-infection, compared to normal WSSV-infected *P. monodon* hemocytes. These results suggested that *Pm*VRP15 is important to WSSV propagation. Confocal laser scanning microscopy study, revealed that *Pm*VRP15 localized at the nuclear membrane. In this study, subcellular fractionation of WSSV-infected hemocytes showed that *Pm*VRP15 was probed in soluble nuclear and chromatin-bound fractions. Taken together, *Pm*VRP15 may function in nucleus as a part of membrane protein or related with membrane protein. It is possible that *Pm*VRP15 involves in nuclear import/export of WSSV. To test this hypothesis, ratio of WSSV DNA in nuclear and cytoplasmic fractions of normal and *Pm*VRP15-silenced hemocytes were compared. Knockdown of *Pm*VRP15 resulted in a lower ratio of WSSV copy number in nuclear to cytoplasmic fractions by 9.3 fold, compared to that of control, indicating that *Pm*VRP15 may involve in nuclear entry of WSSV. For crystallization purpose, r*Pm*VRP15 was expressed in *Saccharomyces cerevisiae* and *Escherichia coli* expression system. Full-length r*Pm*VRP15 was expressed at low level in *S. cerevisiae* (FGY217) but overexpressed in *E. coli* C43 (DE3) at 1-3 h after IPTG-induction. The r*Pm*VRP15 was purified by Ni-NTA and DEAE columns, respectively. MALDI-TOF MS revealed that the molecular weight of *Pm*VRP15 is 15.899 kDa. Circular dichroism spectroscopy has been used to examine secondary structure of *Pm*VRP15. *Pm*VRP15 contains 48.45 % of alpha-helix and 13.57 % of beta-sheet. In addition, screening of *Pm*VRP15 crystallization conditions was performed in order to obtain a crystal for structure determination by X-ray crystallography. Crystals produced in Index^{TM} D12 gave close spots in diffraction, suggesting the presence of protein. This condition will be optimized for further X-ray crystallographic study.

Department: Biochemistry
Field of Study: Biochemistry and Molecular Biology
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Student's Signature Krisadaporn Jaturontakul
Advisor's Signature ....
Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

A	Ampere
bp	Base pair
Da	Dalton
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EST	Expressed sequence tag
F	Forward
FBS	Fetal bovine serum
g	Gram
GFP	Green fluorescence protein
h	Hour, Hours
HPI	Hour post infection
K	Kilo
l	Liter
LB	Luria-Bertani
m	Milli
M	Molar
min	Minute, Minutes
MW	Molecular weight
No.	Number
PAGE	polyacrylamide electrophoresis
PCR	Polymerase chain reaction
PBS	1x phosphate buffered saline

Pm	*Penaeus monodon*
R	Reverse
Rb	Rabbit
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcription
RT	Room temperature
SD	Standard deviation
sec	Seconds
SPF	Specific pathogen free
SPI	Serine proteinase inhibitor
SSH	Suppression subtractive hybridization
ssRNA	Single-stranded RNA
THC	Total hemocyte number
µ	Micro
UV	Ultraviolet
V	Volt
VRP15	Viral responsive protein 15 kDa
WSSV	White spot syndrome virus
YHV	Yellow head virus
YPD	A blend of yeast extract, peptone, and dextrose in optimal proportions for growth of most strains of *S. cerevisiae*

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