รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษา membrane proteome ของเซลล์เม็ดเลือดกุ้งกุลาดำ ชนิดต่างๆในภาวะติดเชื้อไวรัสหัวเหลือง

โดย: ดร. ภัทรอร หาวนพันธ์

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สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

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Phattara-orn Havanapan

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Project Title : Characterization of membrane proteome in various hemocytic cell types of *Penaeus monodon* during Yellow Head Virus infection

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The molecular events mediating for viral infection and host defense mechanism in crustacean remain unclear, especially in virus – hemocyte interaction and their immune responses. We have speculated that distinct types of immune cells play different roles in response to virus entry and different cell mediators. Chemically biotinylation was performed for hyaline (agranular) and granule-containing hemocytes using Biotin-NSH-LC. Biotinylated protein was extracted and resolved by two-dimensional gel electrophoresis. Electro-transferred proteins on a nitrocellulose membrane were probed with streptavidin-HRP complex to detect biotinylated proteins whereas the purified biotinylated proteins were resolved on SDS-PAGE. Relative quantification analysis and tandem mass spectrometry were utilized to identify protein. The 6 and 8 proteins have been identified according to the differential protein expression found only in hyaline and granule-containing hemocytes, respectively, whereas 5 proteins were commonly found in both groups resulting from purification approach. Based on the classification of subcellular localization, these identified proteins were not restricted to the cell surface proteins. Currently, most of them can be revealed as membrane-associated proteins. It might be possible that some of these proteins could be located more than one subcellular localization for specific function. This present work provides the first data on the proteome of hemocyte subtypes by cell surface biotinylation technique. The possible functions of these membrane-associated proteins of hemocyte have been discussed. It was necessary to investigate the interaction of these proteins with shrimp virus to provide novel insight on the roles for these proteins in mediating internalization in hemocyte subtypes.

Keywords: *P. monodon*, Biotinylation, Hyaline, Granule-containing hemocytes

รหัสโครงการ: TRG5480004

ชื่อโครงการ: การศึกษา membrane proteome ของเซลล์เม็ดเลือดกุ้งกุลาดำชนิดต่างๆในภาวะติดเชื้อไวรัสหัวเหลือง

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การทดลองนี้ได้ทำการศึกษาโปรตีนบนผิวเซลล์ของเม็ดเลือดกุ้งกุลาดำชนิดที่ไม่มีแกรนูล (Hyaline hemocytes) และ ชนิดที่มีแกรนูล (semigranular และ granular hemocytes) จากการศึกษาที่ผ่านมาพบว่าเฉพาะเซลล์เม็ดเลือดกุ้งชนิดที่มีแกรนูลเท่านั้น ที่สามารถจับกับอนุภาคของไวรัสหัวเหลืองได้ จึงเป็นไปได้ว่าที่บริเวณผิวเซลล์ของเม็ดเลือดกุ้งชนิดที่มีและไม่มีแกรนูล น่าจะมีการแสดงออกรวมทั้งการตอบสนองของโปรตีน โดยเฉพาะที่เกี่ยวกับระบบภูมิคุ้มกันที่แตกต่างกันออกไป ดังนั้นในการทดลองนี้จึงทำการติดฉลากโปรตีนที่ผิวเซลล์ทั้งสองชนิดด้วยอนุพันธ์ไบโอติน (Biotin-NHS-LC) ซึ่งวิธีการติดฉลากด้วยอนุพันธ์ไบโอตินนี้ เป็นวิธีที่มีความจำเพาะต่อการคัดเลือกเฉพาะโปรตีนส่วนที่อยู่บนผิวเซลล์เท่านั้น โปรตีนที่มีการติดฉลากเรียบร้อยแล้วจะนำมาผ่านกระบวนการแยกโปรตีนแบบสองมิติ และสามารถตรวจพบโปรตีนที่มีการติดฉลากได้ด้วย streptavidin-HRP complex นอกจากนั้นโปรตีนที่มีการติดฉลากส่วนหนึ่งจะถูกทำให้บริสุทธิ์ และนำไปวิเคราะห์บนแผ่นพอลิอะคริลาไมด์ หลังจากนั้นทำการระบุชนิดของโปรตีนที่มีการติดฉลากด้วยแมสสเปคโตรเมทรี ผลวิจัยเบื้องต้นพบว่า มีการแสดงออกของโปรตีนจำนวน 6 และ 8 ชนิดที่พบในเม็ดเลือดกุ้งชนิดที่ไม่มีแกรนูลและมีแกรนูลเท่านั้น ตามลำดับ และโปรตีนจำนวน 5 ชนิดซึ่งสามารถพบได้ทั้งเม็ดเลือดกุ้งทั้งชนิดที่ไม่มีและมีแกรนูล ซึ่งพบว่าโปรตีนทั้งหมดที่สามารถระบุชนิดได้นี้ มิใช่โปรตีนที่อยู่บนผิวเซลล์ แต่อย่างไรก็ตามโปรตีนเหล่านี้มีบทบาทหน้าที่เกี่ยวข้องกับบริเวณที่ผิวเซลล์ จึงเป็นไปได้ว่าโปรตีนเหล่านี้น่าจะมีตำแหน่งที่อยู่ภายในเซลล์ได้มากกว่าหนึ่งตำแหน่ง เพื่อการทำหน้าที่เฉพาะอย่างที่แตกต่างกันออกไป โดยผู้วิจัยหวังว่าข้อมูลต่างๆจากแต่ละส่วนของการวิจัยในครั้งนี้จะนำไปสู่ความรู้ ความเข้าใจในระดับโมเลกุลต่อการติดเชื้อไวรัส และแนวทางในการป้องกันต่อไป

คำหลัก : กุ้งกุลาดำ, เซลล์เม็ดเลือดกุ้ง, แกรนูล, อนุพันธ์ไบโอติน

Introduction

Penaeid shrimp culture is an important Agro-industry worldwide. Thailand became the world's leader in exports due to the increased demand of shrimps in world markets. However, the current production of *Penaeus monodon* (black tiger shrimp) has been reduced significantly due to the diseases. Shrimps are exposed to a variety of opportunistic pathogens during growing out such as viruses, bacteria and fungi. Nevertheless, many diseases especially from viral origin reported around the world, for example, Yellow Head Virus (YHV), White Spot Syndrome Virus (WSSV), Hepatopancreatic Parvo-like virus (HPV) and Taura Syndrome Virus (TSV) have affected the industry. Upon an infection with YHV, the infected shrimps show a yellowish cephalothorax because of the abnormally yellow hepatopancreas. These infected shrimps are weak and swim slowly at the surface of the pond where they quickly die. Physically, the YHV is an enveloped, rod-shaped virus with diameter approximately 40 nm and 170 nm in length and a tubular helical nucleocapsid containing a positive-sense single stranded RNA of approximately 26 kb [1, 2]. Three major YHV structural proteins of gp116, gp64 and p20 have been detected by SDS-PAGE. The gp116 and gp64 have been reported to be glycosylated by sugar moieties. Moreover, gp116, but not gp64, has been shown to be responsible for the binding of virus particle to host cellular receptor and virus-neutralizing activity [3].

The innate immune system of shrimp responses to viral infection consists of cellular and humoral responses. The hemocytes (blood cells), the primary mediators of cellular immune responses in crustacean, play important roles in defense mechanisms. To date, the knowledge and

understanding about molecular response of shrimp-immune system *via* each specific cell type of hemocyte is still unclear. Our research group isolated and characterized at least three types of hemocytic cells by Percoll™ gradient centrifugation, consisted of hyaline (HC), semi-granular (SGC) and granular (GC) cells based on granularity and cell size. Confocal microscopy study at 12 hours post infection revealed YHV particles were detected in SGC and GC. However, no virus could be detected in the hyaline cells. The SGC of penaeid shrimp contains higher virus loads and shows faster infection rates. It is apparently more susceptible to YHV infection even in other crustacean including crayfish [4]. Our preliminary data indicated that the YHV infection selectively affected specific subpopulations of hemocyte. It probably resulted from different type and number of specific binding protein in each cell type. To study of viral binding protein, it generally involved with characterization of membrane and surface protein (protein on the cell surface). In many cases, important function of membrane protein was emphasized on protein receptors that function on signalling from both environment and other cells. These molecular events can result in a cellular response such as signal transduction, proliferation, differentiation, apoptosis, degranulation, etc.

There were many methods to selectively extract membrane protein such as subcellular fractionation, using chemicals and membrane protein labeling. For cell surface biotinylation, biotin can be conjugated to many proteins containing exposed primary amines without altering the biological activities. Cell surface biotinylation has emerged as an important tool for studying the expression and regulation of receptors and transporter, differentiation, distribution of membrane proteins to epithelial cells [5-7]. Recently, the elucidation of membrane proteins was carried out by cell surface biotinylation both in vertebrate and invertebrate [5, 8-10]. For this research project, the

extraction by using chemicals methods which are the most commonly used to extract membrane proteins and cell surface biotinylation were selected to perform. However, the extraction by using chemical methods has some limitation. The lack of protein marker used for verification of purified membrane fraction. In this work, the selectively Sulfo-NHS-LC-biotin labeled proteins from granule-containing hemocytes and hyaline hemocytes were comparatively investigated by using 2-D gel based proteomics and then identified by mass spectrometry. Moreover, the study of viral-binding protein has been successfully done by utilizing different protocols such as VOPBA, biotinylated protein pull-down assay and yeast two hybrid screens in many organisms especially in human [6, 11-13]. There were several reports about the efficiency of the investigation of cellular receptors by VOPBA and a biotinylated protein pull-down assay together with LC-MS/MS [9, 10, 14, 15].

Information of these dataset from these biotinylated hemocytic proteins from the black tiger shrimp were employed not only to study the basic roles of membrane-associated proteins in shrimp innate immunity, but also to discover the novel targets proteins for viral entry into host cells especially granule-containing hemocytes.

Materials and Methods

1. Shrimp preparation

The specific pathogen-free black tiger shrimp, *P. monodon*, were obtained from Shrimp Genetic Improvement Center, Suratthani province, Thailand. The shrimp were reared in aerated artificial seawater with 25 part-per-thousand salinity at 25 ^{o}C. The artificial seawater was changed once daily. The healthy shrimps with average size of 30-35 g by weight were divided into control and YHV-infected group.

2. Hemocyte isolation

The healthy shrimps were injected with normal saline (0.45 M NaCl) whereas YHV-infected shrimps were injected with purified virus diluted (1:1,000) in normal saline. Shrimps were intramuscularly injected with 100 µl (per 25 g shrimp) of normal saline and diluted YHV for control and infected shrimps, respectively, using 26-gauge needle through the lateral surface of the fourth abdominal segment. After 1 hour post infection (hpi), shrimp hemolymph was collected from hemocoel using 21-gauge needle mixed with equal volume of cold modified Alsever's (AS) anticoagulant solution containing 19.3 mM Tri-sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA; pH 7.2 [16] and then kept on ice. For hemocyte isolation, the hemocyte subtypes were divided into two groups that are hyaline hemocytes and granule-containing hemocytes by using Percoll gradient centrifugation described previously by Soderhall and Smith [17]. Briefly, continuous 70% Percoll gradient solution in 0.33 M NaCl was generated by ultracentrifuge at 50,000 X *g* for 42 min. The collected hemolymph was loaded into generated-gradient 70% (v/v)

Percoll and centrifuged at 1,700 X *g* at 4 °C for 30 min. The upper band was collected as hyaline cells and two lower bands were collected as granule-containing hemocytes. All isolated cells were harvested. Percoll material was removed by centrifugation at 1,700 x g at 4 °C for 10 min. These intact cells were washed three times with cold AS solution before biotinylation assay.

3. Cell membrane preparation

3.1 Chemical method

Cell membranes were prepared according to the protocol of Martinez-Barragan and del Angel [18] with minor adaptation. Briefly, the cell pellets were lysed by the addition of 0.2% Triton X-100 in buffer containing 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA pH 8.0 and 1 mM PMSF and subsequently centrifuged at 600 X *g* for 5 min to remove nuclei and cell debris. The supernatant were collected and centrifuged at 6,000 X *g* and subsequently at 20,000 X *g* for 20 min. The crude membrane pellets were resuspended in 0.2% Triton X-100 in previous buffer. The protein concentration of the crude membrane preparation was determined by the Bradford assay. Store sample at -20 °C if not used immediately.

3.2 Biotinylation of membrane proteins

The isolated hemocytes were labeled by incubation with 5 mM sulfosuccinimidyl-6-[biotin-amido]hexanoate (Sulfo-NHS-LC-Biotin) (Pierce, Rockford, IL) in AS solution or in AS solution alone for negative control at 4 °C for 5 min at dark. The biotinylation reaction was quenched by 100 mM glycine-AS with incubation for 5 min. The excess Sulfo-NHS-LC-

Biotin was washed by 100 mM glycine-AS with incubation for 5 min for two times. The intact hemocytes were washed in AS solution, pelleted by centrifugation and solubilized in lysis buffer containing 8 M Urea, 2 M Thiourea, 2% (w/v) CHAPS, 50 mM DTT, protease inhibitor cocktails [GE Healthcare]. The hemocyte pellet was stored at -20 ^{o}C for further experiment. For protein extraction, the hemocyte pellet was further disrupted by ultrasonication for 20 min at 10 ^{o}C. Total hemocytic proteins were precipitated using methanol-acetone method. The precipitated proteins were washed three times with cold acetone. Protein concentration was determined by Bradford's assay using bovine serum albumin as the standard.

3.3 Purification of cell membrane biotinylation

Solubilized biotinylated proteins from granule-containing hemocytes and hyaline hemocytes were purified by affinity chromatography using NeutrAvidin^{TM} Agarose resins (Invitrogen), with minor modifications of the protocol supplied by the manufacturer. Briefly, 2.5 mL columns of NeutrAvidin^{TM} Agarose resins were prepared and extensively washed with PBS for two times. The lysed cells with biotinylated membrane proteins were again solubilized by ultrasonication. Then the solubilized solution was clarified by centrifugation at 13,000 rpm for 10 min at 4 ^{o}C. The clarified cell lysate was added to the NeutrAvidin^{TM} Agarose resins and were captured for 30 min at room temperature with a low rotator speed. The column was washed with PBS containing protease inhibitor cocktail. The bound biotinylated proteins were eluted from the column with 2% SDS, 30 mM biotin, 50 mM phosphate, 100 mM NaCl, 6 M Urea, 2 M Thiourea (pH 12) [19]. The eluted proteins were precipitated

using methanol-acetone method and then washed two times with cold acetone. Protein concentration was determined by Bradford's assay using bovine serum albumin as the standard. The purified biotinylated proteins were resolved on SDS-PAGE.

4. Two-dimensional gel electrophoresis (2-D PAGE) and Two-dimensional immunoblotting

Biotinylated proteins were resolved by 2-D PAGE. The total of 130 µg of proteins derived from eights individual shrimps was loaded onto each IPG strip (7 cm, nonlinear pH 3-10; GE Healthcare). Three IPG strips for each condition, representing 24 individual shrimps, were analyzed. Each strip was passively rehydrated overnight with 130 µg of total proteins that are premixed with a rehydration buffer containing 7 M Urea, 2% (w/v) CHAPS, 2% (v/v) ampholytes (pH 3-10), 120 mM DTT, 40 mM Tris-base and bromophenol blue. The first dimension of 2-DE was carried out in IPGphor IEF system [GE Healthcare). The IEF was performed at 50 mA per strip at 20 ^{o}C using a continuous increase in voltage (up to 5,000 V) to reach 12,100 Vhrs. The focused IPG strips were incubated in an equilibration buffer containing 30% (w/v) glycerol, 20% (w/v) sucrose, 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8), 100 mM DTT, and 0.002% (w/v) bromophenol blue for 15 min. The strips were subsequently incubated for 15 min in equilibration buffer which replaced 100 mM DTT with 250 mM of iodoacetamide. The second dimension was conducted on miniVE Vertical Electrophoresis System [GE Healthcare]. The equilibrated strips were placed onto the top of 12.5% SDS-PAGE gel and then separated with a constant voltage at 120 V for 2 hrs. Some gels were Colloidal coomassie blue G (CBG)-stained. The 2-D immunoblotting was

carried out to detect the biotinylated proteins. For some other gels, the resolved proteins were transferred to PVDF membrane [GE Healthcare] using a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad, Richmond, CA). The membranes were presoaked in absolute methanol and equilibrated in Tris-glycine transfer buffer (39 mM glycine, 0.04% SDS, 10% methanol, and 48 mM Tris-HCl). Unstained membranes were prepared for hybridization by incubation with blocking solution [5% BSA in TBS buffer (20 mM Tris-HCl, and 150 mM NaCl; pH 7.4] for 2 hrs at room temperature to prevent nonspecific binding. They were then washed and incubated with streptavidin conjugated with horseradish peroxidase (Invitrogen, Carlsbad, CA) (1: 7,000 dilution) in TBS containing 1% (w/v) skim milk and 0.1% Tween 20 for 1.50 hrs at room temperature. The membranes were washed three times with TBS containing 0.1% Tween 20. After three washes, immunoreactive spots were visualized with a chemiluminescent substrate using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography. Gels and blots were digitized by using Densitometer [GE Healthcare]. Visualized patterns were directly compared to comparable gel CBG-stain patterns. Gels were kept at 4 ^{o}C for spot excision and protein identification.

5. SDS-PAGE and Western blot analysis

The purified biotinylated proteins and the efficiency of purification were checked by SDS-PAGE and western blot with streptavidin-HRP complex. The 15 µg of purified biotinylated proteins were subjected to electrophoresis in 12.5% acrylamide gel. After running at constant 120 V, the gels were either stained with CBG or electrotransferred onto

nitrocellulose membranes. The protocols followed semi-dry transfer was as same as in 2-D Immunoblotting (as aforementioned). To compare the level of protein expression of control and YHV-infected hemocyte subtypes at 1 hpi, equal amount (10 μg/ lane) of total extracted proteins from granule-containing hemocytes and hyaline hemocytes were loaded in each lane of 12.5% SDS-PAGE gel. The resolved proteins were then transferred onto a nitrocellulose membrane (Whatman; Dassel, Germany) using a Mini Trans-Blot electrophoresis transfer cell. Non-specific bindings were blocked with 5% BSA in 0.5% TTBS buffer (0.5% Tween, 20 mM Tris-HCl, and 150 mM NaCl; pH 7.4) for 2 hrs at room temperature. Thereafter, the membranes were incubated with rabbit polyclonal anti-A2M, anti-SPI, or anti-β-tubulin at 4 ^{o}C overnight with dilution factors of 1:10000 in 2.5% skim milk/0.5% TTBS. After three times of washing with 0.5% TTBS, the membrane was incubated at room temperature for 1.5 hrs with secondary antibody anti-rabbit conjugated with horseradish peroxidase 1:10000 in 2.5% skim milk/0.5% TTBS. The immunoreactive protein bands were visualized by SuperSignal West Pico chemiliminescent substrate and autoradiography. Band intensities were quantitated by Image Master densitometer (GE Healthcare).

6. Image analysis by Melanie software

Protein spots on a CBG-stained gel were aligned with immunoreactive spots on a 2-D immunoblotting by using Image Master 2D Platinum software version 5.0 (GeneBio, Geneva) and rechecked manually. The protein spots from 2-D gels which found only in granule-containing hemocytes and hyaline hemocytes were excised. For protein bands from

SDS-PAGE, the bands which were found only in granule-containing hemocytes, hyaline hemocytes and commonly found in both groups were excised. These excised proteins were subjected to perform in-gel digestion and mass spectrometry analysis, respectively.

7. In-gel digestion

Briefly, the gel pieces (1*1 mm^2) were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate and alkylated in the dark with 100 mM iodoacetamide (IAA) in 10 mM AB. After alkylation, the gel pieces were dehydrated twice with 100% ACN. The MS-grade modified trypsin solution (10 ng/μl trypsin in 25 mM AB) was added and incubated at 37 ^{O}C overnight. To extract peptide digestion products, 100 μl of 50% ACN in 5% trifluoroacetic acid (TFA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. The extracted peptides were dried by vacuum centrifugation. The lyophilized peptides can be stored at -20 ^{O}C before performing MS/MS.

8. Protein identification by nano-LC-ESI-MS/MS

The in-gel tryptic digested samples which dissolved in 0.1% formic acid were subjected to nano-LC coupled to ESI MS/MS (nano-LC-ESI-MS/MS). The MS/MS data were outputted as the searchable *.pkl* file. The resulting file was then searched by Mascot search engine (Matrix Science, Boston, MA) using NCBInr database. Parameters for the MASCOT search were peptide mass tolerance of 1 kDa; MS/MS ion mass tolerance of 1 Da; maximally one missed cleavage; and tryptic digestion. Variable modifications included methionine oxidation

and cysteine carbamidomethylation. Only matched proteins with significant scores (p <0.05) were reported.

9. Bioinformatics analysis

The identified proteins were further classified according to their subcellular localization. Thereafter, sequences of the identified proteins were subjected to analyze for their subcellular localization reported using UniProt Protein Knowledgeable Database (http://www.uniprot.org).

10. Immunofluorescence detection of biotin-labeled proteins

To observe the signal of biotinylated proteins on the cell surface, the unlabeled (negative control) and biotin-labeled hemocytes which were isolated by Percoll gradient centrifugation were maintained in L-15 medium for 1 h. The hemocyte culture was fixed in 10% formaldehyde (10% formaldehyde in 0.45 M NaCl) for 20 min. Some unlabeled hemocytes, the fixed cell was permeabilized with 0.1% Triton X-100 (0.1% Triton X-100 in 1X PBS; pH 7.0). The hemocyte culture was incubated with blocking solution (10% fetal bovine serum in PBS). Subsequently, hemocyte was incubated with streptavidin Alexaflour- 488 conjugated secondary antibody (Invitrogen). To detect the fluorescence signal, the cells were stained with DAPI containing anti-fade permount (Invitrogen) before scanning by confocal microscope (Olympus Fluoview 1000).

11. Determination of Yellow head virus binding proteins

11.1 Virus overlay protein binding assay (VOPBA)

To identify biotinylated hemocytic proteins involved in YHV binding, a VOPBA method were carried out [20]. The membrane proteins on 2-D gels were transferred to a nitrocellulose membrane. The membrane was incubated with purified YHV diluted (1:100) in normal saline at 4 ^{o}C overnight with rocking. The membrane was subsequently incubated with anti-gp116 YHV mouse monoclonal antibody, followed by incubation with peroxidase-conjugated goat anti-mouse antibody. The antigen-antibody complexs were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography. Subsequently, the biotinylated proteins were detected using Streptavidin-HRP complex on the same membrane.

11.2 Biotinylation pull-down assay

Total hemocytes were biotin-labeled for 5 min on ice. The biotinylated hemocytes were solubilized in ice-cold 0.1% NP-40/PBS buffer. The insoluble materials were discarded after centrifugation and protein extracts in the soluble fraction were incubated with purified YHV diluted (1:50) in normal saline for 12 hr at 4 ºC. YHV bound protein pellets were collected, washed, and eluted using 0.1% NP-40/PBS with 1M NaCl for 2 hrs at 4 ºC. Eluates were separated and analyzed with Western blotting analysis using peroxidase-conjugated streptavidin. This complex was visualized using Supersignal detection reagent (Pierce). The biotinylated protein bands of interest were identified by Liquid Chromatography-Mass spectrometry (LC-MS/MS).

Results

1. Extraction of membrane protein fraction by using Chemical method

The membrane protein fraction was isolated from other parts of cellular fractions by different speed of centrifugation as shown in Figure 1. However, the amount of fractionated membrane proteins was very low when compared to total amount of shrimp hemocytic proteins. The high amounts of individual shrimps were used in this experiment. Moreover, there was no specific protein marker for detection of shrimp membrane protein fraction. Therefore, this method is not proper for membrane protein extraction in this work. The biotinylation method can solve these problems because this membrane impermeable biotin reagent is specific for selective labeling of cell surface proteins.

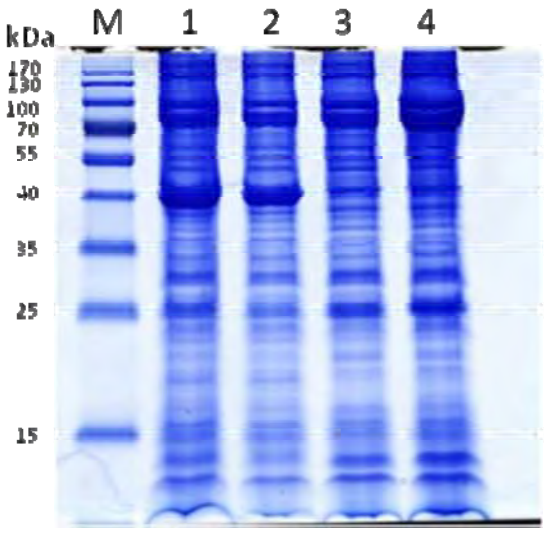

Figure 1: Subcellular fractionation of *P. monodon* hemocytes by Chemical method. Lane M: Protein molecular mass marker, Lane 1: Membrane fraction from shrimp no.1-10, Lane 2: Membrane fraction from shrimp no.11-20, Lane 3: Cytosolic fraction from shrimp no.1-10, Lane 4: Cytosolic fraction from shrimp no.11-20. Each lane contains 15 µg proteins.

2. Hemocyte isolation

After centrifugation, at least three bands of shrimp hemocytes were observed after separation as shown in Figure 2. These three bands were observed under microscopic in order to characterize each subtype according to cell size and granule-containing cell. The first band was identified as hyaline cells or agranular cells which is the smallest size among all three subtypes. The morphology of hyaline cells is round, flat and smooth surface with a large nucleus in the middle of the cell. The second band was identified as semigranular cells which contained the small granule within the cell whereas the last band was identified as granular cell which contained large granules in cytoplasm.

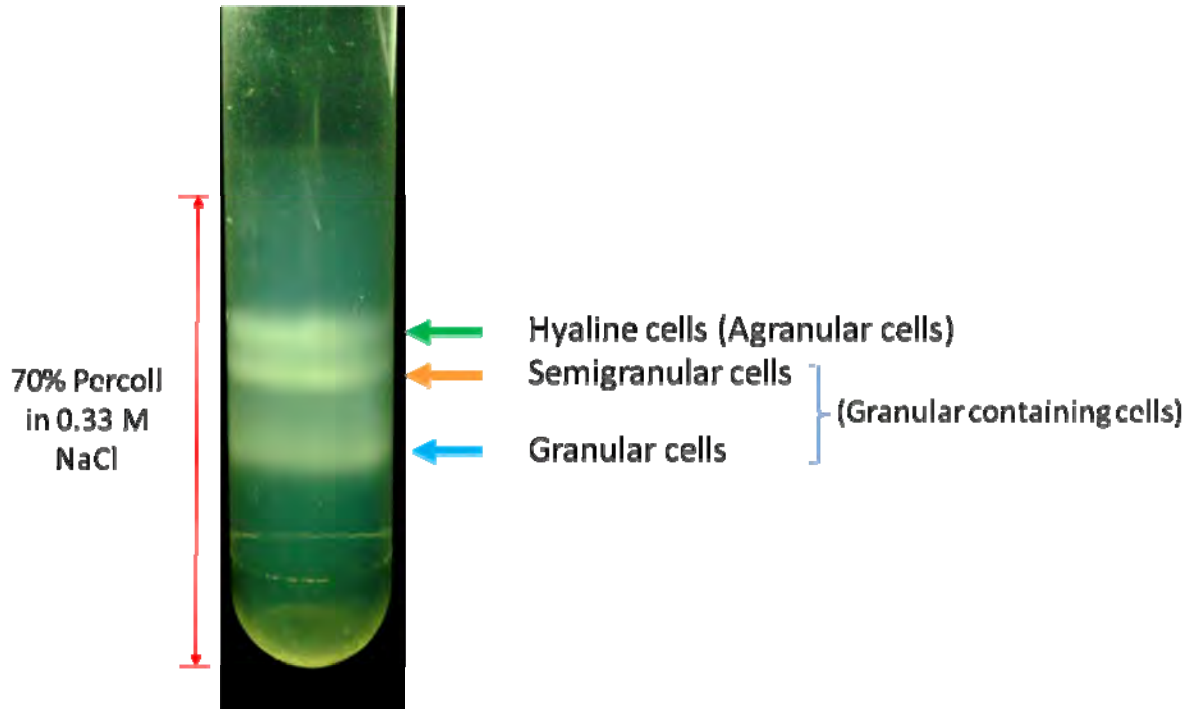

Figure 2: Hemocyte isolation by Percoll gradient centrifugation

3. Verification of successful *in vitro* biotinylation

In our experiment, 5 mM of Sulfo-NHS-LC-Biotin is the most optimal concentration for shrimp hemocyte protein biotinylation. For negative control, unlabeled total hemocytic proteins incubated with streptavidin-HRP complex clearly showed that there were no the endogenous biotin-containing polypeptides (immunoreactive band) for SDS-PAGE as shown in Figure 3 whereas 2-D blot showed only one positive spot with an apparent molecular mass of 85 kDa and a pI of 6.0 (data not shown). This spot was not included in the analysis of biotinylated proteins.

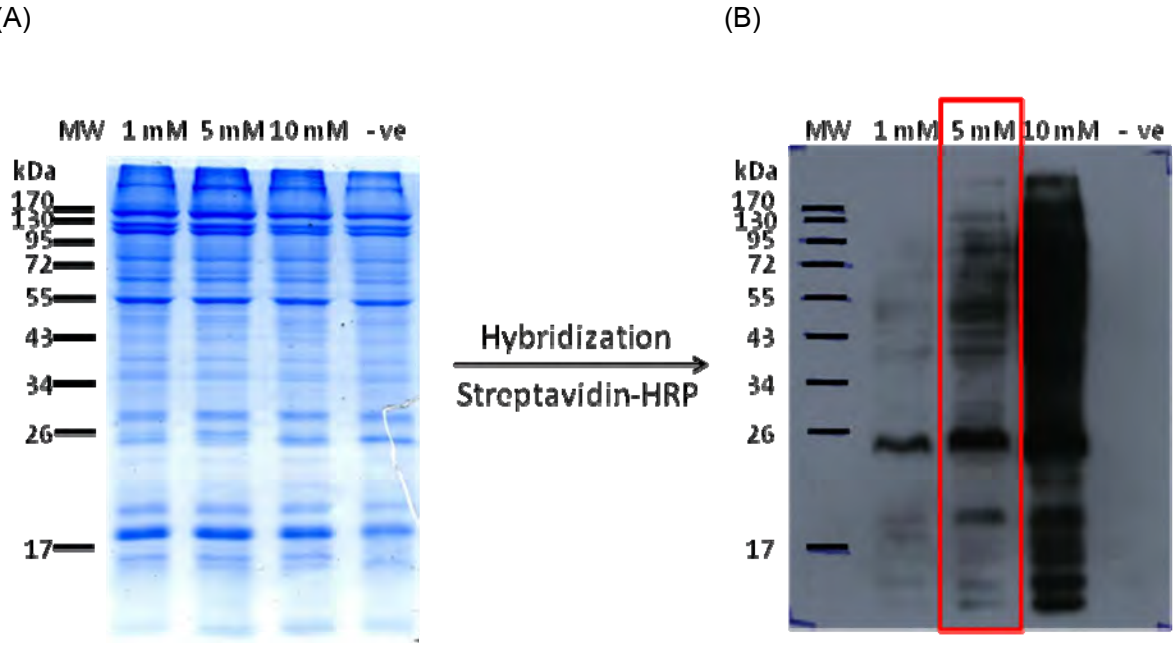

Figure 3: Visualization of biotinylated surface proteins of shrimp hemocytes. (A) SDS-PAGE analysis of hemocytic proteins. Each lane showed Sulfo-NHS-LC-Biotin labeled hemocytes with different concentration (1, 5 and 10 mM Sulfo-NHS-LC-Biotin, respectively). (B) Detection of biotinylated surface proteins. Surface proteins from shrimp hemocytes were

biotinylated, solubilized, resolved by SDS-PAGE, and then transferred to nitrocellulose membranes. They were visualized by hybridization with streptavidin-HRP complex.

The efficiency of each hemocyte subtypes biotinylation was verified by immunofluorescence staining. The images showed that most of biotin (approximately 95%) was bound to the boundary of each hemocyte subtypes.

(Fig.3)

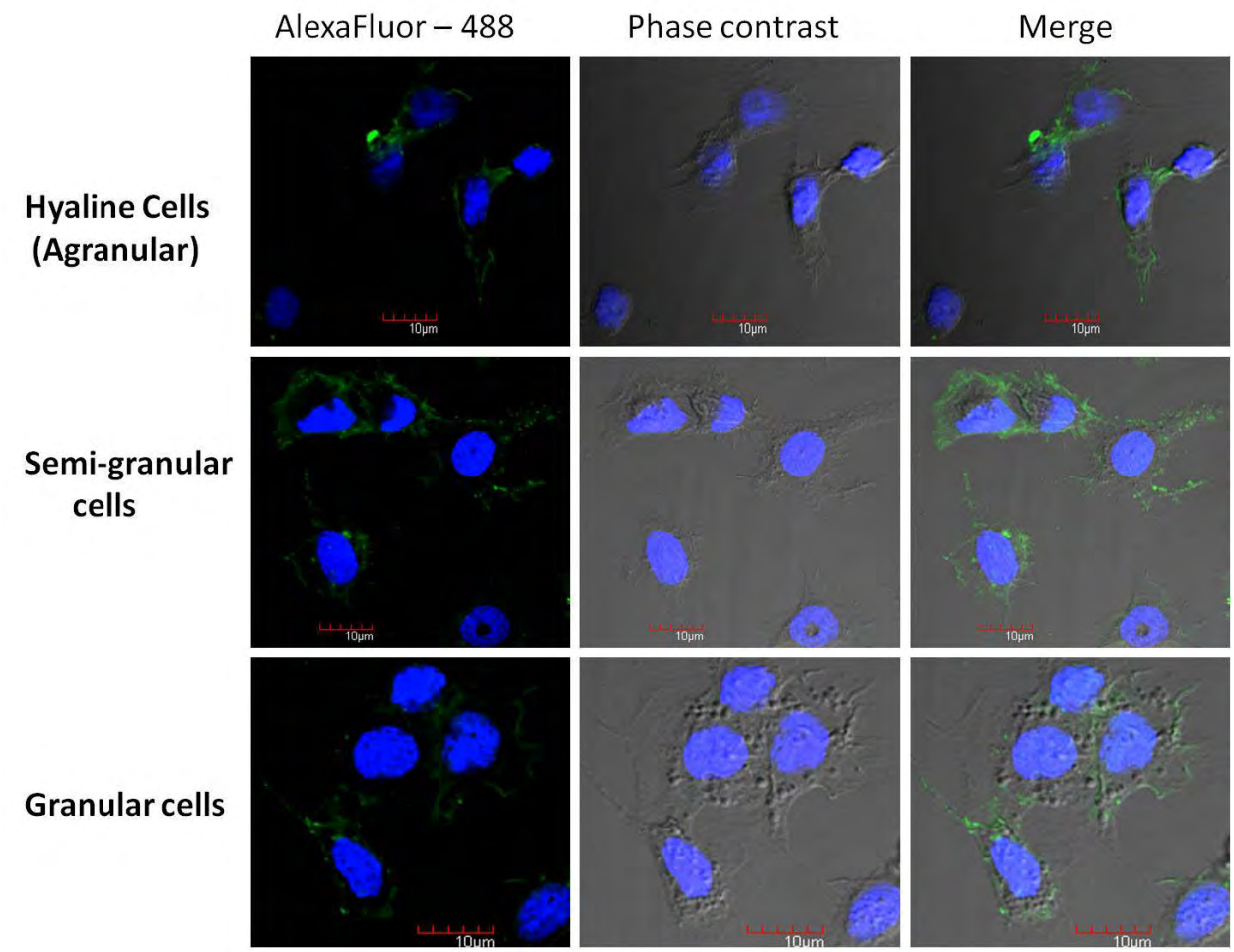

Figure 3: Localization of biotinylated proteins within isolated shrimp hemocytes. After hemocyte isolation by Percoll gradient centrifugation to be three subtypes, each subtype of hemocytes were biotinylated by 5 mM Sulfo-NHS-LC-Biotin for 5 min at 4 ^{o}C. Subsequently, the biotinylated hemocytes stained with Streptavidin conjugated with Alexa

Flour® 488 whereas nuclei were stained with to-pro-3 iodide fluorescence dye (blue). The images were visualized and captured with a confocal microscope (FV1000, Olympus) with the magnification power 60X for all panels.

In addition, verification of specific biotin labeling of hemocyte membrane protein was determined in membrane disrupted cell (Triton X-100 treated cell). The image also confirmed that the endogenous biotin-containing polypeptides can be stained within the permeabilized cells (Fig. 4). Therefore, this membrane impermeable biotin reagent should be suitable for the specific labeling of hemocyte surface protein.

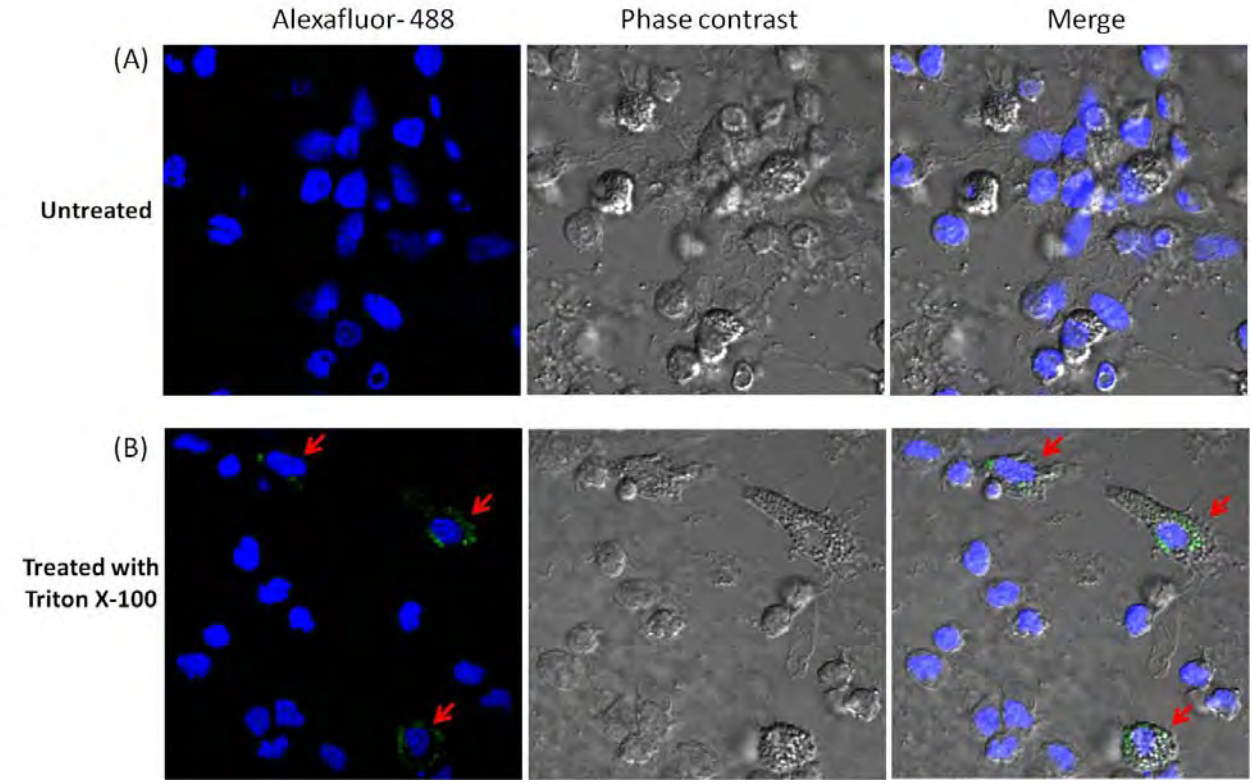

Figure 4: Localization of non-biotin labeling of shrimp hemocytes (Negative control). The intact total hemocytes without treatment of Triton X-100 (A) and Triton X-100 treated shrimp hemocytes (B) were prepared and then stained with Streptavidin conjugated with Alexa Flour® 488 whereas nuclei were stained with to-pro-3 iodide fluorescene dye (blue). The

images were visualized and captured with a laser scanning microscope (FV1000, Olympus) (original magnification power of 60X for all panels). The lysed cells after Triton X-100 treatment together with endogenous biotin signal were observed as indicated by red arrows.

4. Biotinylation of membrane proteins

The proteome maps of granule-containing hemocytes and hyaline hemocytes were successfully constructed by using 7-cm; NL pH 3-10 IPG strip, followed by 12.5% SDS-PAGE (Fig. 5). Each condition consisted of three replicated gels. Proteins resolved in each gel were derived from a pooled sample of eight individual shrimps. Thus, the data of each condition represented the information for a total 24 shrimps.

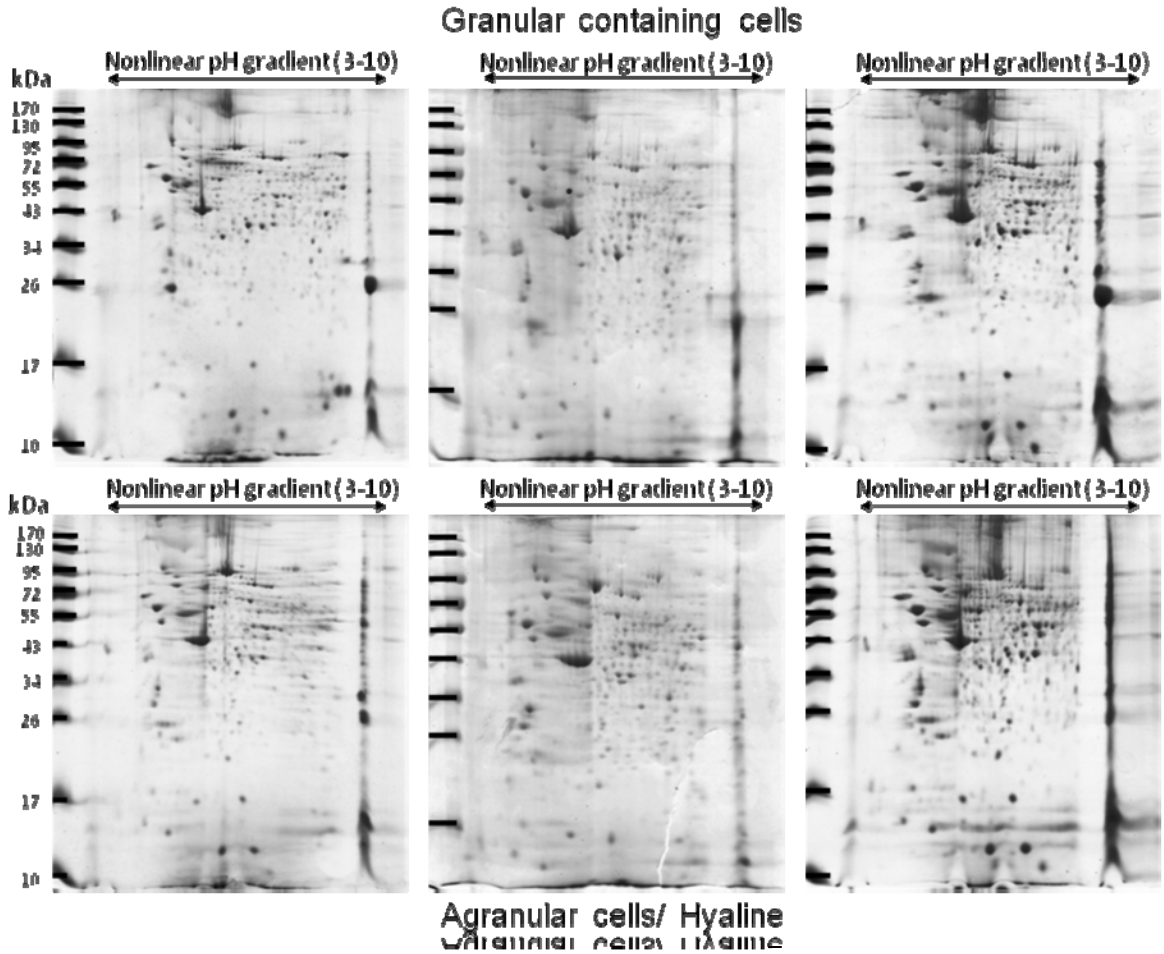

Figure 5: 2-D PAGE analysis of proteins from granule-containing cells (three upper gels) and agranular cells (three lower gels). 100 µg proteins from each condition were solubilized and resolved by 2-D PAGE using IPG in the first dimension. Each gel represented 8 individual healthy shrimp.

Subsequently, resolved proteins were transferred to nitrocellulose membranes. Approximately 60 and 80 biotinylated protein spots could be visualized on 2-D blots of granule-containing hemocytes and hyaline hemocytes, respectively (Fig. 6).

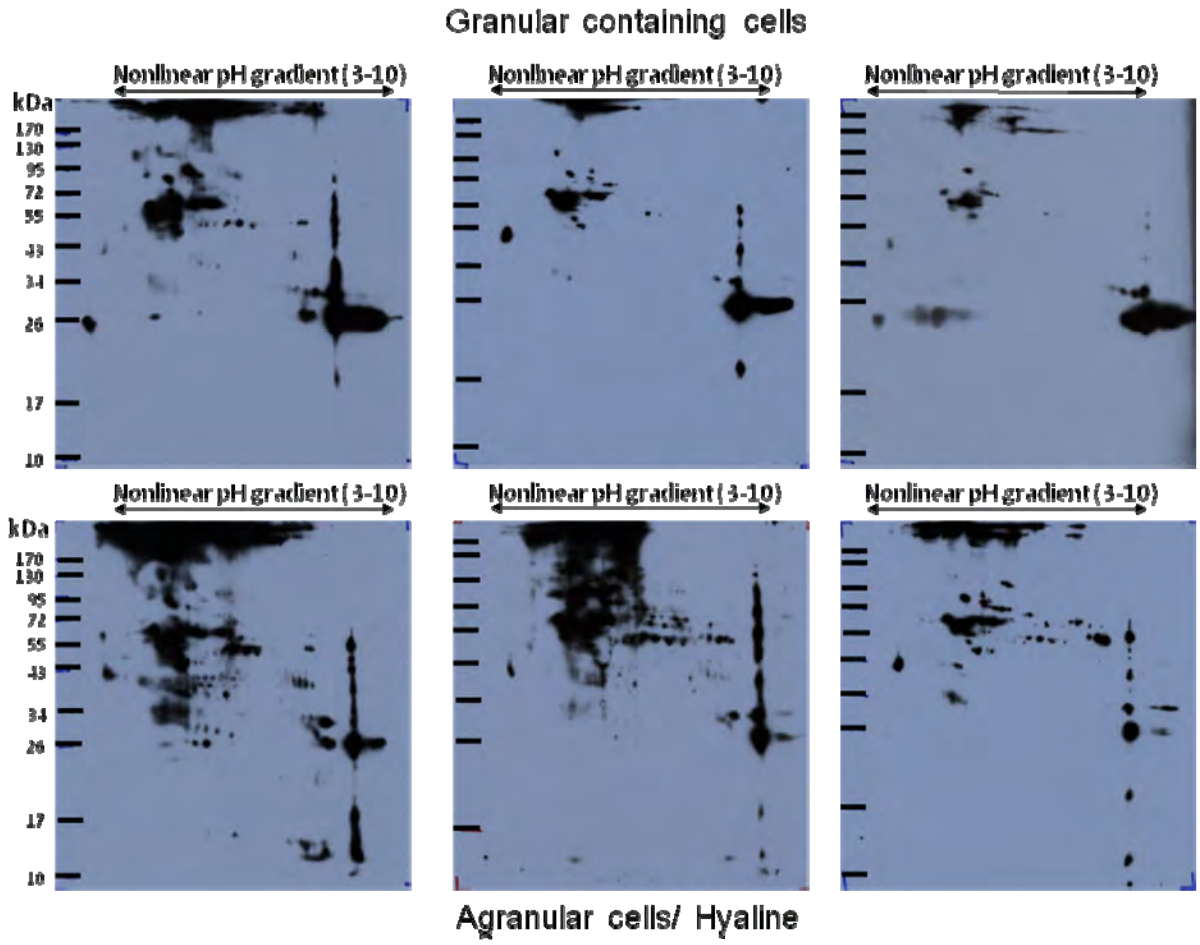

Figure 6: Detection of biotinylated proteins from granule-containing hemocytes (n=3; three upper gels) and agranular cells (n=3; three lower gels). Surface proteins from shrimp hemocytes were biotinylated, solubilized, resolved by 2-D PAGE, and then transferred to nitrocellulose membranes. They were visualized by hybridization with streptavidin-HRP complex.

Comparative analysis of the biotinylated proteins from granule-containing hemocytes and hyaline hemocytes was then performed and revealed that 2 and 24 spots on 2-D blots were particularly found only in granule-containing hemocytes and hyaline hemocytes, respectively;

whereas approximately 60 spots were commonly found in both groups. When the visualized patterns were directly compared to comparable gel CBG-stain patterns, 2 spots from granule-containing hemocytes and only 14 spots from hyaline hemocytes could be matched on 2-D gels, excised and identified by nano-LC-ESI-MS/MS (Fig. 7). Most biotinylated proteins, especially from hyaline hemocytes, had no match in the CBG stained 2-D pattern of cell lysate. It is evident that biotinylated proteins which visualized in CBG stained 2- D gels represented the low abundance in the whole cell level. Therefore, this approach is able to obtain a selective and enhanced visualization of low abundance proteins through biotinylation of intact cells. Several the resolved biotinylated proteins showed adjacent spots indicating isoforms that undergo post-translational modification (glycosylation, phosphorylation etc.). The 16 identified spots represent 8 unique and 6 unknown proteins. The numbers corresponding to the protein identities were shown in Table 1. The observed molecular mass and *p*I values were in good agreement with that of predicted. Protein spots identification revealed that Hemocyte kazal-type proteinase inhibitor (KPI) (spot#111) and alpha-2-macroglobulin (A2M) (spot#101) were exclusively found in granule-containing hemocytes. This result might be indicating those proteins are cell specific proteins and possibly related to immune regulation process. In addition, the identified proteins (6 unique proteins) which found only in hyaline hemocytes played role on many biological processes including immune-related function.

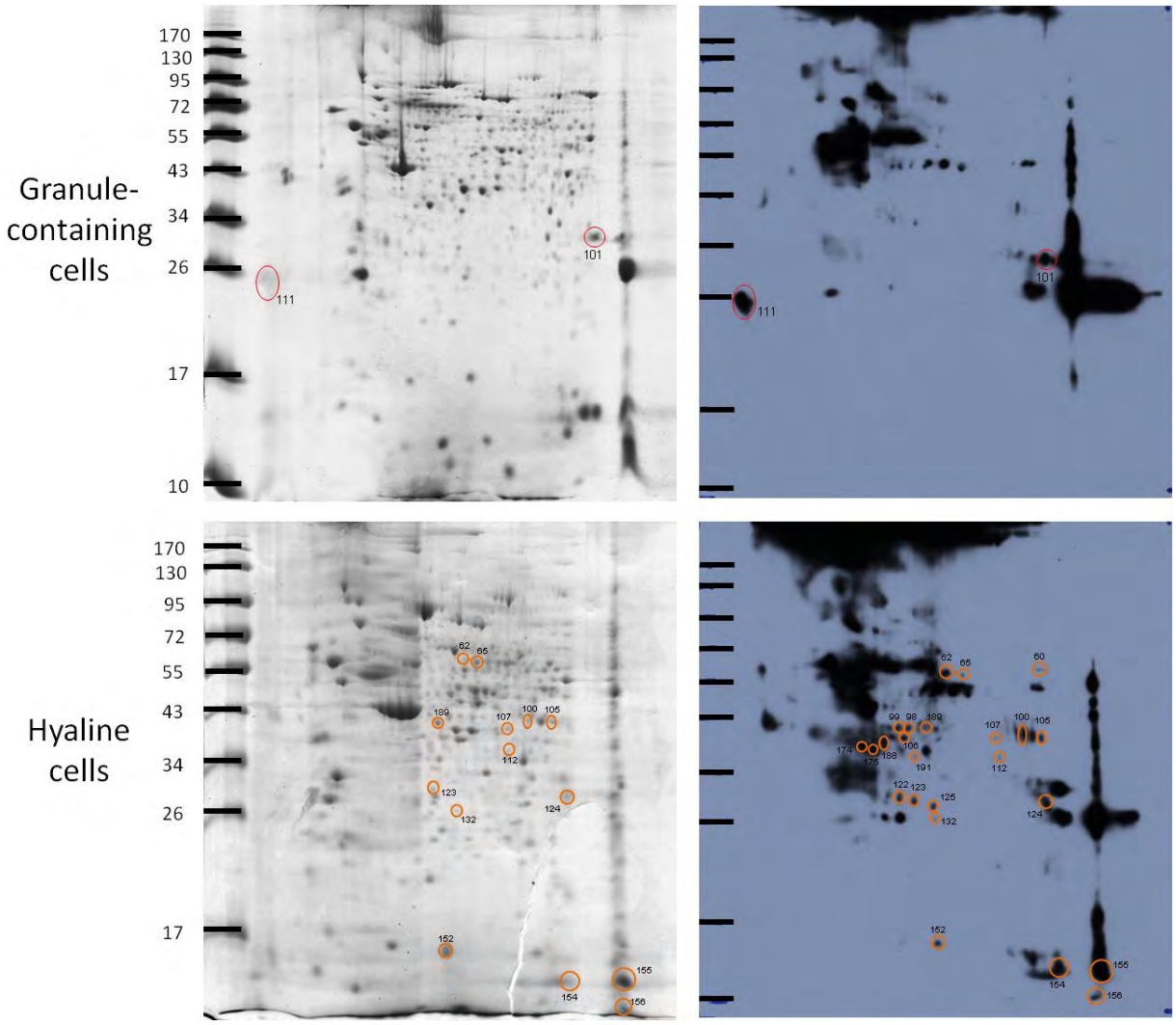

Figure 7: The representative 2-D gels and 2-D blots from granule-containing hemocytes (upper) and hyaline hemocytes (lower). From 2-D blots, the protein spots that found only in each groups were labeled with circle containing 2 protein spots of granule-containing hemocytes and 24 protein spots of hyaline hemocytes. The comparison between 2-D gels and 2-D blots to match the position of the interested protein spots was performed. Only 14 protein spots of hyaline cells and 2 protein spots of granular containing cells were manually excised, in-gel digested and subjected to perform nano-LC-ESI-MS/MS.

Table 1: Lists of differentially biotinylated proteins found only in granule-containing hemocytes and hyaline hemocytes by biotinylation method.

Spot no.	NCBI ID	Proteins (Species)	Score	No. of matched peptides	Predicted MW (kDa)/pI	Observed MW (kDa)/pI	% seq coverage	Subcellular localization
Proteins identified only in granule-containing hemocytes								
101	gi|33087164	hemocyte kazal-type proteinase inhibitor (*Penaeus monodon*)	551	14	30.5/8.5	30/8.2	49	Secreted
111	gi|60549971	alpha-2-macroglobulin (*Penaeus monodon*)	84	4	32.7/5.0	25/3.6	17	Secreted
Proteins identified only in hyaline hemocytes								
65	gi|157137024	Chaperonin (*Aedes aeypti*)	173	8	60.3/6.1	60/6.0	9	Cytoplasm
100	gi|46561746	Fructose-bisphosphate aldolase (*Homalodisca vitripennis*)	108	2	40.0/7.6	42/6.6	6	Cytoplasm, nucleus, Membrane
105	gi|270008484	hypothetical protein TcasGA2_TC014998 (*Tribolium castaneum*)	54	1	40.1/7.6	42/6.9	3	
152	gi|91094039	similar to Cofilin/actin-depolymerizing factor homolog (*Tribolium castaneum*)	135	4	17.1/6.2	15/5.7	16	Cytoplasm, Cytoskeleton, Nucleus
154	gi|157137721	Histone H2A (*Aedes aegypti*)	134	4	13.4/10.9	13/7.9	35	Nucleus
155	gi|122001	Histone H2A	142	3	13.4/10.7	13/9.6	25	Nucleus
189	gi|16716569	protease, serine (*Mus musculus*)	63	1	26.8/4.8	40/5.7	8	Secreted

5. Purification of biotinylated membrane proteins

Total extracted proteins from granule-containing hemocytes and hyaline hemocytes including the negative control of both groups were resolved on SDS-PAGE. A column of NeutrAvidin™ Agarose resins was utilized to bind biotinylated proteins. The bound biotinylated proteins were eluted from the column with 2% SDS, 30 mM biotin, 50 mM phosphate, 100 mM NaCl, 6 M Urea, 2 M Thiourea (pH 12). They were resolved by SDS-PAGE, followed by CBG staining. The eluted resin-bound proteins compared to total extracted proteins showed that the eluates were found to be widely distributed in the SDS-PAGE with various MWs (10-200 kDa) (Fig.

8A). This technique can be observed that the biotinylated proteins were captured on the streptavidin-sepharose resin and the non-biotinylated proteins was washed out during the washing step. The resolved proteins were transferred to nitrocellulose membranes and then hybridized with streptavidin-HRP complex to confirm the pattern of biotinylated proteins. The profile of biotinylated proteins from granule-containing hemocytes and hyaline hemocytes that were visualized by hybridization was similar to the profile obtained from MS-compatible silver-stained gels (Fig. 8B). For negative control of both granule-containing hemocytes and hyaline hemocytes, the immunoreactive bands could not be observed that meant there were no any biotin-labeled proteins detected. The biotinylated bands from the granule-containing hemocytes and hyaline hemocytes were apparently similar to each other; however some bands were specifically found in each group. These observed bands were identified by nano-LC-ESI-MS/MS. Table 2 showed 5 and 2 identified proteins were found only in the granule-containing hemocytes and hyaline hemocytes, respectively; whereas 4 proteins which commonly found in both groups. These 14 identified proteins represent totally 11 unique proteins. Most purified biotinylated proteins were identified as hypothetical and unknown proteins. Histones were found in multiple bands indicating isoforms (H2A, H3, and H4). Hemocyanin (approximately 10 kDa), suggested to be protein cleavage which showed much lower molecular mass than the predicted molecular mass (approximately 80 kDa); however we found both full-length and fragment of hemocyanin.

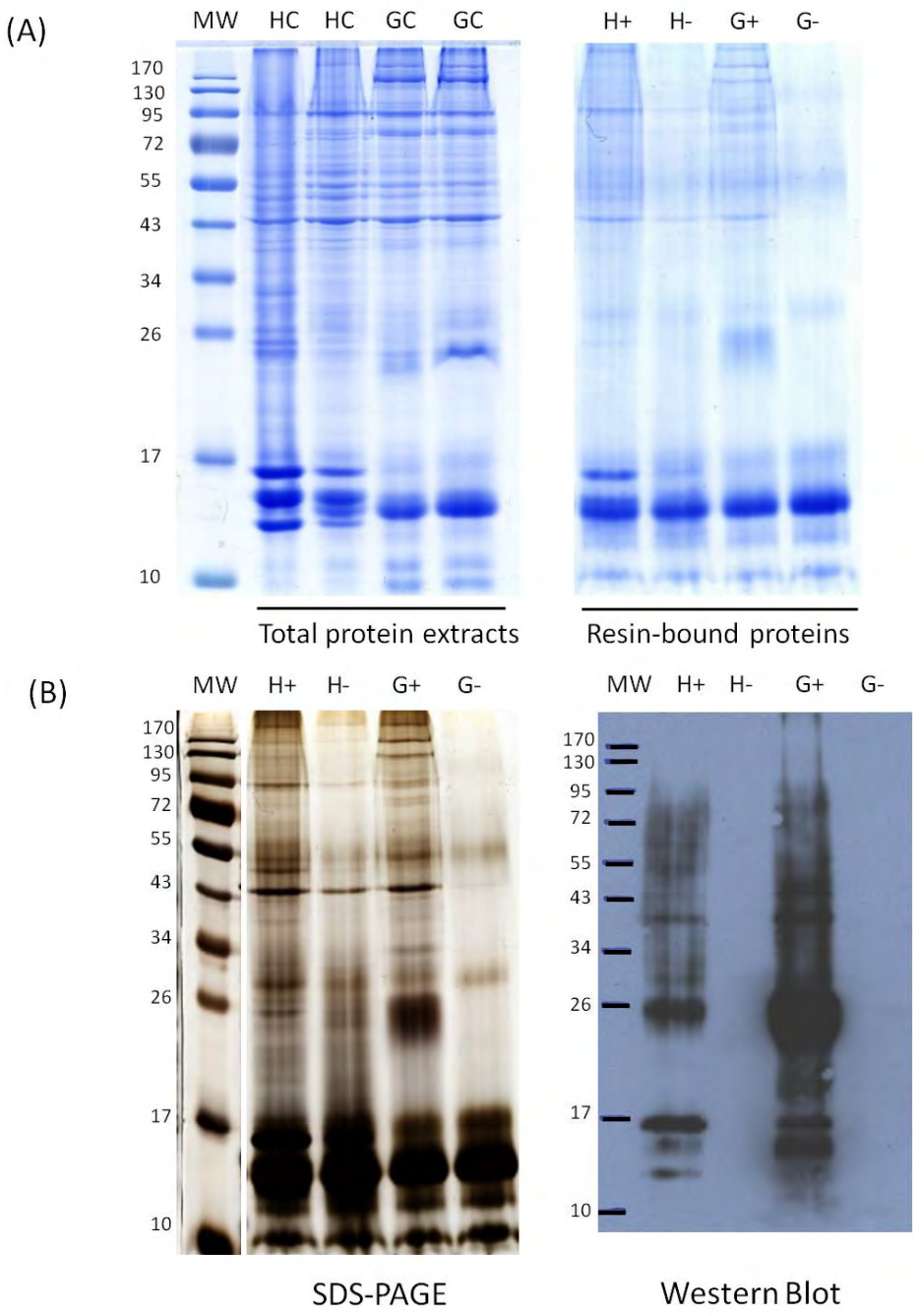

Figure 8: Visualization of purified biotinylated proteins from hyaline hemocytes (HC) and granule- containing hemocytes (GC). (A) CBG-stained SDS-PAGE of protein extracts and biotin-labeled (+) and non-labeled (-) proteins in the fraction of proteins bound to NeutrAvidin™ Agarose Resins from HC and GC. (B) Similarity of biotinylation patterns of

proteins from HC and GC as visualized by silver-stained image and blot using streptavidin-HRP conjugate.

Table 2: Lists of purified biotinylated proteins found only in granule-containing hemocytes, hyaline hemocytes and found in both groups.

NCBI ID	Proteins (Species)	Score	No. of matched peptides	Predicted MW (kDa)/pI	% seq coverage	Subcellular localization
Proteins identified only in granule-containing hemocytes						
gil145904597	Alpha 2 macroglobulin	505	13	169.7/5.4	10	Secreted
gil15718457	Peroxinectin	288	11	88.0/8.5	18	Secreted
gil5565792	Prophenoloxidase	210	8	78.9/5.8	15	Secreted
gil229459067	Crustin Pm4	95	2	25.0/9.0	8	Secreted
gil170029184	Zinc finger MYM-type protein1	49	1	82.1/8.7	1	Nucleus
Proteins identified only in hyaline hemocytes						
gil340728427	tuberin-like	52	2	118.1/7.9	2	Cytoplasm, Membrane
gil13446744	Elongation factor 1 alpha	159	4	14.6/7.0	34	Nucleus
Proteins identified of both groups of *P. monodon* hemocytes						
gil7582388	Hemocyanin subunit	50	2	83.7/5.3	3	Secreted
gil33694274	Transglutaminase	764	26	85.5/5.5	32	Cytoplasm, Secreted
gil33114094	Histone H3	186	8	15.4/11.3	31	Nucleus
gil170572153	Histone H2A	54	2	13.4/10.7	25	Nucleus
gil281323596	Histone H4	53	2	6.0/10.9	33	Nucleus
gil16612121	Hemocyanin	121	4	51.1/5.1	12	Secreted
gil10304437	Beta actin	440	15	42.2/5.3	35	Cytoskeleton

6. Identification of biotinylated proteins

A comparative proteomic analysis was performed between the sample of granule-containing hemocytes and hyaline hemocytes labeled with biotin. The classification by subcellular localization was done according to the SwissProt/UniProt databases. Subcellular localization of the identified proteins was secreted, cytosolic, cytoskeleton, nuclear and membrane proteins. However,

according to currently available data concerning their biological function, most of the identified proteins of granule-containing hemocytes and hyaline hemocytes were found to be membrane-associated proteins. As a result, proteins identified only in hyaline hemocytes showed various subcellular localizations within cells. Considering the subcellular localization of the proteins identified in each group from both approaches (biotinylation and purification of biotinylated proteins), we observed that most of identified proteins which found only in granule-containing hemocytes were annotated as secreted (externally-bound) proteins. Interestingly, alpha-2-macroglobulin (A2M) showed the redundant protein identified and confirmed by these two approaches. A2M were predicted by SignalP 4.0 server to have 18 residue signal sequences. However, A2M identified by these two approaches displayed different molecular mass. The mature A2M found in purification method had molecular mass approximately 170 kDa (Fig. 8) whereas its fragment with molecular mass approximately 25 kDa was detected in biotinylation method (Fig. 7 spot#111). Our Western blotting results (Fig. 9) showed the identified KPI was the kazal-type serine proteinase inhibitor 2 (SPI*Pm2*) from *P. monodon*. Because YHV specifically interact with granule-containing hemocytes of *P. monodon*, we then further hypothesized that A2M and KPI found only in 2-D blot of granule-containing hemocytes should be also up-regulated in the YHV-infected granule-containing hemocytes. The comparative protein expression levels of A2M and KPI between YHV-infected and healthy groups were investigated by 1-D Western blot analysis. β-tubulin served as the loading control. The band intensities of A2M and KPI were normalized with that of β-tubulin. Figure 9 illustrated that *P. monodon* A2M expression was induced in granule-containing hemocytes compared between YHV-infected and control groups about 1.2 folds change

after 1 hpi whereas the level of A2M expression decreased about 0.4 folds change in infected hyaline hemocytes. The comparative expression level between YHV-infected and control hyaline hemocytes and granule-containing hemocytes of KPI is as same as of A2M.

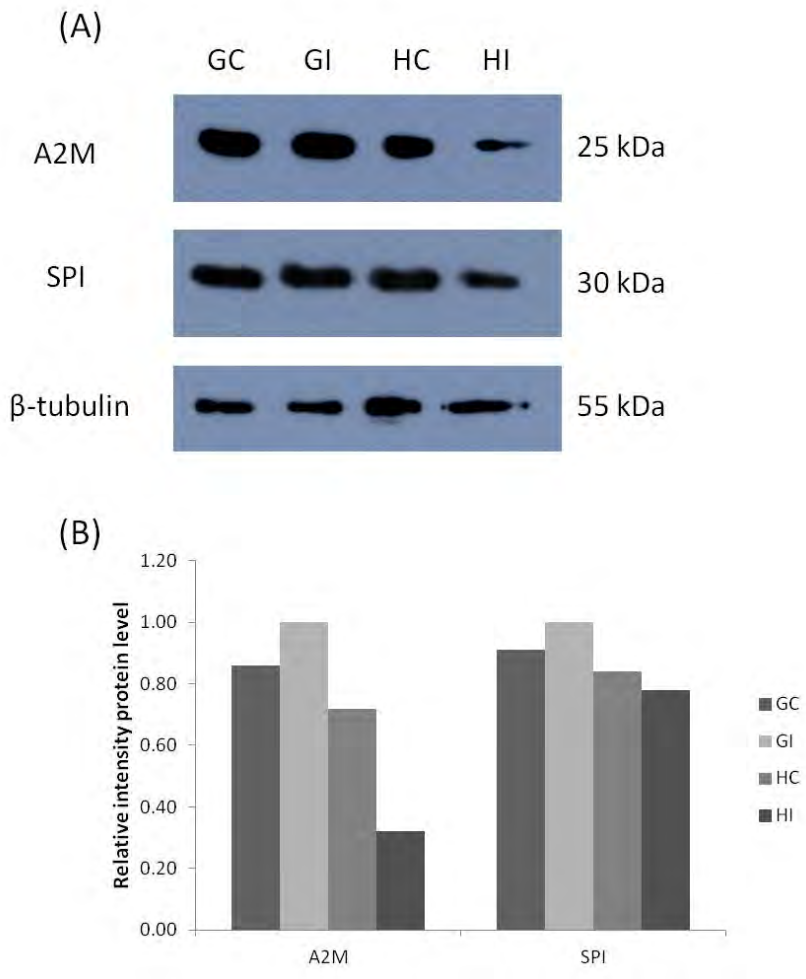

Figure 11: Validation of the proteomic data by Western blot analysis. (A) For A2M and SPI*Pm2* by comparing between among control Granule-containing hemocytes (GC), YHV-infected granule-containing hemocytes (GI) at 1 hour post infection, control hyaline hemocytes (HC) and YHV-infected hyaline hemocytes (HI) at 1 hour post infection. β-tubulin served as the internal control. The band intensities of A2M and SPI were normalized with that of β-tubulin (B).

Moreover, crustin*Pm4* was being the most abundant band in 1-D blot of granule-containing hemocytes. Crustin*Pm4* expression level from 2-D gel was significant increased approximately 2.90 folds change in granule-containing hemocytes which corresponding to the increase of 2-D blot hybridized with HRP-linked streptavidin approximately 2.82 folds change (Fig. 10). Histone H2A was found only in hyaline hemocytes by biotinylation method whereas it was found in both groups by purification method. The other identified proteins found commonly between granule-containing hemocytes and hyaline hemocytes were secreted proteins such as hemocyanin and transglutaminase. However, several protein bands were unidentified due to the lack of shrimp database.

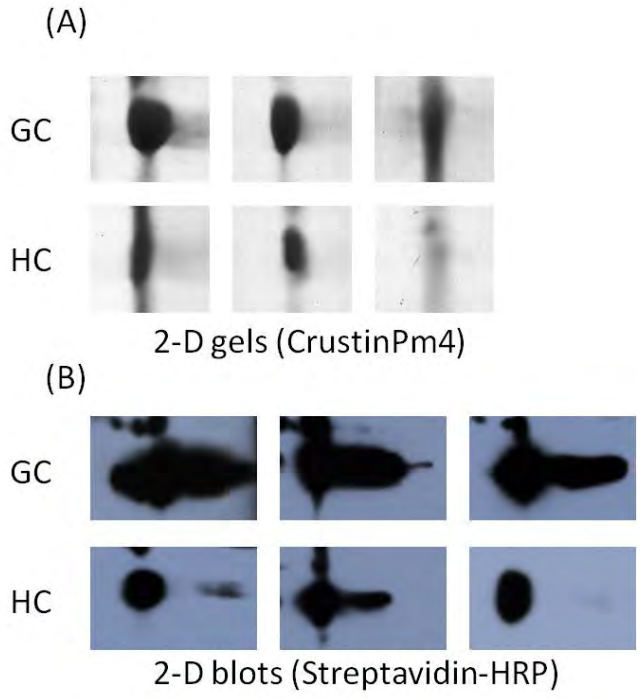

Figure 10: Magnified images of Crustin*Pm*4 compared to granule-containing hemocytes (GC) and hyaline hemocytes (HC). The expression of Crustin*Pm4* showed down-regulation both in 2-D gels (A) and blots (B).

7. Determination of Yellow head virus binding proteins

7.1 VOPBA

For conducting 1-D VOPBA, the result was shown in Figure 11 revealed that there were the immunoreactive bands for biotin-labeled granule-containing hemocytes and hyaline hemocytes. These bands were confirmed by using streptavidin-HRP complex. Some bands were found to be the biotinylated proteins.

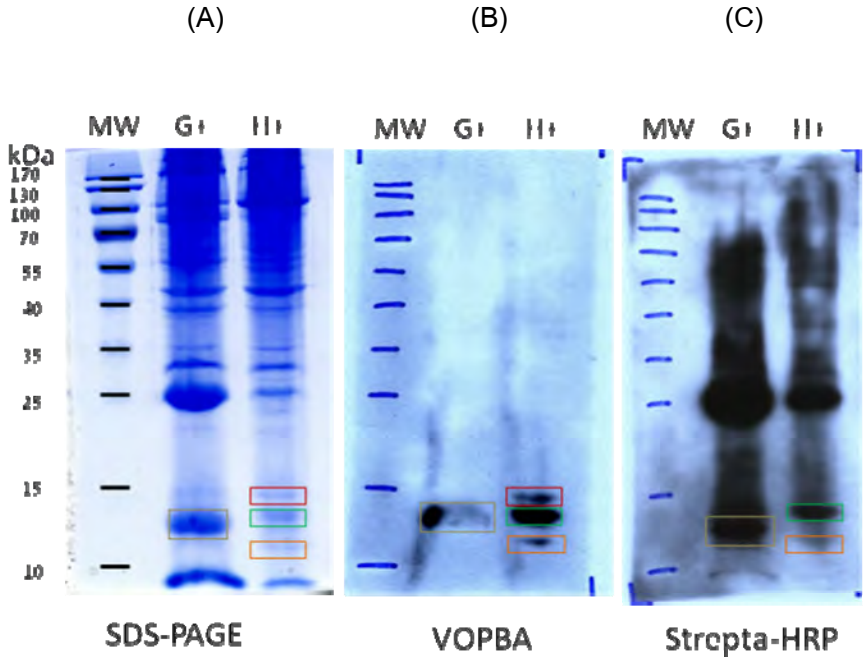

Figure 11: Results from 1- D VOPBA show the binding of YHV gp116 to biotinylated proteins from granule containing hemocytes and hyaline hemocytes. (A) Coomassie blue G staining of granule containing hemocytes and hyaline hemocytes. (B) blot of proteins from granule containing hemocytes and hyaline hemocytes incubated with purified YHV then

probed with anti-gp116 antibody. (C) blot of streptavidin-HRP complex showed the biotinylated proteins.

When 1- D VOPBA were successfully constructed, 2-D VOPBA were further performed as shown in Fig. 12. This immunoreactive spot (approximately 14 kDa) of both granule containing hemocytes and hyaline hemocytes was confirmed together with the result of 1-D VOPBA. However, the expression of this spot was very faint in hyaline hemocytes. This protein spot were subjected to analyze by nano-LC-ESI-MS/MS. From the searchable file, this protein was found to be crustin-like protein from *P. monodon* with predicted MW and pI of 15.4 and 8.23. This crustin-like protein should be crustin*Pm1* because its observed MW and pI of 14.0 and 8.5 was agreed with the reported crustin*Pm1*. However, this mass spectrometric data will be confirmed by 2-D immunoblotting by using anti-crustin*Pm1* antibody.

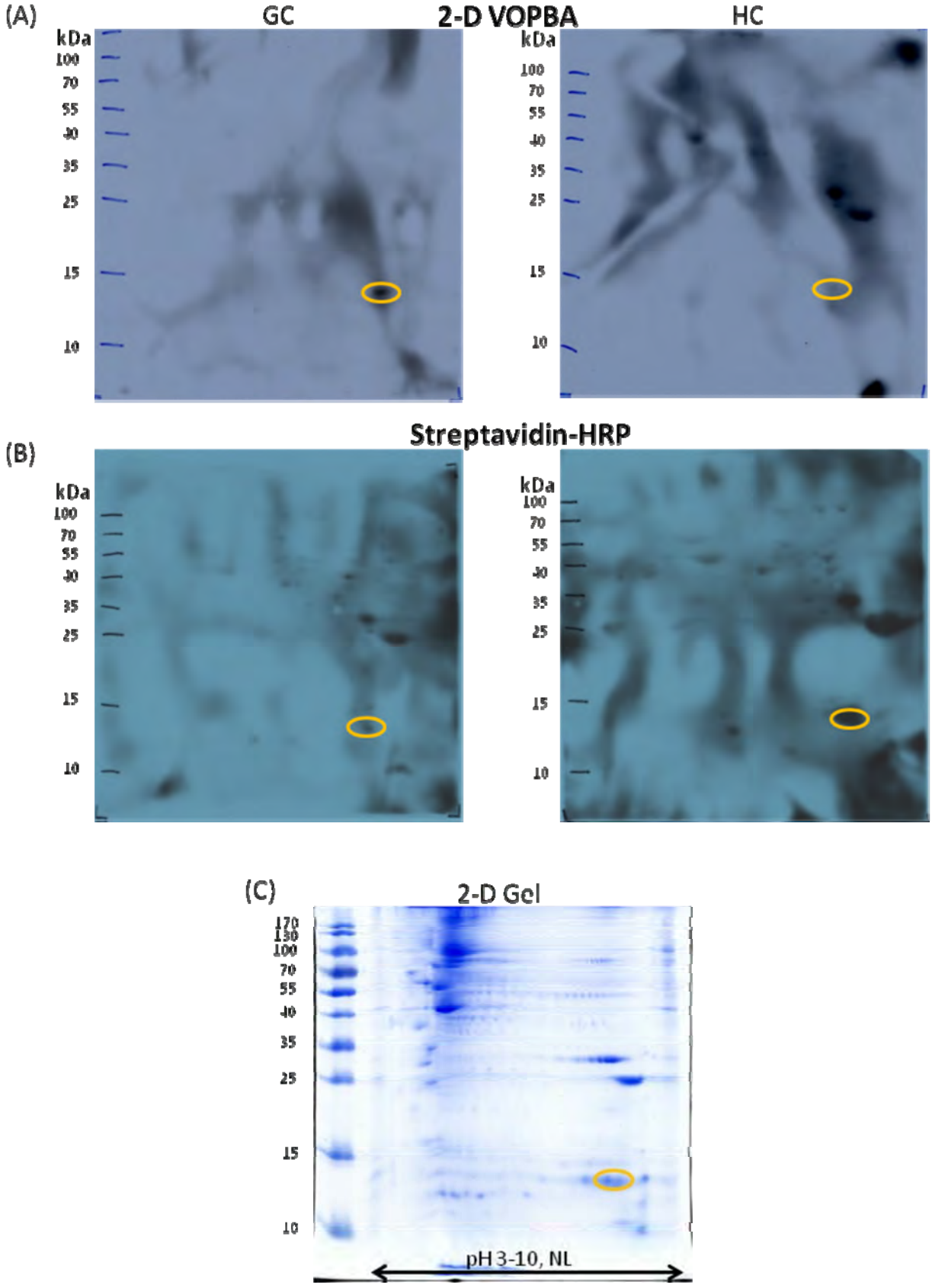
(A)
GC
2-D VOPBA
HC
kDa
100
70
55
40
35
25
15
10
(B)
Streptavidin-HRP
(C)
2-D Gel
170
130
pH 3-10, NL

Figure 12: 2-D VOPBA show the binding of YHV gp116 to biotinylated proteins (results from Streptavidin-HRP complex) of approximately 14 kDa from granule-containing hemocytes (GC) and hyaline hemocytes (HC) as shown in the circle. (A) blot of proteins from granule containing hemocytes and hyaline hemocytes incubated with purified YHV then probed with anti-gp116 antibody. (B) blot of streptavidin-HRP complex showed the biotinylated proteins. (C) Coomassie blue G staining of granule containing hemocytes resolved by 2-D PAGE. The position of protein spot between 2-D blot and 2-D gel were matched as shown in the circle.

7.2 Binding of purified YHV to cell surface hemocytic proteins

The pull down assay was employed to assess YHV gp116 interaction with cell surface proteins. The cell surface proteins of intact total hemocytes were selectively labeled with biotin prior to incubation with purified YHV. The biotinylated proteins were solubilized in 0.1% NP-40/PBS buffer. At this concentration of NP-40, it was particularly lysed the cell membrane not including nuclear membrane (Fig. 13).

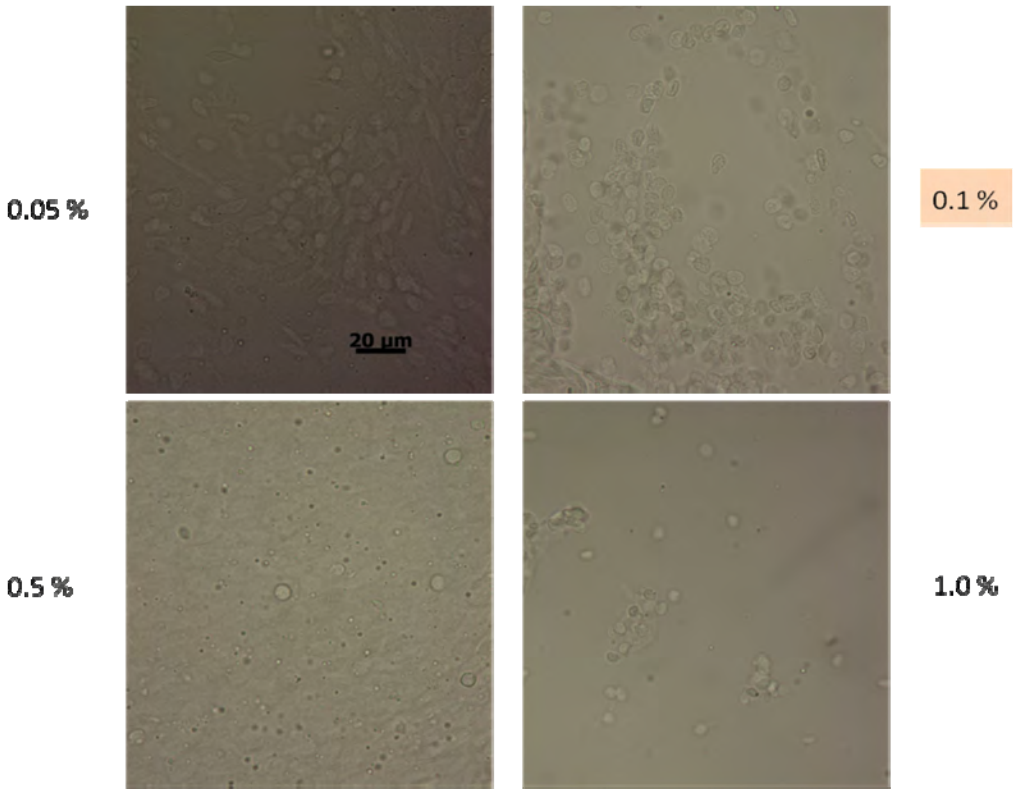

Figure 13: The optimization of NP-40 concentration used for cell membrane solubilization. The 0.1% NP-40 was found to be suitable for shrimp hemocytic membrane proteins.

The purified YHV-bound proteins were eluted and resolved on SDS-PAGE with Western blot analysis using peroxidase-conjugated streptavidin. One interesting purified YHV-binding protein with approximately size of 14 kDa (Fig. 14) which was corresponding to the result from 2-D VOPBA. The specificity was confirmed when the 14 kDa proteins were not detected when without incubation with purified YHV (negative control). This protein might be identified as crustin*Pm1*. However, it is necessary to confirm this protein identification by Western blot analysis.

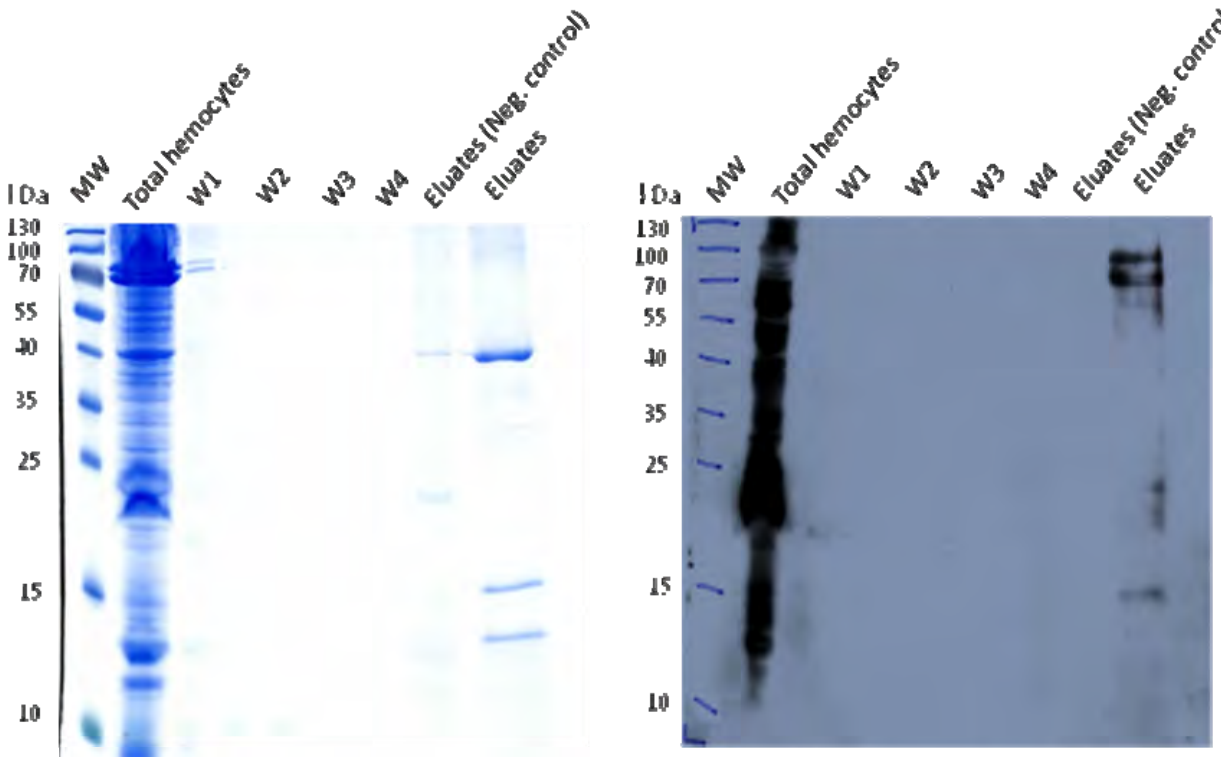

Figure 15: Western blotting analysis of eluates from pull down assay using biotinylated cell surface hemocytic proteins and/or purified YHV. Biotinylated proteins in 0.1 % NP-40/PBS buffer were incubated with or without purified YHV. Purified YHV-bound protein pellets were collected, washed, and eluted using 0.1% NP-40/PBS with 1 M NaCl. Eluates were analyzed on SDS-PAGE with Western blotting analysis using peroxidase-conjugated streptavidin. Purified YHV interacts with shrimp surface hemocytic proteins, with approximately size of 14 kDa.

Discussion

To date, several receptor proteins for viruses, especially from vertebrate, have been identified and elucidated [21, 22]. Currently, we have a fairly detailed knowledge of specific viral binding proteins from invertebrate, including a 65-kDa protein for yellow head virus [3], the small GTP-binding protein Rab7 for white spot syndrome virus [20], and laminin receptor for taura syndrome virus, infectious myonecrosis virus and yellow head virus [23, 24] based on different assays. The methodology used in this study was conducted by cell surface in vitro biotinylation with Sulfo-NHS-LC-biotin, efficient purification by streptavidin affinity chromatography, quantitative analysis, and identification. Sulfo-NHS-LC-biotin was utilized for the selective labeling of surface membrane proteins because it is water-soluble; thus it is not permeable across hydrophobic lipid bilayers. In penaeid shrimp, this is the first time that this method has been used to establish the differential molecular profile between granule-containing hemocytes and hyaline hemocytes and attempt to describe the functions of these cellular mediators and the mechanisms of shrimp immune response involved in the internalization of viral infection via each hemocyte subtypes.

Due to the dynamic protein expression and limitation of crustacean database for searching by Mascot, therefore most proteins could not be matched and identified on particular CBG-stained 2-D gel of the whole cell lysate and SDS-gel of purified biotinylated proteins. Total 6 and 8 identified proteins were differential found in granule-containing hemocytes and hyaline hemocytes, respectively, by biotinylation of membrane proteins together with purification of biotinylated proteins. Almost identified proteins were not localized in membrane compartment or well known as

surface receptor (e.g. CD146, Toll-like receptor). It is possible that the expression level of these proteins in the cell membrane might be too low to be detected, being covered by other more abundant proteins such as secreted proteins [25]. Moreover, another limitation of biotin-labeling was that the lack of primary amines in the extracellular domain and the inaccessibility due to the protein structure which may prevent biotinylation [26]. However, the identified proteins in this study were mostly found as membrane-associated proteins.

For granule-containing hemocytes, the differentially identified proteins was interesting as aforementioned, these proteins might be play roles directly involved in cellular binding or mediating of shrimp virus internalization into the permissive hemocytes. For example, A2M, commonly found based on two techniques, has been described as proteinase inhibitors and played an important role in controlling and regulating the prophenoloxidase (proPO) system that leads proPO to active form PO resulting in melanization [27]. *P. monodon* A2M contains receptor-binding domain and 18 residues signal sequence [28]; functions in the binding to a cell surface receptor for receptor-mediated endocytosis [29]. From previous results, they reported A2M was stored in the granules of granular hemocytes of several crustaceans [30, 31]. As same as in proPO, it was stored in the granular and semi-granular hemocytes. From the horseshoe crab *L. polyphemus* hemocyte, A2M detected on the surface of hyaline hemocytes and granule-containing hemocytes could indicate the occurrence of A2M-protease complexes linked to the membrane receptor and subsequent clearance [32]. According to Western blotting, A2M and KPI level showed up-regulated in the YHV-infected granule-containing hemocytes whereas both of them showed faint bands of YHV-infected hyaline hemocytes. Previously, P. monodon A2M expression in hemocytes increased significantly

after 12-48 h peptidolycan injection [23]. Not only A2M plays role in the innate immune system, but also A2M stored and induced in YHV-infected granule-containing hemocytes might have the potential function in virus internalization. In swine origin influenza A virus infection, salivary A2M could reduce the viral – induced hemaglutination and infectivity. In case of crustacean, A2M may play crucial role in natural inhibitor of virus infection via endocytosis.

One of identified WSSV-binding proteins from the *P. monodon* hemocyte membrane that bind to VP28 is hemocyte kazal-type proteinase inhibitor (KPI) based on VOPBA. Of the 9 KPI cDNA clones found in the EST database, SPI*Pm2* cDNA clone is the most abundant in the hemocyte cDNA libraries [33]. The SPI*Pm2* expression level of granule-containing hemocytes was up-regulated when the shrimps was challenged with YHV at 1 hpi as same as that in response to WSSV reported by Rimphanitchayakit et.al [34]. They found that SPI*Pm2* was expressed and stored in the granules of semi-granular and granular hemocytes but not hyaline hemocytes. They have also shown that SPI*Pm2* binds both WSSV and shrimp hemocyte membrane and involved in antiviral defense against WSSV. From biotinylation method, SPI*Pm2* was found only in granule-containing hemocytes. It is assumed that SPI*Pm2* perform its defensive role by capable of neutralization and inhibition of the viral proteinase. For crustin, it is crustacean antimicrobial peptides (AMPs) and posses a signal sequence at the n-terminus [35]. Our purification of biotinylated proteins showed that crustin*Pm4* was found only in granule-containing hemocytes whereas biotinylation method illustrated that the up-regulated expression of crustin*Pm4* in granule-containing hemocytes approximately 3 folds change compared to hyaline hemocytes both 2-D gels and blots. In crustacean, AMPs, consisting of penaeidin [36], and carcinin [37], are synthesized and

stored in the granular hemocytes and released by exocytosis upon microbial invasion whereas most crustins reported mainly in the hemocytes [38]. Evidently, peptides have many functions in modulating immunity and involving in infections and inflammation [39], whilst specific role in viral defense mechanism remains unclear. Peroxinectin posses both cell adhesion and peroxidase activity which is generated concomitant with activation of the proPO system [40]. Integrin, cell membrane-associated adhesion molecule and common receptor, recognize motifs such as KGD (integrin-binding motif) which found at the C-terminal of prawn peroxinectin [41]. During cell adhesion, Peroxinectin is synthesized and stored in semi-granular and granular hemocytes in an inactive form [42]. Peroxinectin has multiple functions which is essential in crustacean cellular defense for encapsulation and phagocytosis [43].

Chaperonin was the differential identified proteins found only in hyaline hemocytes. Chaperonin, previously considered to be expressed as a cytoplasmic protein whose function was to assist the correct protein folding, has recently been found outside the cells either as secreted protein or localized to the cell surface of a variety of cell type [44]. In any case, the interaction with other surface proteins is probable. The role of heat shock proteins (HSPs) on the cell surface is becoming better elucidated. It has been shown that HSP 60 is a putative endogenous ligand of the toll-like receptor-4 complex [45]. A signaling complex of receptors including HSPs 70 and 90, chemokine receptor4, and growth differentiation factor 5 is formed during immune system recognition of bacterial lipopolysaccharide [46]. Thus, it was plausible that HSPs and some receptors interact at the cell surface involving in receptor-mediated functions. Other identified protein in HC, fructose-bisphosphate aldolase has been found to be localized to the surface of

several bacteria where they bind host molecules and exhibit non-glycolytic functions [47]. For serine protease, they have well characterized roles in diverse cellular activities. Membrane-anchored S1 serine proteases may function as key regulators of cellular signaling events on plasma membrane with potential roles in mediating cell surface proteolysis associated with cancer and other disease [48]. We found three core histones either in HC (i.e. H2A) by biotinylation method or in both granule-containing hemocytes and hyaline hemocytes (i.e. H2A, H3, and H4) by purification but not found in granule-containing hemocytes only. Histone, primarily locates and function in the nucleus [49], however, increasing current study reported that histone can localize in other cellular compartment such as cytoplasm and the cell surface [50, 51]. Previous study showed that histone had ability to directly cross cell plasma membrane and mediate penetration process acting as carriers for the delivery of macromolecules into living mammalian cells [52]. In addition, histone may act as a direct cellular entry mediator or as a bridging protein for accumulation with an additional host cell surface protein. Transglutaminase and hemocyanin, found both two groups, are secreted proteins and play important role in host defense via innate immune system. Our published data demonstrated that the interaction between C-terminal hemocyanin and ERK1/2 led to playing important role in molecular immune response upon taura syndrome virus-infected *P. vannamei*. Activated ERK1/2 phosphorylate many substrates in all cellular compartment such as membrane, cytoskeleton, nuclear proteins [53]. Some amounts of transglutaminase are present on the cell surface and in the extracellular matrix [54]. Tissue transglutaminase, found on the cell surface, mediates adhesion and spreading of cells on the 42-kDa fibronectin fragment [55].

Regarding these identified proteins in recent study, most of them had no signal sequences. However, their functions were revealed on the cell surface, it remains unknown how it is exported to the cell surface. It might be possible that some of these proteins could be located more than one subcellular localization. In addition, there was some evidence of the receptor function of typical non-cell surface proteins increased, especially, proteins with multi-functional such as Ku70 and Ku80 [56]. Obviously, further studies about these membrane-associated proteins with surface membrane proteins of each hemocyte subtype are needed to confirm if these proteins play some important role on virus internalization and the regulation of shrimp hemocytes.

In summary, this present wok provides the first data on the differential proteome of granule-containing hemocytes (consisting of semi-granular and granular cells) and hyaline hemocytes. The proteins identified were commonly occurring proteins as well as membrane-associated proteins. Most of them were involved in cell adhesion including the externally-bound proteins and proteins associated with the plasma membrane. The changes at the cell surface of each hemocyte subtype in response to pathogen infection will lead to better understanding of host defense processes and further expanded for therapeutic product.

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2.2 Suparat Taengchaiyaphum, Atchara Paemanee, Nuanwan Phungthanom, **Phattara-orn Havanapan**, Apinunt Udomkit, Sittiruk Roytrakul, Kallaya Sritunyalucksana and Chartchai Krittanai. 2011. Phosphoproteomic analysis of *Penaeus monodon* hemocytes; the role of granular cell in early state of YHV infection (poster presentation). 6th International Symposium of the Protein Society of Thailand, 31st August – 2nd September 2011. Chulabhorn Research Institute Conference Center.

2.3 Phattara-orn Havanapan, Suparat Taengchaiyaphum, Nuanwan Phungthanom, and Chartchai Krittanai. 2012. Study of biotinylated proteins from hyaline and granular containing cells of Penaeus monodon (poster presentation). 7th International Symposium of the Protein Society of Thailand, 29th – 31st August 2012. Chulabhorn Research Institute Conference Center.

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