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Differential proteomic profiles and characterizations between hyalinocytes and granulocytes in ivory shell *Babylonia areolata*

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ABSTRACT

The haemocytes of the ivory shell, *Babylonia areolata* are classified by morphologic observation into the following types: hyalinocytes (H) and granulocytes (G). Haemocytes comprise diverse cell types with morphological and functional heterogene and play indispensable roles in immunological homeostasis of invertebrates. In the present study, two types of haemocytes were morphologically identified and separated as H and G by Percoll density gradient centrifugation. The differentially expressed proteins were investigated between H and G using mass spectrometry. The results showed that total quantitative proteins between H and G samples were 1644, the number of up-regulated proteins in G was 215, and the number of down-regulated proteins in G was 378. Among them, cathepsin, p38 MAPK, toll-interacting protein-like and beta-adrenergic receptor kinase 2-like were up-regulated in G; alpha-2-macroglobulin-like protein, C-type lectin, galectin-2-1, galectin-3, β-1,3-glucan-binding protein, ferritin, mega-hemocyanin, mucin-17-like, mucin-5AC-like and catalytic subunit of protein kinase A were down-regulated in G. The results showed that the most significantly enriched KEGG pathways were the pathways related to ribosome, phagosome, endocytosis, carbon metabolism, protein processing in endoplasmic reticulum and oxidative phosphorylation. For phagosome and endocytosis pathway, the number of down-regulation proteins in G was more than that of up-regulation proteins. For lysosome pathway, the number of up-regulation proteins in G was more than that of down-regulation proteins. These results suggested that two subpopulation haemocytes perform the different immune functions in *B. areolata*.

1. Introduction

In invertebrates, innate immunity plays a crucial role in responding to infectious pathogens because of lacking an adaptive immune system [1]. In innate immunity, the circulating haemocytes are involved in both humoral and cellular immunity in invertebrates, including synthesis of antimicrobial peptides, melanization, encapsulation and phagocytosis [2]. At present, reactions of humoral immunity are well understood in insects, however, cellular immune reactions are not extensively explored yet [3]. Cellular immunity is mediated by the circulating haemocytes in invertebrate and plays an essential role in immobilizing or destroying invasive pathogens. Mollusc immunity relies exclusively on innate immunological mechanisms through humoral and cell-mediated responses insured by haemocytes. Mollusks have ineffective immune globulins and depend more on their cellular defense than vertebrates [4]. Haemocytes are the main defense cells of molluscs and play a dominant role in shellfish immunity [5]. However, compared with insects, immunity functions of mollusc haemocytes are still poorly understood.

The haemocytes of molluscs are known to be responsible for many functions, including chemotaxis, cellular recognition, attachment, aggregation, phagocytosis, elimination by oxidation of non-self particles, killing or elimination of invading microorganisms and non-self particles such as bacteria and parasites [6]. In addition, haemocytes are involved in cellular defense [7,8], digestion and nutrient transport [7], excretion [9], and shell repair [10].

The haemocytes comprise a diversity of haemocytes types with functional and structural heterogeneity, and different types of haemocytes play different roles in host defense reactions [8]. The morphological classification of different haemocytes in mollusk has been studied

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since the 1970s, however, the immune function of the different sub-populations is still far from well understood. In previous studies, the haemocytes were characterized mainly by morphological and cytochemical feature, such as the cell size, nucleo-cytoplasmic (N:C) ratio, enzyme content and cytoplasmic complexity [8]. Although three haemocyte sub-populations (hyalinocytes, granulocytes and plasmatocytes) have been reported in mollusk [8,11], there is no consistent classification even in the same species, and a clear definition of different haemocytes with related function is still in urgent need.

For the argument of classification of circulating haemocytes, there are two main representative viewpoints: functionally different haemocytes types and a single haemocytes type [8,12]. For example, in mussel *Mytilus edulis* and *Mytilus galloprovincialis*, though two types of were described dependent upon morphological criteria, they were suspected to be the same haemocytes type in two different stages (hyalinocytes at young stage and granulocytes at old stage) [13,14]. Similarly, in oyster *Crassostrea rhizophorae*, a haemocytes maturation process was from blast-like cells, hyalinocytes to granulocytes and was inferred for circulating haemocytes [12]. In mollusk species, it is now commonly accepted that two main haemocyte types are generally accepted as granular haemocytes (granulocytes) containing intracytoplasmic granules and agranular haemocytes (hyalinocytes) containing few or no granules [15]. Currently, most of the investigations are focused on morphological description and the functions of haemocytes, however, molecular characterization of haemocyte sub-populations are still very limited. Therefore, it is obvious that the accuracy classification of haemocyte subpopulation should be studied with the cooperation of morphological and functional characterization together with molecular markers. Proteins participate in almost all cellular processes, information of which regarding activity and interaction, is central to understanding a biological system, however, no prior work evaluated the differences in proteins profiles between different haemocyte sub-populations in *Babylonia areolata*. Fortunately, the proteomics information can provide a rich resource for identification of immune-relevant proteins and investigation of molecular mechanism of mollusc immunity, and many progresses have been made on the immune defense mechanism of molluscs. These progresses may be helpful in characterizing different cell sub-sets at the molecular level.

The development of high-throughput mass spectrometry (MS) techniques overcomes the weakness of 2D electrophoresis in proteome investigations. MS cooperated with sophisticated bioinformatics tools has expanded the understanding of molecular mechanisms within biological systems [16]. Proteomics has been applied to the studies of several aspects within the aquaculture. The review by Rodrigues et al. [17] shows the power of proteomics techniques to deal with various aquaculture-related questions such as animal welfare, safety, quality, health and nutrition. In marine organisms, proteomics approaches are increasingly applied to monitor responses to challenges, for example environmental changes (stress response and adaptation) and diseases [18]. Although previous proteomics researches have been applied in the concerning marine invertebrates such as sponges, oysters, mussels, clams, scallops, shrimps and crabs [19]. However, little work has been investigated in *B. areolate* haemocyte.

B. areolata (Mollusca, Gastropoda, Neogastropoda, Babyloniidae) are common species in China [20], which have been the most promising economic marine gastropod in this century, because of its wide distribution along most parts of Asian coastlines, delicate flavor, and high market reception [21,22]. However, the slow growth rate of *B. areolata* and the potential for disease outbreaks remain a concern for farmers. To better defense infectious diseases, most researches are focused on the diagnosis of diseases and producing disease resistant strains. The latter strategy depends on the development of the knowledge of molluscan immunology. Because little is known concerning the different sub-population haemocyte immunity of *B. areolate,* investigation of the *B. areolata* immune system is important. The function and the expression of immune-related proteins varied among the different haemocyte types. In our previous study, two types of haemocytes, granulocytes and hyalinocytes, were identified in *B. areolata* according to the presence and abundance of cytoplasmic granules, staining properties and nuclear cytoplasmic ratio [20,23].

In the present study, the Percoll density gradient separation was employed to study the haemocytes of *B. areolate*, the differentially expressed proteins were investigated between hyalinocytes and granulocytes using MS-based proteomics approach. The main aims were: to separate the sub-populations of haemocytes based on their morphological and physical features, to characterize their corresponding immune functions, to understand the possible cell lineage of circulating haemocytes, to provide protein information and explore the expression profiles of immune-related proteins in different sub-populations, and to contribute to further understanding of their cellular functions. To the best of our knowledge, there are no comprehensive proteomic studies of the different sub-population haemocytein *B. areolata.* The effectiveness of LC-MS/MS and the Uniprot database for identification proteins from a non-model organism such as *B. areolata* would be checked.

2. Materials and methods

2.1. Animals

The adults of *B. areolata* (8–10 cm shell length) were collected from Dongshan Haitian Aquaculture Co., Ltd, Fujian Province. Snails were maintained in flow-through water (27.1 ± 1.1 °C, salinity 29.8 ± 0.9, pH 8.0 ± 0.6, oxygenated and free-flowing water). A layer of fine calcareous sand was added to allow burrowing. They were fed daily with oyster and chopped fresh fish.

2.2. Sampling of haemolymph

Sampling of haemolymph was performed as previously described [20]. By touching the foot with the point of a micropipette tip, the snail was forced to retract deeply into its shell and extruded haemolymph [24]. Approximately 100 µl of haemolymph per snail was extracted from adult *B. areolata* specimens. The haemolymph was immediately mixed with pre-chilled anticoagulant (Anticoagulants ZA: the solution consists of glucose 2.05 g, sodium citrate (2H2O) 0.80 g, NaCl 0.42 g, HEPES 10 Mm in 100 ml distilled water; 10% citric acid adjusted to pH6.1 (112 °C sterilization) at a 1:1 vol/volume ratio, pooled into sterilized 1.5 ml Eppendorf tubes. Staining was carried out using improved Wright's stain. The staining method was performed as previously described [23]. The haemolymph/anticoagulant mixture was added the same volume of 100% methanol, fixed for 6 min, an 8 µl haemolymph was placed on a glass slide, blow-dried with electric blower, stained for 12 min, washed with double distilled water, air-dried, and then examined using light microscopy.

2.3. Enrichment of different haemocytes types with Percoll

Percoll (Pharmacia, 1.130 g ml^{-1}) was used for density gradient centrifugation. The commercial Percoll solution was adjusted by adding NaCl solution to a final concentration of 0.43% (w/v). The 38% (v/v) Percoll solution were prepared in ZA. The haemocytes re-suspended in ZA (about 2–5 × 10^5 cells/mL) were layered onto the top of a 38% Percoll solution. 2 ml of preseparated haemocytes were put onto the layer and was centrifuged at 1300 g for 45 min at 4 °C, the haemocytes presented at the bottom, the interface and the upper layer of the gradient were collected and washed with ZA, and designated as sub-population granulocytes (G), interface including granulocytes and hyalinocytes (HG) and hyalinocytes (H), respectively. Then the collected G, HG and H subpopulations, and then HG were layered onto the top of the 35% Percoll solution for the second separation separately. This second gradient was then centrifuged at 1300 g for 45 min at 4 °C. Separated cells were harvested from gradients with Pasteur pipettes (Eppendorf).

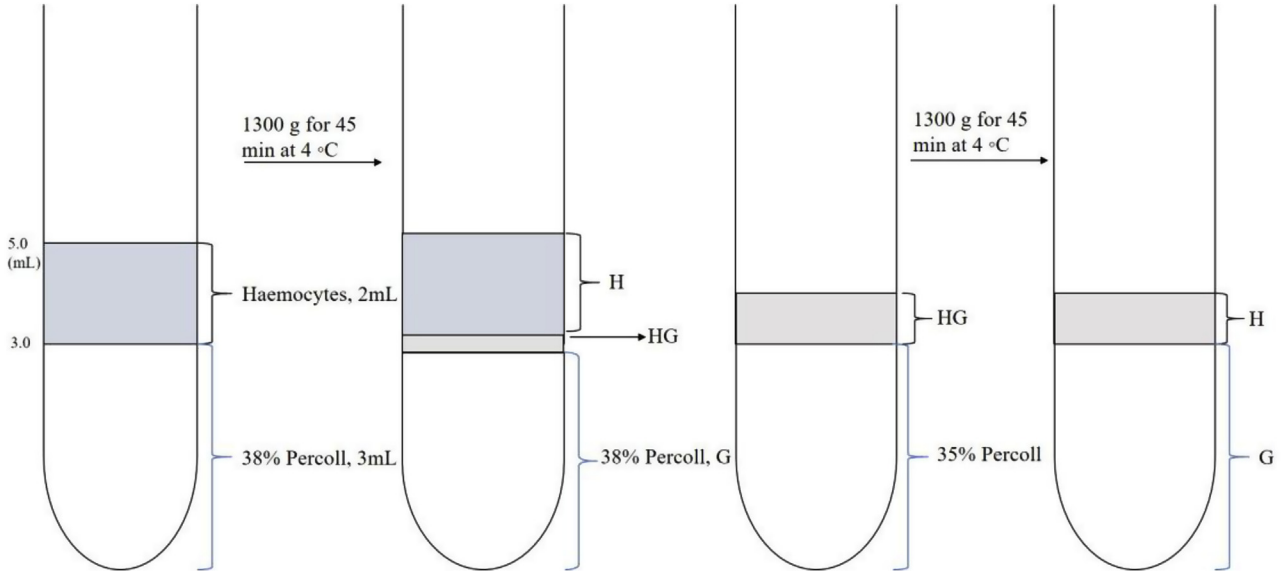

Fig. 1. Schematic representation of density gradient separation with Percoll used to isolate the different haemocytes of *Babylonia areolata*. The haemocyte separation procedure included two centrifugation steps with two different gradients. The positions of the enriched haemocytes bands generated after density gradient centrifugation were indicated for 38% and 35% Percoll. After first time centrifugation, hyalinocyte (H) and granulocytes (G) two layers were harvested from hemocytes by the first density gradient centrifugation in 38% Percoll. Only HG haemocytes bands were run through the 35% Percoll gradient.

The sub-populations of the same layer in each gradient were collected together, washed with ZA, and checked by the Trypan blue test for their viabilities. Then the collected haemocyte sub-populations were stained by Wright's stain and morphologically checked under microscope, 10 fields were counted for each slide, and 10 slides were counted to calculate the purification rate of haemocyte sub-populations. Finally, the haemocyte sub-populations were used for proteins isolation. The entire process was described as shown in Fig. 1.

2.4. Proteomic sample preparation

The frozen samples were ground in liquid nitrogen. Added 2 ml lysis buffer (8 M urea, 2% SDS, 1x Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland)), followed by sonication on ice and centrifugation at 13 000 rpm for 10 min at 4 °C. Then transferred the supernatant to a new centrifuge tube. For H and G samples, proteins were precipitated using ice-cold acetone at −20 °C overnight, and then the precipitations were cleaned with 50% acetone and 50% ethanol three times.

2.5. Protein digestion

Protein concentration was measured using the BCA protein assay, and then transferred 100 μg proteins per sample into a new Eppendorf tube and adjusted to a final volume of 100 μL with 8 M urea. Then added 11 μL of 1 M Dithiothreitol (DTT), incubated sample at 37 °C for 1 h, then transferred sample into 10 K ultrafiltration tube (Millipore). Samples were centrifuged by adding 100 mM Triethylamonium bicarbonat (TEAB) for three times to remove urea. Added 120 μL of 55 mM iodoacetamide (IAA) to the samples, incubated for 20 min and protected from light at room temperature. Then trypsin digestion was performed with sequence-grade modified trypsin (Promega, Madison, WI) overnight.

2.6. Nano-HPLC-MS/MS analysis

Each haemocyte sample was divided into three equal parts, re-suspended with 30 μl solvent C (C: water with 0.1% formic acid) respectively, then the samples were separated using nano LC and analyzed by on-line electrospray tandem mass spectrometry (MS). The sample was carried out on an Easy-nLC 1000 system (Thermo Fisher Scientific, MA, USA), connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA), equipped with an on-line Nano-electrospray ion source. 10 μl peptide sample of haemocyte was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2 cm), with a flow of 10 μl/min for 3 min, subsequently separated peptide sample on the analytical column (Acclaim PepMap C18, 75 μm × 15 cm) with a linear gradient, from 3% D (D: ACN with 0.1% formic acid) to 32% D in 120 min. Re-equilibrated the column at initial conditions for 10 min, maintained the column flow rate at 300 nL/min. The electrospray voltage of 2 kV versus the inlet of the mass spectrometer was used.

The mass spectrometer was performed under data dependent acquisition mode, and automatically switched under MS and MS/MS mode. MS1 mass resolution was set as 35 K with m/z 350–1550, MS/MS resolution was set as 17.5 K under HCD mode, the dynamic exclusion time was set as 20 s.

2.7. Data analysis

Tandem mass spectra were processed by MaxQuant v1.5. PEAKS DB was set up to search the *B. areolate* transcriptome database (we have performed transcriptome *B. areolate* haemocyte), assuming the digestion enzyme was Trypsin. PEAKS DB was searched with a parent ion tolerance of 7.0 ppm. A fixed modification was set as Carbamidomethylation. Oxidation (M) and Acetylation (Protein N-term) were specified as variable modifications. Peptides were filter by 1% FDR. Intensity based absolute quantification (iBAQ) was used for peptide and protein abundance calculation. Different expressed proteins (DPEs) were filtered, for quantitative analysis, the *t*-test was conducted, and protein with at least 2.0-fold changes between H and G were compared and considered upregulated or downregulated.

2.8. Bioinformatics analysis of GO and KEGG pathway

Gene Ontology (GO; http://www.geneontology.org)) project is a major bioinformatics initiative with the aim of standardizing representation of genes and gene product attributes across all species and databases. GO annotation is a process of scoring the similarity between target sequences, the sequences were aligned, determined the reliability of the GO term. Only the GO term that satisfies the preset score may be

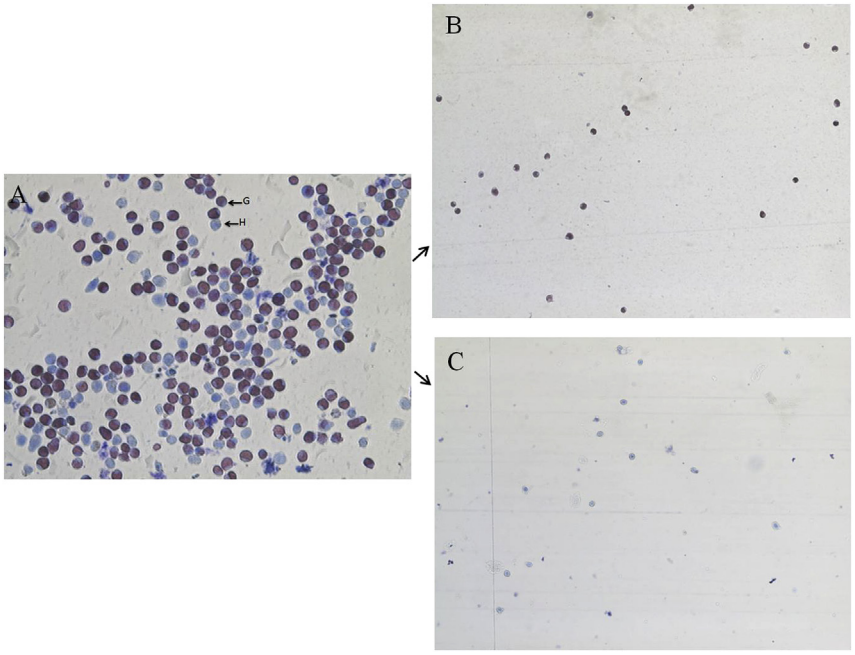

Fig. 2. Photographs of hemocytes from the *Babylonia areolata*
A. Light micrographs of haemocytes with Wright's stain (400×), G-granulocyte, H- hyalinocytes; B. Granulocytes fractions from the *Babylonia areolata* haemocytes using Percoll density gradient centrifugation (200×); C. Hyalinocytes from the *Babylonia areolata* haemocytes using Percoll density gradient centrifugation (200×).

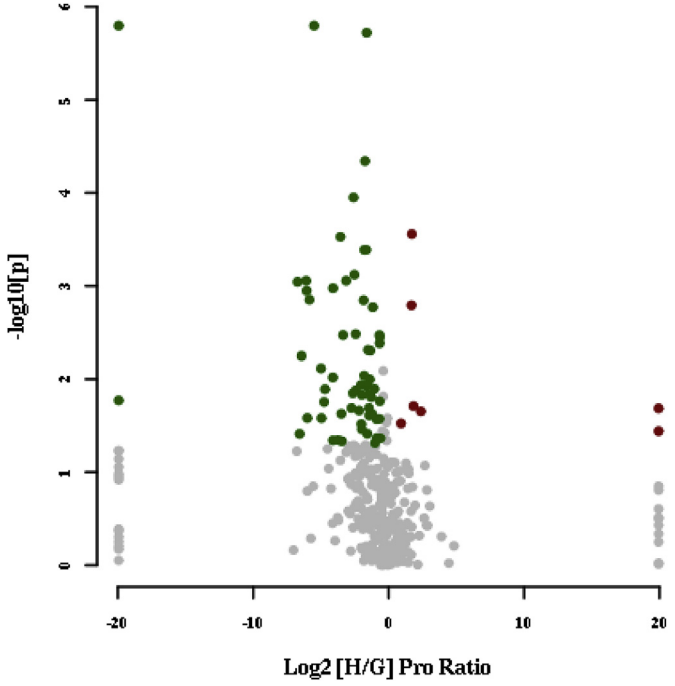

Fig. 3. Number of proteins between H and G samples. That showed the number of the up-regulated proteins when the abscissa is greater than 0, and less than 0 showed the number of down regulated proteins.

annotated. Fisher's exact test was performed to evaluate the significance test of protein enrichment of each GO term. The protein sequence was aligned with the KEGG database. The significance test of protein enrichment of each pathway was calculated by Fisher's exact test.

3. Results

3.1. Haemocytes of B. areolata

The following major haemocyte types were identified in *B. areolata*: H and G. In granulocytes, the cytoplasm was purplish red with Wright's staining, but it was blue in hyalinocytes (Fig. 2 A).

3.2. Density gradient centrifugation with Percoll

Highly enriched populations of two haemocyte subpopulations (Fig. 2B and C) were obtained by density gradient centrifugation with Percoll. G fraction accumulated in the layer near the bottom of centrifuge tube after 38% Percoll centrifugation (Fig. 1). Over 95% of the cells in this fraction were G, with only a small proportion (< 5%) of H. HG fraction was comprised approximately 30% haemocytes, with about 25% H and 5% G, after the second step by 35% Percoll centrifugation. Thus, collected fraction of G comprised more than 95% of G, fraction of H comprised more than 87% of H (Fig. 1).

3.3. Proteins identified and quantified proteins using label-free proteomic analysis

A total of 1345 proteins (FDR < 1%, at least two unique peptides) were identified from H samples, 1179 proteins were identified from G samples, the number of identified peptides was 4211 in H, it was 3645 in G. Algorithm was used, determined protein quantitative indices and protein abundances based on MS maps, normalized data of different samples to eliminate systematic errors, then analyzed the significant difference level of protein by statistical test. The number of quantified proteins was 1251 in sample H, it was 1102 in G, the number of quantified peptides was 3916 in H, it was 3401 in G. Total quantitative proteins between H and G samples were 1644, the number of up-regulated proteins in G was 215, the number of down-regulated proteins in G was 378. The total number of quantitative proteins was union between two samples, proteins that only were detected in one sample were specific protein. The number of proteins between H and G samples was shown in Fig. 3. That showed the number of the up-regulated proteins when the abscissa is greater than 0, and less than 0 showed the number of down regulated proteins.

3.4. Analysis of the significant differences in protein abundance

The differentially expressed proteins with important physiological functions between G and H with protein volumes calculated are showed in Supplementary Tables 1–2. Supplementary Table 1 showed a

significant portion of up-regulated proteins in G, Supplementary Table 2 showed a significant portion of down-regulated proteins in G.

In the present study, cathepsin C, Cathepsin F, cathepsin Z-like isoform X2, p38 MAPK, CD151 antigen-like, CD63 antigen-like, engulfment and cell motility protein 1-like isoform X2, toll-interacting protein-like, beta-adrenergic receptor kinase 2-like, calcium/calmodulin-dependent protein kinase (CaM kinase) II were up-regulated in G (Supplementary Table 1).

In this study, alpha-2-macroglobulin-like protein, C-type lectin (CTLs), galectin-2-1, galectin-3,β-1,3-glucan-binding protein, ferritin, ferritin light chain, oocyte isoform-like isoform X1, yolk ferritin-like, snail yolk ferritin, IgG Fc-binding protein-like, mega-hemocyanin, mucin-17-like, mucin-5AC-like, catalytic subunit of protein kinase A, 15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like isoform X1, histone H2B 1/2-like, cathepsin D, lysosome-associated membrane glycoprotein 5-like, peroxiredoxin-2-like were down-regulated in G (Supplementary Table 2).

3.5. Annotation of KEGG pathway

In KEGG pathway enrichment analysis, the KEGG pathway is considered the unit, and all qualitative proteins are considered the background. The statistical assessment of the enriched top 20 KEGG pathways is shown in Fig. 4. The relationship between KEGG pathway and the number of protein sequence was showed in Fig. 5 and Table 1. Table 1 results showed that mostly enriched KEGG pathways were the pathways related to ribosome, phagosome, endocytosis, carbon metabolism, protein processing in endoplasmic reticulum, oxidative phosphorylation, RNA transport, biosynthesis of amino acids, glycolysis/gluconeogenesis, ECM-receptor interaction, in that order. The results showed that the ribosome pathway is the pathway that is annotated the most to protein sequence, phagosome and endocytosis successively. What we are interested in is the immune-related pathways, and the identified proteins in the immune-related signaling pathways are as follows:

Phagosome: in present study, some proteins were identified and involved in phagosome including cathepsin L, V-type H + -transporting ATPase subunit A, V-type H + -transporting ATPase subunit B, V-type H + -transporting ATPase subunit C, V-type H + -transporting ATPase subunit D, V-type H + -transporting ATPase subunit E, V-type H + -transporting ATPase subunit F, V-type H + -transporting ATPase subunit G, complement component 3, Ras-related C3 botulinum toxin substrate 1, thrombospondin 2/3/4/5, actin beta/gamma 1, integrin beta 1, integrin beta 2, lysosomal-associated membrane protein 1/2, tubulin alpha, tubulin beta, Ras-related protein Rab-5A, Ras-related protein Rab-5B, Ras-related protein Rab-5C, Ras-related protein Rab-7A, calnexin, calreticulin, dynein heavy chain 1, dynein intermediate chain, dynein light intermediate chain 1, myeloperoxidase, protein transport protein SEC61 subunit alpha, early endosome antigen 1, and syntaxin 12/13.

Endocytosis: in this study, some proteins were identified and involved in endocytosis including beta-adrenergic-receptor kinase,

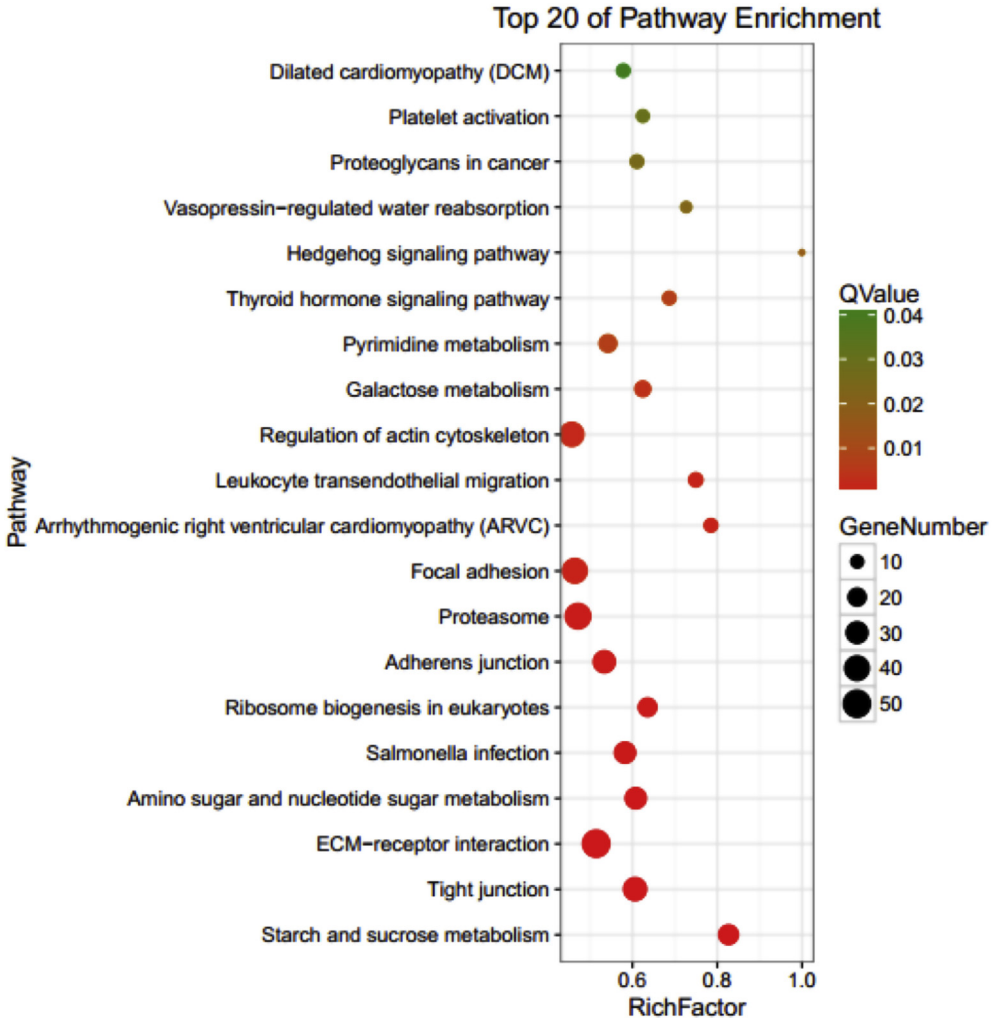

Fig. 4. Top 20 KEGG pathway enrichment of differentially expressed proteins between H and G samples.

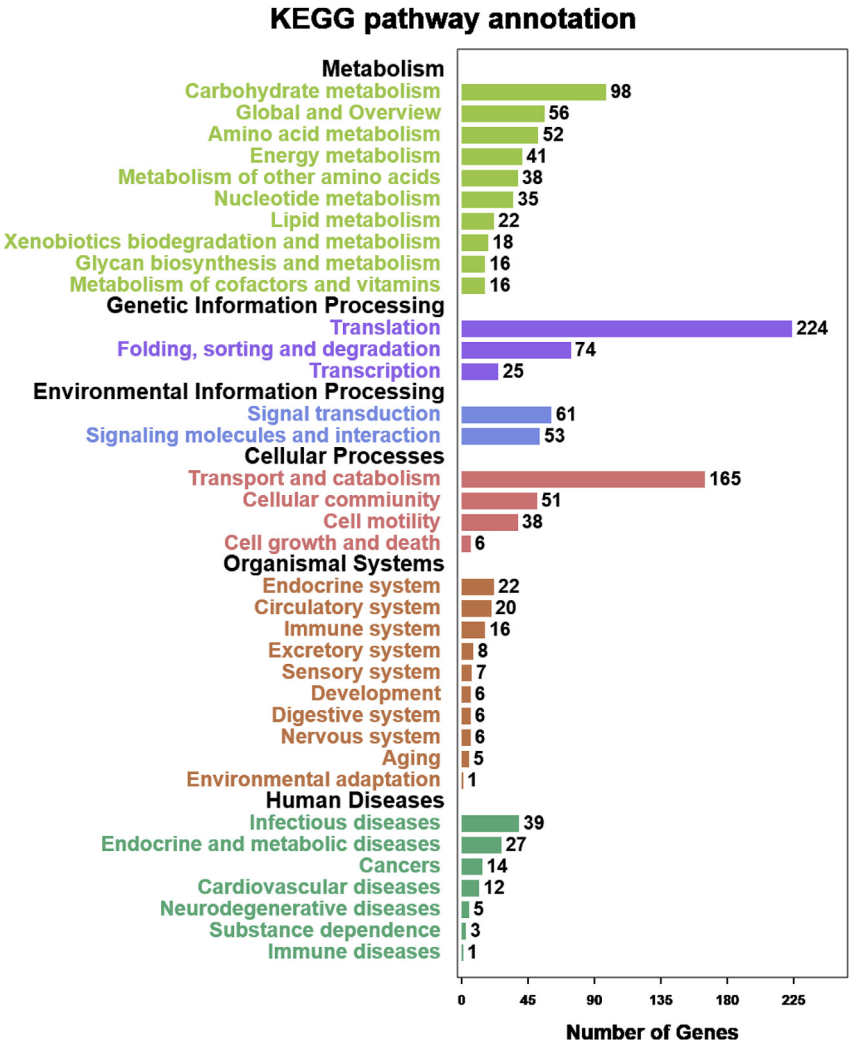

Fig. 5. The relationship between KEGG pathway annotation and the number of protein sequence.

dynamin GTPase, GTPase HRas, heat shock 70 kDa protein 1/8, cell division control protein 42, Ras homolog gene family, member A, clathrin light chain B, clathrin heavy chain, fibroblast growth factor receptor 3, actin related protein 2/3 complex, subunit 5, actin related protein 2/3 complex, subunit 4, actin related protein 2/3 complex, subunit 3, actin related protein 2/3 complex, subunit 1A/1B, actin related protein 2/3 complex, subunit 2, Ras-related protein Rab-35, Ras-related protein Rab-5B, Ras-related protein Rab-5C, Ras-related protein Rab-7A, Ras-related protein Rab-8A, Ras-related protein Rab-10, Ras-related protein Rab-11A, Ras-related protein Rab-11B, ADP-ribosylation factor 1, ADP-ribosylation factor 3, ADP-ribosylation factor 5, ADP-ribosylation factor 6, capping protein (actin filament) muscle Z-line, alpha, kinesin family member 5, endophilin-A, endophilin-B, AP-2 complex subunit alpha, AP-2 complex subunit beta-1, AP-2 complex subunit mu-1, charged multivesicular body protein 4, vacuolar protein-sorting-associated protein 4, vacuolar protein sorting-associated protein VTA1, vacuolar protein sorting-associated protein VTA1, SH3 domain-containing kinase-binding protein 1, EH domain-containing protein 3, early endosome antigen 1, EH domain-containing protein 1, Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein, sorting nexin-1/2, sorting nexin-3/12, vacuolar protein sorting-associated protein 26, vacuolar protein sorting-associated protein 29, vacuolar protein sorting-associated protein 35, and vacuolar protein sorting-associated protein IST1.

Lysosome: in present study, some proteins were identified and involved in lysosome including alpha-galactosidase, alpha-N-acetylgalactosaminidase, cathepsin C, cathepsin B, cathepsin L, cathepsin F, cathepsin D, V-type H + -transporting ATPase subunit d, V-type H + -transporting ATPase subunit a, clathrin light chain B, clathrin heavy chain, ATP-binding cassette, subfamily A (ABC1), member 2, CD63 antigen, lysosomal-associated membrane protein 1/2, CD164 antigen, cathepsin X, lysosomal alpha-mannosidase, hexosaminidase, saposin, ganglioside GM2 activator, Niemann-Pick C1 protein, AP-1 complex subunit gamma-1, AP-1 complex subunit beta-1, AP-1 complex subunit mu, ADP-ribosylation factor-binding protein GGA, cathepsin A (carboxypeptidase C), Niemann-Pick C2 protein, and sulfatase modifying factor 1.

Wnt signaling pathway: in this study, some proteins were identified and involved in Wnt signaling pathway including low density lipoprotein receptor-related protein 5/6, S-phase kinase-associated protein 1, casein kinase II subunit alpha, protein kinase A, serine/threonine-protein phosphatase 2B catalytic subunit, Ras-related C3 botulinum toxin substrate 1, presenilin 1, calcyclin binding protein, Ras homolog gene family, member A, calcium/calmodulin-dependent protein kinase (CaM kinase) II, serine/threonine-protein phosphatase 2B regulatory subunit, Ras-related C3 botulinum toxin substrate 2, and Ras-related C3 botulinum toxin substrate 3.

MAPK signaling pathway: in present study, some proteins were identified and involved in MAPK signaling pathway including GTPase HRas, heat shock 70 kDa protein 1/8, protein kinase A, serine/threonine-protein phosphatase 2B catalytic subunit, Ras-related protein Rap-1A, growth factor receptor-binding protein 2, ribosomal protein S6

Table 1
Top KEGG pathway.

	Pathway name	Number of proteins (2948)	Pathway ID
1	Ribosome	627	ko03010
2	**Phagosome**	297	ko04145
3	**Endocytosis**	267	ko04144
4	Carbon metabolism	219	ko01200
5	Protein processing in endoplasmic reticulum	209	ko04141
6	Oxidative phosphorylation	167	ko00190
7	RNA transport	135	ko03013
8	Biosynthesis of amino acids	124	ko01230
9	Glycolysis/Gluconeogenesis	108	ko00010
10	ECM-receptor interaction	97	ko04512
11	Spliceosome	95	ko03040
12	Proteasome	91	ko03050
13	**Lysosome**	86	ko04142
14	Focal adhesion	86	ko04510
15	Gap junction	84	ko04540
16	Regulation of actin cytoskeleton	83	ko04810
17	Citrate cycle (TCA cycle)	76	ko00020
18	Pyruvate metabolism	72	ko00620
19	mRNA surveillance pathway	72	ko03015
20	Oocyte meiosis	72	ko04114
21	**Wnt signaling pathway**	71	ko04310
22	**MAPK signaling pathway**	65	ko04010
42	**Peroxisome**	43	ko04146
68	**PI3K-Akt signaling pathway**	27	ko04151
96	**Jak-STAT signaling pathway**	19	ko04630
97	**Inflammatory mediator regulation of TRP channels**	19	ko04750
123	**NOD-like receptor signaling pathway**	15	ko04621
153	**Toll-like receptor signaling pathway**	9	ko04620

kinase alpha-1/2/3/6, Ras-related C3 botulinum toxin substrate 1, cell division control protein 42, mitogen-activated protein kinase kinase 6, mitogen-activated protein kinase 8 interacting protein 3, GTPase NRas, Ras-related protein Rap-1B, Ras-related C3 botulinum toxin substrate 2, and Ras-related C3 botulinum toxin substrate 3.

3.6. Gene Ontology (GO) functional annotations

Proteins with high similarity were annotated with GO terms, GO describes genes in the organism and the attributes of gene products from three aspects, namely biological processes (BPs), molecular functions (MFs), and cellular component (CCs). The identified proteins were analyzed by GO annotations in G and H, the GO functional classification was shown in Fig. 6. Using the BP term, the number of proteins participating in cellular process and those involved in single organism processes was the first and second largest groups, respectively. Using the MF term, the number of proteins involved in binding and related to catalytic activity was the first and second largest groups, respectively. Using the CC term, the number of proteins involved in cells and cell part were the first largest, followed by those related to organelles, the macromolecular complex respectively (Fig. 6).

3.7. KEGG pathway enrichment analysis of differentially expressed proteins (DEPs)

In organisms, different proteins are coordinated to complete a series of biochemical reactions, in which they perform their biological functions. Therefore, pathway analysis is the most direct and necessary method to obtain a systematic and comprehensive understanding of cellular processes, phenotype, the occurrence and mechanism of diseases, and mechanisms of drug actions. The KEGG pathway database (www.kegg.jp) was accessed using the KEGG automatic annotation server. The differentially expressed proteins were analyzed by KEGG in two haemocyte sub-populations, statistical assessment of the significantly enriched KEGG pathways is shown in Fig. 7. The results showed that the largest number of differences of the most significantly enriched KEGG pathways between up-regulation and down-regulation in G were the pathways related to phagosome, ECM-receptor interaction, proteasome and endocytosis, which the number of down-regulation proteins was more than up-regulation proteins in G. In addition, in some KEGG pathways, such as lysosome, mRNA surveillance pathway, insulin resistance, glyoxylate and dicarboxylate metabolism, valine, leucine and isoleucine degradation, the number of up-regulation proteins was more than down-regulation proteins in G (Fig. 7). Interestingly, the significant portion of down-regulated proteins in G were PI3K-Akt signaling pathway, cysteine and methionine metabolism, aminoacyl-tRNA biosynthesis, vascular smooth muscle contraction, gap junction, calcium signaling pathway and alcoholism (Fig. 7). The immune-related signaling pathways are as follows:

For phagosome pathway, 4 proteins were up-regulated in G, including Ras-related C3 botulinum toxin substrate 1, actin beta/gamma 1, and dynein intermediate chain; 71 proteins were down-regulated in G (Fig. 7), including V-type H + -transporting ATPase subunit C, V-type H + -transporting ATPase subunit E, V-type H + -transporting ATPase subunit G, V-type H + -transporting ATPase subunit a, thrombospondin 2/3/4/5, actin beta/gamma 1, integrin beta 1, integrin beta 1, Ras-related protein Rab-7A, dynein intermediate chain, and protein transport protein SEC61 subunit alpha (Supplementary Fig. 1).

For endocytosis pathway, 16 proteins were up-regulated in G, including beta-adrenergic-receptor kinase, cell division control protein 42, clathrin light chain B, actin related protein 2/3 complex, subunit 2, Ras-related protein Rab-35, SH3 domain-containing kinase-binding protein 1, and sorting nexin-3/12; 49 proteins were down-regulated in G (Fig. 7), including GTPase HRas, fibroblast growth factor receptor 3, Ras-related protein Rab-7A, Ras-related protein Rab-8A, Ras-related protein Rab-11B, ADP-ribosylation factor 1, kinesin family member 5, endophilin-B, AP-2 complex subunit alpha, AP-2 complex subunit mu-1, EH domain-containing protein 3, and EH domain-containing protein 1 (Supplementary Fig. 2).

For lysosome pathway, 16 proteins were up-regulated in G, including alpha-N-acetylgalactosaminidase, cathepsin C, cathepsin F, clathrin light chain B, CD63 antigen, cathepsin X, lysosomal alpha-mannosidase, and saposin; 8 proteins were down-regulated in G (Fig. 7), including cathepsin D, V-type H + -transporting ATPase subunit a, and ganglioside GM2 activator (Supplementary Fig. 3).

For MAPK signaling pathway, 4 proteins were up-regulated in G, including Ras-related C3 botulinum toxin substrate 1, Ras-related C3 botulinum toxin substrate 3, and cell division control protein 42; 16 proteins were down-regulated in G (Fig. 7), including GTPase HRas, protein kinase A, Ras-related protein Rap-1A, GTPase NRas, and Ras-related protein Rap-1B.

For JAK-STAT signaling pathway, 2 proteins were up-regulated in G, including tyrosine-protein phosphatase non-receptor type 2; 5 proteins were down-regulated in G (Fig. 7), including GTPase HRas and glial fibrillary acidic protein.

For Wnt signaling pathway, 6 proteins were up-regulated in G, including Ras-related C3 botulinum toxin substrate 1, calcium/calmodulin-dependent protein kinase (CaM kinase) II, and Ras-related C3 botulinum toxin substrate 3; 6 proteins were down-regulated in G (Fig. 7), including low density lipoprotein receptor-related protein 5/6 and protein kinase A.

For peroxisome pathway, one protein was up-regulated in G, which was alanine-glyoxylate transaminase/serine-glyoxylate transaminase/serine-pyruvate transaminase; 12 proteins were down-regulated in G, including isocitrate dehydrogenase, isocitrate dehydrogenase, long-chain acyl-CoA synthetase, and superoxide dismutase Cu–Zn family.

For ECM-receptor interaction pathway, one protein was up-regulated in G, which was integrin alpha 6; 49 proteins were down-regulated in G (Fig. 7), including thrombospondin 2/3/4/5, laminin

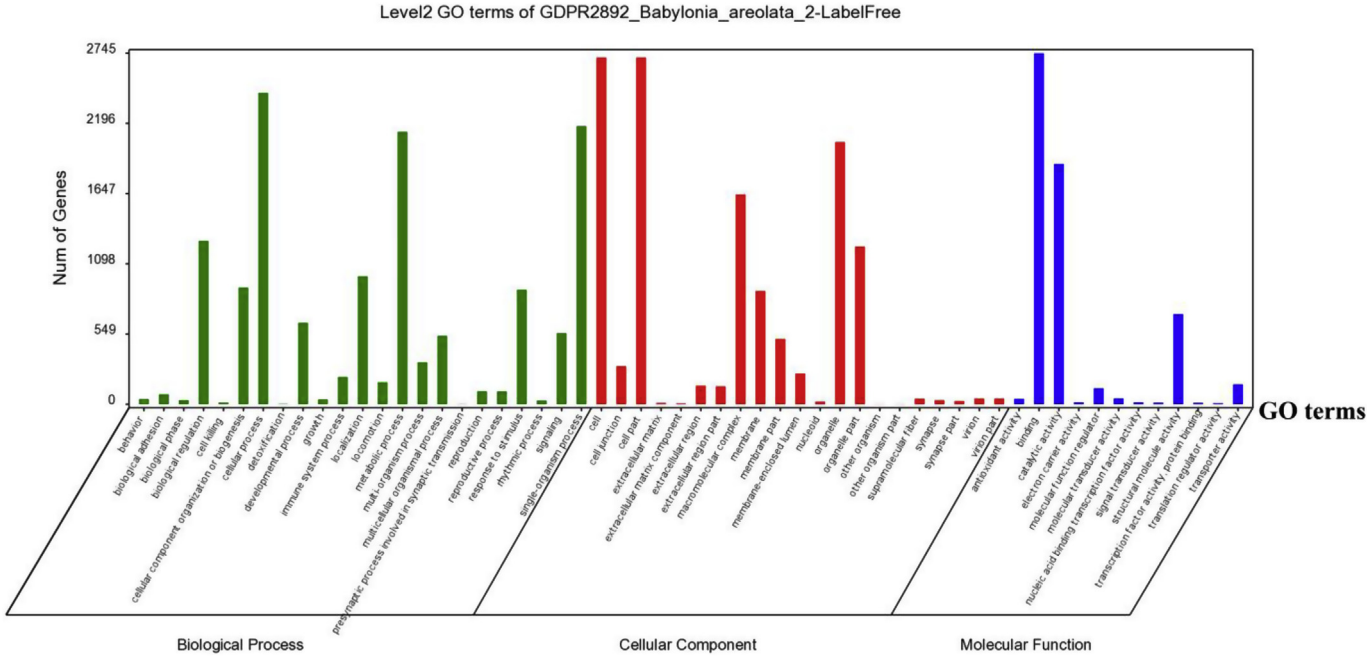

Fig. 6. GO functional classification analysis of identified protein.

(gamma 1), laminin (beta 1), collagen (type IV, alpha), laminin (alpha 3/5), reelin, and integrin alpha 4.

For PI3K-Akt signaling pathway, 4 proteins were down-regulated in G, including GTPase HRas, GTPase NRas, and integrin alpha 4 (Fig. 7).

Fig. 8 showed that the most significantly enriched up-regulated KEGG pathways in G, the pathways related to starch and sucrose metabolism, other glycan degradation, nicotinate and nicotinamide metabolism, mRNA surveillance pathway and lysosome (q value < 0.005). Fig. 9 showed that the most significantly enriched down-regulated KEGG pathways in G, the pathways related to amino sugar and nucleotide sugar metabolism, focal adhesion, tight junction, proteasome, salmonella infection, ECM-receptor interaction and ribosome biogenesis in eukaryotes (q value < 0.005).

3.8. GO enrichment analysis of DEPs

The GO annotation of the target protein set allowed the classification of these proteins according to BP, MF, and CC. The proportion of proteins in each class may indicate the effects of each of the H and G on each GO class. The differentially expressed proteins were analyzed by GO annotations, statistical analysis of the significantly enriched GO terms from data of the H and G is shown in Fig. 10. Using the BP term, the number of proteins was counted respectively, which participated in metabolism processes, cellular processes and single organism. Using the MF term, the number of proteins, mainly involved in binding and related to catalytic activity, was determined, respectively. Using the CC term, the number of proteins, involved in cells and cell part, followed by those related to organelles, the macromolecular complex and the membrane, was determined, respectively. The results showed that most of these DEPs exhibited binding functions (Fig. 10).

4. Discussion

Haemocytes of *B. areolata* are classified into H and G according to the number and size of granules that haemocytes contain and their biological function. Previously, little direct research has been performed to invest immunological function with different types of haemocyte in gastropods, respectively. In the present work, the methodology to separate *B. areolata* haemocyte subpopulations was developed, using a two-steps Percoll density gradient centrifugation. It will help to carry out more detailed studies in future to understand the connection and the immunological function between G and H in gastropods. It could also help researchers to research that the haematopoietic tissue how to develop and differentiate into different types of haemocyte in gastropods.

Unlike gene sequences, proteins, as performers of gene function, determine phenotypes, which can be thought of as snapshots of genome expression. The proteome can be considered closer to the phenotype on which natural selection acts than the transcriptome [25]. This makes high-throughput MS identification of proteins from non-model organisms *B. areolata* more efficient, a proteomics technique is a worthy consideration for the study of non-model species [26].

4.1. Proteins associated with immune responses between G and H in B. areolata

4.1.1. Proteins associated with pattern recognition receptors (PRRs)

Innate immunity is the first line of defence against infections, and nonself recognition of pathogen-associated molecular patterns (PAMPs) through host pattern recognition receptors (PRRs) that are not present in higher animals is considered the initial step of the innate immunity, and then triggers downstream immune reactions [27]. PRRs are essential in innate immunity, especially in invertebrates [28]. The previous studies suggested the higher abundance of representative PRRs in invertebrates than in vertebrates [29]. These PRRs can specially bind PAMPs present on the surface of microorganism, mainly carbohydrate-related molecules, including lipopolysaccharide (LPS), peptidoglycan (PGN) and β-1,3-glucan (βG), which can activate signal transduction and transcription [30].Subsequently, these are recognised by a series of well-conserved PRRs, and activated PRRs directly or indirectly trigger cellular or humoral responses [31], including the cell-mediated responses such as phagocytosis and encapsulation, and the humoral responses including the production of antimicrobial peptide (AMP) synthesis, prophenoloxidase (proPO) system activation and toxin or reactive oxygen species production, are triggered by the interactions between PRRs with PAMPs to prevent from infection at an early stage [32].

PRRs, including peptidoglycan-recognition proteins, LPS-binding

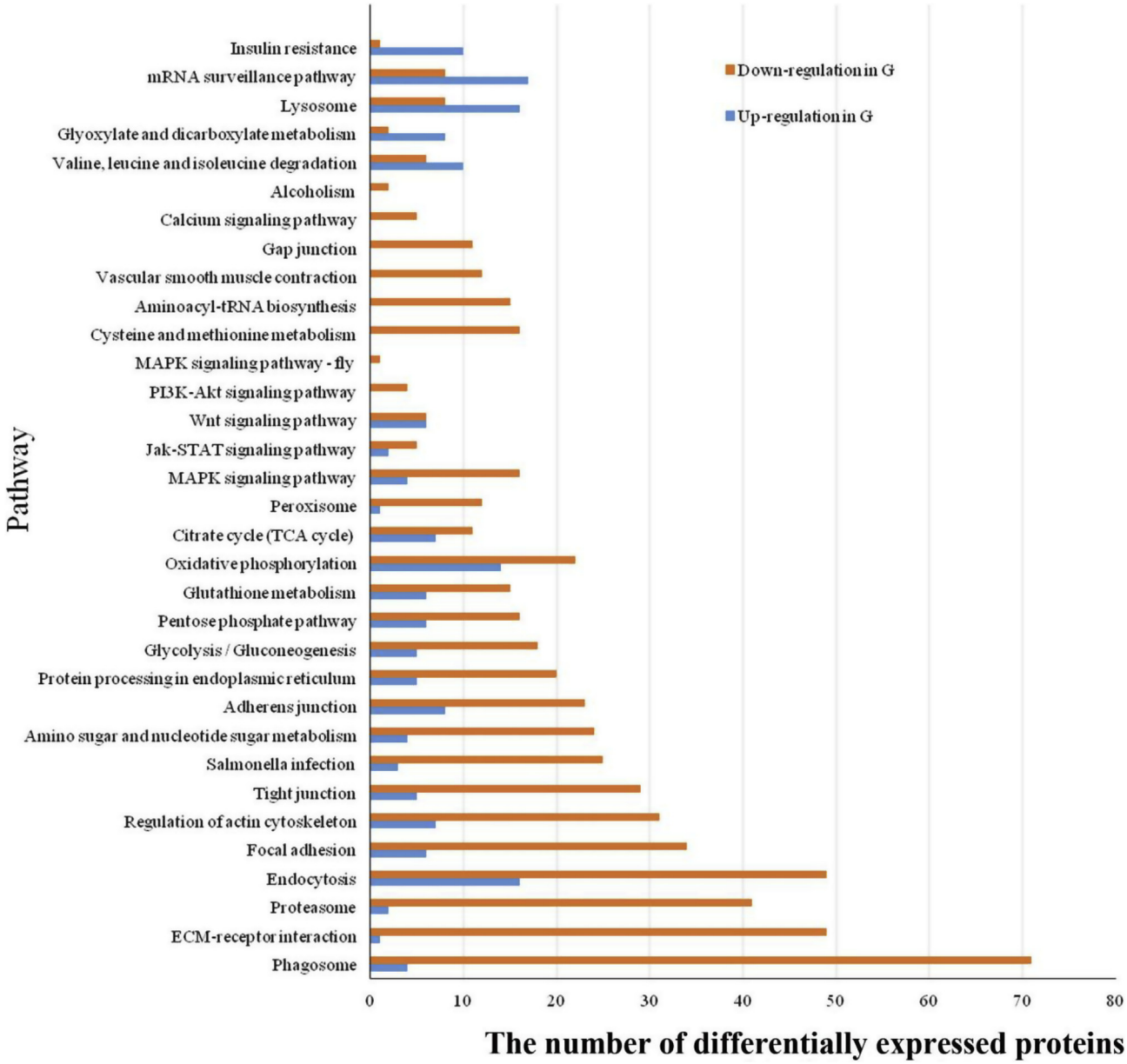

Fig. 7. Significantly enriched KEGG pathways analysis for differentially abundant proteins.

proteins, fibrinogen-related proteins, Toll-like receptors, β-1,3-glucan-binding proteins, C-type lectins (CTLs) and galactoside binding lectins (galectins), were identified in a variety of mollusc species [33]. In this study, some proteins were identified and involved in PRRs and PAMPs, including C-type lectin, galectin-2-1, galectin-3, and β-1,3-glucan-binding protein (Supplementary Tables 1–2). The detailed description was as follows:

Lectins: Because of lacking adaptive immunity in invertebrates, lectins act as determinants of phagocytosis [34]. In phagocytic recognition models, it is proposed that phagocytosis is related to the ability of lectins to recognise antigens [35]. In crustaceans, lectins have a function in initial responses against foreign pathogen invasion. Lectins are classified into six categories according to their structures and functions: C-, L-, P-, I-, R-, and S-type lectins [30,36]. Lectins are important non-Toll-like receptor (TLR) PRRs [37]. Lectin activity is advantageous within the immune system, both for self/nonself discrimination, and for autologous cellular interactions between components of the immune system [38].

In this study, proteins ID c255306_g2_orf1, c254024_g1_orf1, Unigene0049230, and Unigene0055947, were identified as C-type lectin (CTLs) (Supplementary Table 2), and those proteins exhibited down-regulation in G. CTLs functioned as PRR in innate immunity increased much attention [39]. These CTLs are involved in various biological processes, including cytotoxic effects [40], antibacterial activity [41], promotion of phagocytosis [42], production of reactive oxygen species [43], and nodule formation [44]. CfLec-1 and -2 (C-type lectin in *Chlamys farreri*) not only specifically bind different PAMPs but also increase the opsonization of haemocytes [45,46]. A novel C-type lectin was identified and characterized from the razor clam *Sinonovacula constricta* and named as ScCTL [39], and it was suggested that ScCTL carried out important functions in protecting cells from pathogenic infection in *S. constricta* [39]. These CTLs show either antibacterial activity or bacterial binding abilities, and regulate the activation of various immune responses [39]. Therefore, in this study, the result suggested that two sub-population haemocytes in *B. areolata* may reflect the different roles in regulating phagocytosis.

Galectins (Gals): Proteins ID Unigene0045980 and c265854_g1_orf1 were identified as galectin-2-1 and galectin-3,

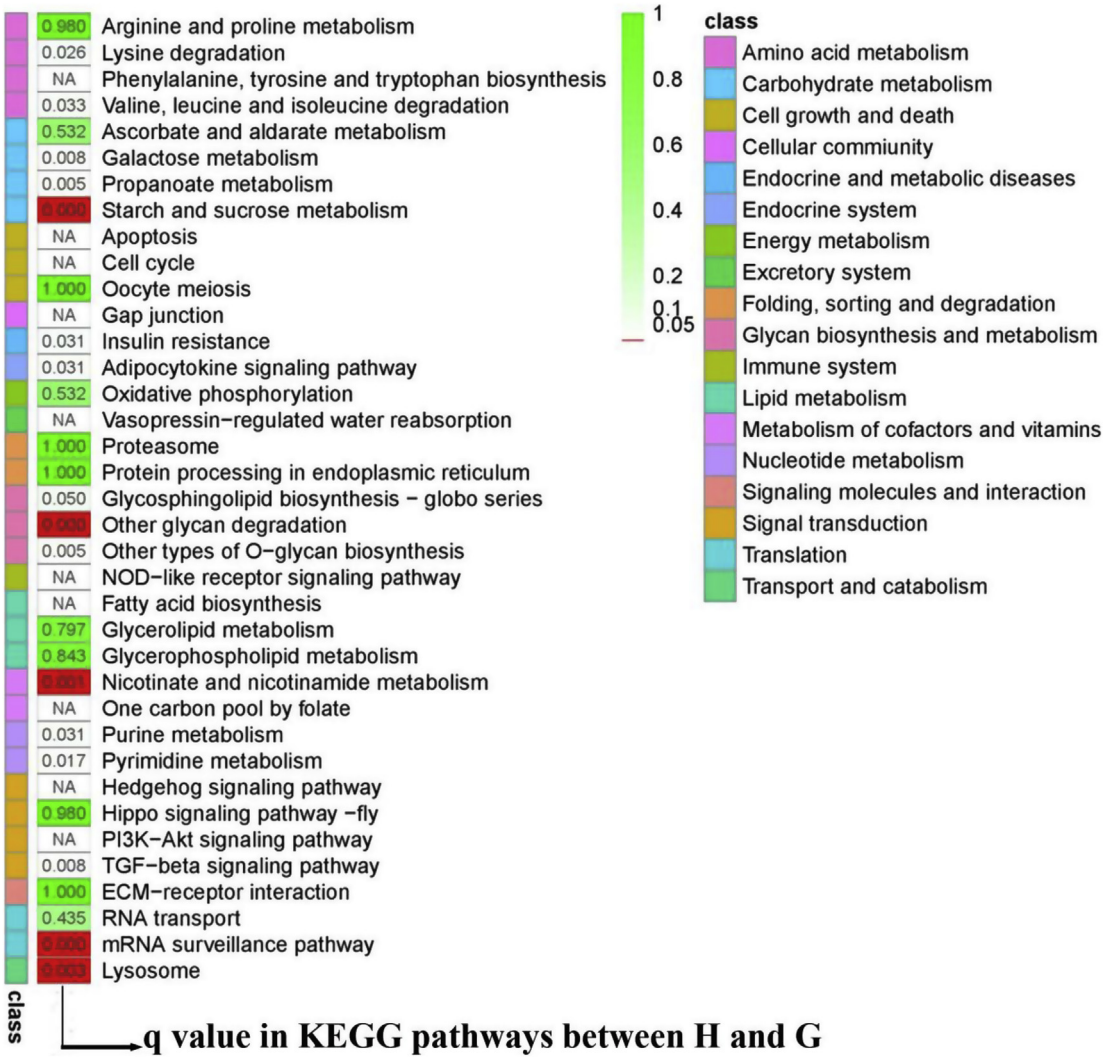

Fig. 8. Significantly enriched up-regulated KEGG pathways in G (q value).

respectively, and intensity based absolute quantification (iBAQ) were 3663400 and 2693500 in H, however those two proteins were 0 in G, and exhibited down-regulation in G (Supplementary Table 2). Galectins were involved in regulation of both innate and adaptive immunity [47–49]. Galectins are S-type lectins with an affinity for β-galactosides, which play the key roles in innate immune response of invertebrate [50]. The roles of Gals have been further supported by their ability to directly recognise microbial pathogens [47]. Gals bind glycans on the surface of pathogenic microbes and parasitic worms, mediates recognition and effector functions in innate immunity [51], indicating their roles as PRRs [52]. Thus, the potential role of Gals as PRRs has become an area of increased attention. Gals could be a good candidate for disease control in aquaculture which suffers losses due to bacterial and viral diseases [53].

Gals were involved in various processes in innate immunity [54]. Previous studies have shown that Gals characterized from aquatic animals have shown potent anti-bacterial, anti-fungal antiviral, and anti protozoan activity [55]. Recently, a variety of Gals have been cloned and named in shellfish. Three newly identified Gals homologues from triangle sail mussel (*Hyriopsis cumingii*) function as potential PRRs [48]. Quadruple-CRD-containing Gals (carbohydrate recognition domain (CRD)), first identified in eastern oyster haemocytes of *Crassostrea virginica* but later in other species, appear to function in the recognition and phagocytosis of microorganisms [56]. Gals also had been found in the haemocytes of *Pinctada fucata* [57]. Zhao et al. [48] results preliminarily demonstrated that the Gals of *Hyriopsis cumingii* can bind to microbial polysaccharides and regulate AMPs expression, indicating that may serve as potential PPRs to participate in innate immune responses [48]. ScGal2 (a novel Gal in razor clam *S. constricta*) plays an indispensable role in innate immunity in razor clam, and likely is involved in immune recognition and clearance processes [52]. Gal-3 can affect the course of an infection by influencing cells in the innate immune system such as neutrophils, monocytes/macrophages and dendritic cells [58]. Meanwhile, Gal-3 exhibits proinflammatory effects through anti-apoptotic activity toward macrophages [59]. Huang et al. [49] study showed that rCgGal-2 and rCgGal-3 (recombinant single- and four-CRD-containing Gals in oyster *Crassostrea gigas*) were found to recruit haemocytes and promote encapsulation. Similar function in cellular adhesion and the enhancement of encapsulation were investigated in the rAiGal2 from *A. irradians* [60].

β-1, 3-glucanase related protein (BGRP): proteins ID c268781_g2_orf1 was identified asβ-1,3-glucan-binding protein, iBAQ was 969130 in H, however the protein was 0 in G, and exhibited down-regulation in G (Supplementary Table 2). BGRP (previously named as lipopolysaccharide or β-1,3-glucan binding protein (LGBP) in crustaceans, identified from the plasma of marine mussel *Perna viridis* [61]) is a representative PRR family, which has been identified ubiquitous in crustaceans [62]. BGRP family was found to be necessary for Prophenoloxidase (proPO) activation in decapod crustaceans by recognizing the invading microorganisms [63], and proPO activating system is an

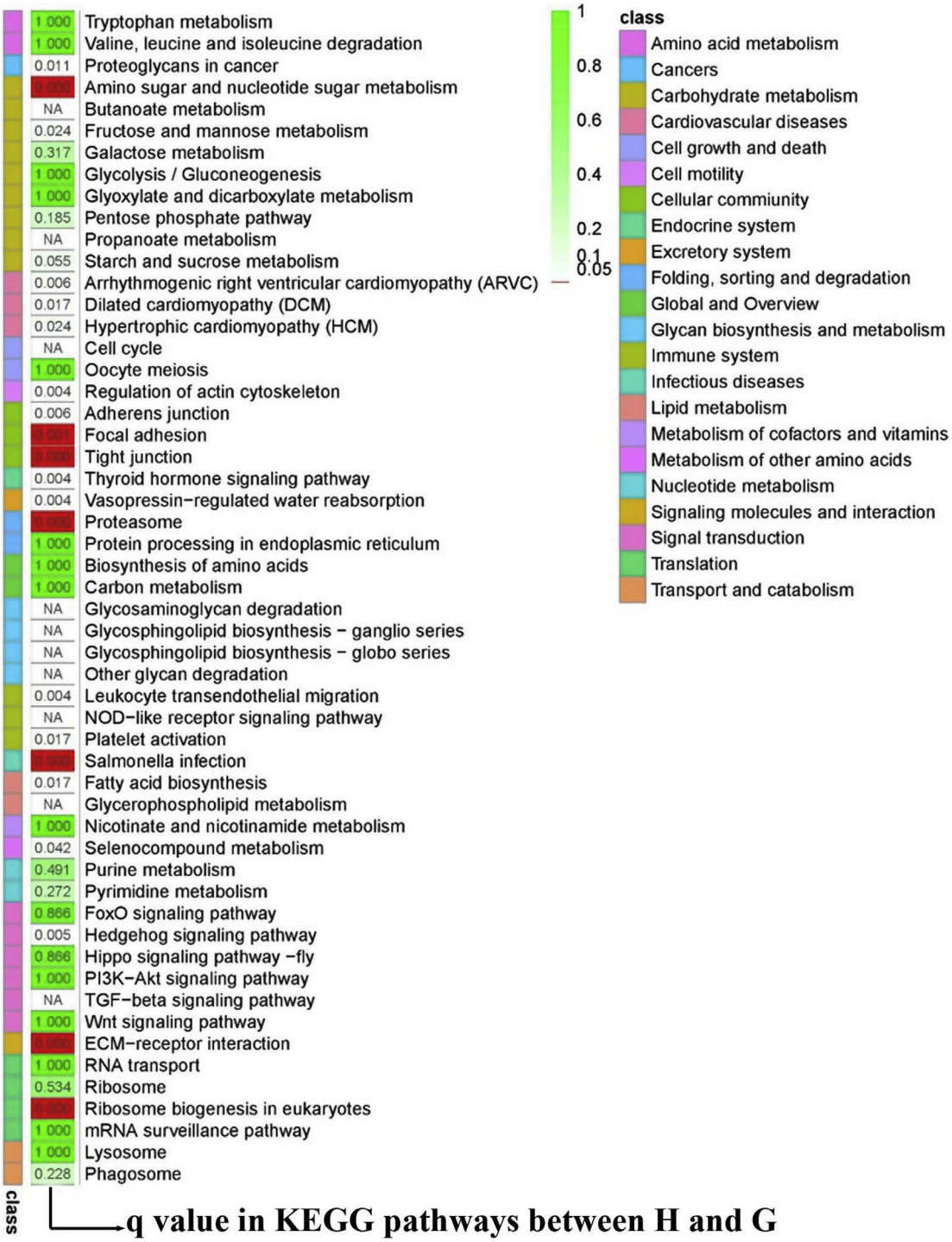

Fig. 9. Significantly enriched down-regulated KEGG pathways in G (q value).

important immune response for arthropods.

C-type lectin, galectin-2-1, galectin-3, and β-1,3-glucan-binding protein were down-regulated in G, and the results suggested that two haemocyte subpopulations involved in PRRs were different.

4.1.2. Other important proteins associated with immune responses

p38 MAPK: proteins ID Unigene0012914 was identified as p38 MAPK, and iBAQ was 0 in H, however the protein (1270500 in G) exhibited up-regulation in G (Supplementary Table 1). The p38 has been shown to be involved in a lot of cellular functions, such as the responses to stress and inflammation [64]. Mitogen-activated protein kinase (MAPK) signaling pathways are highly conserved and play important roles in cellular responses to extracellular stimuli [65]. In invertebrates, expression profiles of the p38 MAPK signaling pathway from Chinese shrimp *Fenneropenaeus chinensis* in response to viral and bacterial infections was studied [64]. Recent research in *Litopenaeus vannamei* showed that p38 MAPK plays an important role in defending against bacterial infections [66], cellular response to inflammatory cytokines, pathogenic infection and environmental stress [64]. These results suggested that p38 MAPK pathways play a key role in the defense against bacterial or viral infections in invertebrates [64].

Toll-interacting protein (Tollip): proteins ID c244687_g1_orf1

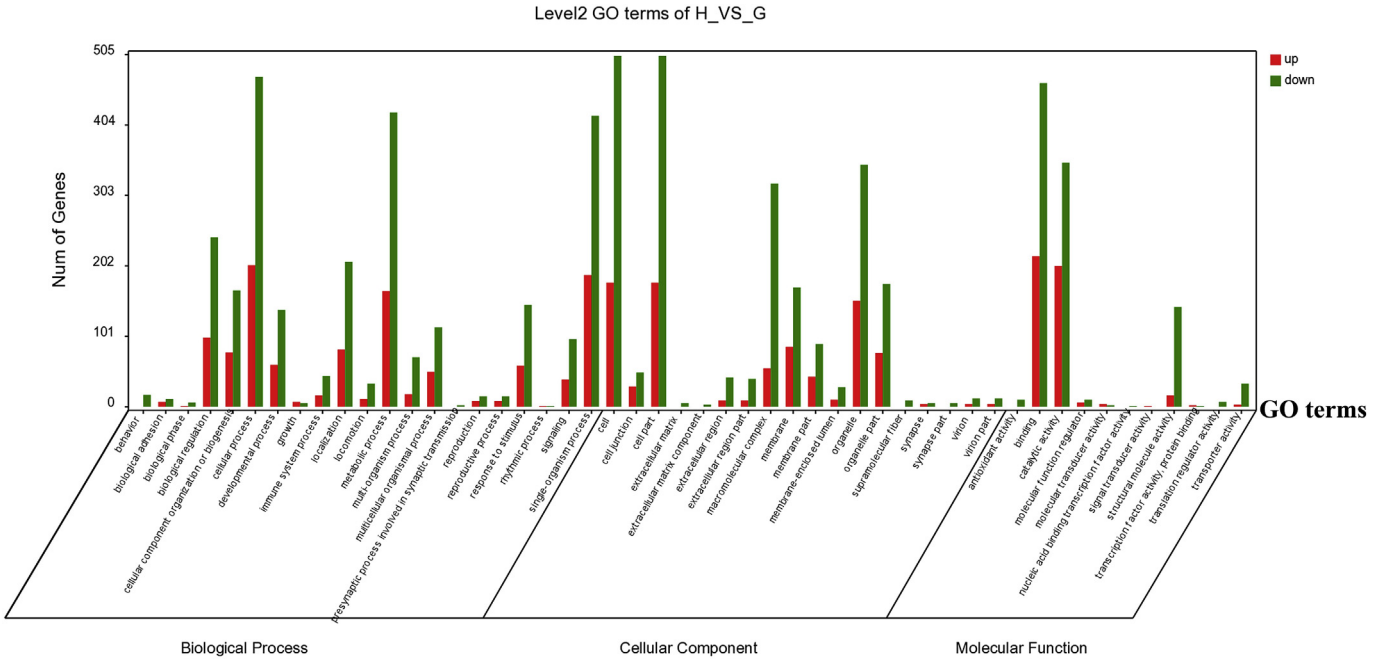

Fig. 10. GO taxonomy of differentially expressed proteins.

was identified as toll-interacting protein-like, and iBAQ was 0 in H, however the protein (464480 in G) exhibited up-regulation in G (Supplementary Table 1). Tollip is a mediator participated in the TLRs signaling pathway which is key for innate immune system [67]. Tollip plays an important role in preventing harmful inflammatory responses [68].

β2 adrenergic receptor (β2AR): proteins ID c262377_g1_orf1 was identified as beta-adrenergic receptor kinase 2-like, and iBAQ was 0 in H, however the protein (271260 in G) exhibited up-regulation in G (Supplementary Table 1). β2AR activation has been reported to be involved in anti-inflammatory properties [69], which can induce microglial NADPH oxidase activation and dopaminergic neurotoxicity through an ERK-dependent/Protein Kinase A-independent pathway [69]. Activation of β2AR, like most G-protein-coupled receptors, induces to an increase in cAMP following the activation of adenylate cyclase by Gs-proteins, and results in the stimulation of protein kinase A (PKA) [70].

4.2. Calcium/calmodulin-dependent protein kinase (CaM kinase) II

In this study, for Wnt signaling pathway, calcium/calmodulin-dependent protein kinase (CaM kinase) II were up-regulated in G (Supplementary Table 1). Calmodulin is a versatile calcium-binding protein [71], which is an important signaling molecule in host-pathogen interactions when retrovirus was studied in mammalian cells [72]. Calmodulin can regulate other proteins involved in immune functions in vertebrates, such as autophagy, programmed cell death, inflammation, and immune response [73]. Li et al. [74] found that calmodulin was up-regulated when the Chinese mitten crab (*Eriocheir sinensis*) was stimulated with pathogenic bacteria, and the result suggested that calmodulin change was related to stress and immune response.

Alpha-2 macroglobulin (α2M): proteins ID Unigene0017207 was identified as alpha-2-macroglobulin-like protein, and iBAQ was 5259800 in H, however the protein (845530 in G) was down-regulated 6.22-fold in G (Supplementary Table 2). The α2M is a widely protease inhibitor and an evolutionarily conserved molecule in innate immune system [75], which has been characterized in *Chlamys farreri*, *Hyriopsis cumingii*, *Cristaria plicata,* and *Littorina littorea* [75]. The α2M characterization and function in haemocyte phagocytosis of *Vibrio alginolyticus* in the pearl oyster *Pinctada fucata* was studied [75]. These results suggested that α2M is an important molecule in the innate immune system. Moreover, α 2 M was indispensable for activation of the proPO system [76].

Hemocyanins: proteins ID c267020_g5_orf1, c267138_g4_orf1, and c261258_g5_orf1 were identified as mega-hemocyanin, which were down-regulated 3.94, 4.36, 6.03-fold in G, respectively (Supplementary Table 2). Hemocyanins are involved in immune functions [77], and molluscan hemocyanins can activate the classical pathway of the human complement system through natural antibodies [78]. However, in invertebrates, more recently studies have described the molluscan *Concholepas concholepas* hemocyanin [CCH] [79] and the limpet *Fissurella latimarginata* hemocyanin [FLH] [80]. These hemocyanins possess immunogenicity and antitumor immunostimulatory properties, equal to or better than those of the keyhole limpet hemocyanin (KLH, from the gastropod *Megathura crenulata*) [81]. In present study, hemocyanins was down-regulated in G, and the results contribute to understand the location and change of hemocyanin production.

Protein kinase A (PKA): Protein ID Unigene0010301 was identified as catalytic subunit of protein kinase A, which was down-regulated in G (Supplementary Table 2). Protein kinase A (PKA), participated in regulation of immune response of invertebrates [82,83], is a serine/threonine kinase that regulates many cellular process in invertebrate haemocyte activity as well as humoral immune response. PKA substrates participated in immune activation including transcription factors, phospholipases and members of the MAPK pathway [83]. PKA can regulate Src kinases through modulation of Csk, and suggested that PKA participated in the fine-tuning of immune receptor signaling in lipid rafts [83] and the activation of the marine mussel *Mytilus galloprovincialis* haemocytes by bacterial lipopolysaccharide (LPS) and interleukin-2 [82]. A cyclic AMP signaling pathway was involved in modulation of phagocytosis in oyster *Crassostrea gigas* haemocytes by noradrenaline [84]. Ottaviani et al. [85] revealed the cAMP-PKA pathway involved in regulating changes in cell shape of mollusc *M. galloprovincialis* haemocytes which were induced by recombinant interleukin-8, and cAMP–PKA pathway was involved in the regulation of haemocyte adhesion ability [86].PKA was involved in the regulation of *G. mellonella* haemocytes adhesion ability in vitro [87] and non-self

cellular response in vivo [86], the results suggested that PKA was involved in the cellular immune response regulation. Fortunately, in this study, PKA activity changes concurrently in different haemocyte types upon immune challenge have been identified.

Ferritin: Proteins ID Unigene0046578, c254631_g1_orf1, c264074_g2_orf1, and c264746_g1_orf1 were identified as ferritin, ferritin light chain, oocyte isoform-like isoform X1, yolk ferritin-like, snail yolk ferritin, and those proteins were down-regulated in G (Supplementary Table 2). As an important iron storage protein, ferritin plays a crucial role in the iron-withholding defense system [88]. Kong et al. [89] study showed that secreted ferritins in the Chinese mitten crab *Eriocheir sinensis* were up-regulated after bacterial or fungi challenge. Recently, ferritin has been characterized from various of seawater mollusks including *Crassostrea gigas*, *Haliotis discus discus*, *Meretrix meretrix*, *Haliotis rufescens*, *Saccostrea cucullate*, *Argopecten irradians*, *Hyriopsis schlegelii* and so on, exhibiting its roles in antioxidative capacity and immune defense against bacterial infection [90].

In this study, p38 MAPK, Toll-interacting protein, β2 adrenergic receptor and Calcium/calmodulin-dependent protein kinase (CaM kinase) II exhibited up-regulation in G, alpha-2 macroglobulin, hemocyanins, protein kinase A, and ferritin were down-regulated in G, and the result suggested that there were different immune regulatory pathways in H and G.

4.3. Differentially expressed proteins associated with immune-related KEGG pathway enrichment analysis in G and H

4.3.1. V-ATPase

For lysosome pathway, V-type H + -transporting ATPase subunit a was down-regulated in G. V + ATPases are the main proteins responsible for acidification of intracellular compartments of secretory vesicles, lysosomes, endosomes, plasma membranes and Golgi vesicles [91]. V + ATPases also play an important role in endocytic trafficking, protein sorting, protein degradation, fusion of secretory vesicles with plasma membranes, signaling, and in endosomal and exocytosis [91]. In this study, V-type H + -transporting ATPase subunit a, showed the differential expressions in two sub-population haemocytes. Therefore, in *B. areolata,* two sub-population haemocyte proteins may reflect their different roles in regulating cellular homeostasis. Coyne [92] found that haemocytes need a normal energy supply to maintain cellular functions, especially immune-related functions. The previous studies showed the differential expressions of V + ATPase after host immune stimulation [93], and there is a functional connection between endosomal trafficking and V + ATPase in Drosophila. It was suggested that the acidification of intracellular compartments in cells is key for NOTCH signaling. There was an increased expression of V + ATPase B in the neutrophils of adult zebrafish, *D. rerio*, in the chemically induced inflammation [94]. A probiotic-supplemented diet influenced the differential expressions of V + ATPase B in *H. midae* haemocytes [95]. Thus, V-type H + -transporting ATPase may be a suitable candidate for further studies to understand the immune response of *B. areolata*.

4.3.2. V-ATPase and Rab protein associated with the process of phagosome maturation and endocytosis pathway

In this study, V-ATPase and Rab were identified to participate in the process of phagosome maturation. V-ATPase is a crucial protein in phagosome activity [96], which was delivered to the phagosome for membrane acidification. The reason is that cathepsins, which are key proteins present in phagosomes and need for degradation of phagocytosed particles, are only activated at low pH levels [97]. In this study, for lysosome pathway, cathepsin C, cathepsin F, and cathepsin Z-like isoform X2, were up-regulated in G (Supplementary Table 1), and cathepsin D and V-type H + -transporting ATPase subunit a, were down-regulated in G (Supplementary Table 2). Therefore, the proteins of two sub-population haemocyte may reflect their different roles in phagosome activity in *B. areolata*.

For phagosome pathway, Ras-related C3 botulinum toxin substrate 1 was up-regulated in G (Supplementary Table 1); V-type H + -transporting ATPase subunit C, V-type H + -transporting ATPase subunit E, V-type H + -transporting ATPase subunit G, V-type H + -transporting ATPase subunit a, and Ras-related protein Rab-7A were down-regulated in G (Supplementary Table 2).

Rab proteins are the central node in cell, which can regulate trafficking of organelles, including phagosomes. The phagosome maturation is the result of interactions between the phagosome and various intracellular compartments [98]. Rab proteins are crucial molecules for phagosome activity, and multiple Rab proteins have been identified from the early stage of phagosome formation to the regulation of phagosome maturation [99]. Rab 7 is known as a crucial regulator in phagosome maturation [100]. Both Rab 5 and Rab 7 are present on both bacteria- and apoptotic cell-containing phagosomes [101].

For endocytosis pathway, Ras-related protein Rab-35 was up-regulated in G (Supplementary Table 1). Ras-related protein Rab-7A, Ras-related protein Rab-8A, and Ras-related protein Rab-11B were down-regulated in G (Supplementary Table 2). Since endocytic trafficking is central to normal cellular physiology, and Ras-related Rab GTPases (or simply Rabs) are involved in membrane trafficking and vesicle transportation in eukaryotic cells [102]. Rab and associated proteins are the critical regulators of endocytosis [103]. The membranes of endocytic and exocytic vesicles present different Rab proteins [104]. Rab 4, Rab 5, Rab 7, Rab 11, Rab 22, and Rab 25 are some of the Rab proteins associated with endocytic compartments [95]. The role of Rab in exocytic, endocytic and intracellular trafficking explains their importance in the immune system since it counteracts pathogenic bacteria and viral infection [105]. In this study, the result suggested that two type sub-population haemocytes have different roles in the process of phagosome maturation and endocytosis pathway.

4.3.3. Rab proteins associated with the process of defensing against infection and other pathways

Similar to our finding, the differential expressions of Rab proteins have been investigated in previous studies in host after challenged with pathogenic bacteria. For example, in the Chinese mitten crab, *E. sinensis*, the expression levels of Rab 1 and Rab 3 in haemocytes were significantly up-regulated after infected with the pathogenic bacterium *Vibrio angullarium* [106]. The expression level of Rab was up-regulated in shrimp *Penaeus japonicas* resistant to WSSV (white spot syndrome virus), and the results suggested that Rab is involved in anti-viral immunity [107]. Rab play a role in the phagocytic activity of the haemocytes in *P. japonicas*, [108]. Over-expression of Rab increased the resistance of Red drum *Sciaenops ocellatus* to intracellular invasion by *Edwardsiella tarda*, and the result suggested that Rab plays a key role in immune defense against infection by pathogenic bacteria in Red drum [109].

In the present study, for MAPK signaling pathway, Ras-related C3 botulinum toxin substrate 1, and Ras-related C3 botulinum toxin substrates, were up-regulated in G; GTPase HRas, GTPase NRas, Ras-related protein Rap-1A, and Ras-related protein Rap-1B, were down-regulated in G. For JAK-STAT signaling pathway, GTPase HRas was down-regulated in G. For Wnt signaling pathway, Ras-related C3 botulinum toxin substrate 1, calcium/calmodulin-dependent protein kinase (CaM kinase) II, and Ras-related C3 botulinum toxin substrate 3, were up-regulated in G. For PI3K-Akt signaling pathway, GTPase HRas and GTPase NRas were down-regulated in G.

GTPases have been involved in many cellular processes in all eukaryotic organisms, such as cell proliferation, vesicle transporting, cytoskeleton organization, biosynthesis, and response to stimulus [106].

A comparison of the results in our study, ras-related C3 botulinum toxin substrate 1, ras-related C3 botulinum toxin substrate 2, ras-related protein Rab-14 isoform X1, ras-related protein Rab-24-like isoform X2, ras-related protein Rab-39B-like isoform X2, and RAB protein were up-regulated in G (Supplementary Table 1); ras-related protein Rab-11B-

like isoform X2, ras-related protein Rab-7a, ras-related protein Rab-8A-like, Ras, and Ras protein Rap 1b were down-regulated in G (Supplementary Table 2). In this study, the results may be supported by Pei et al. [110], who addressed that there are two pools of Rab proteins in the cell: one poolis newly synthesised, and the other poolrecycles between the cytosol and target membranes. It was suggested that these two pools of Rab proteins may have different roles: a rapid pool of Rab responds to the instant requirements of a specific pathway, and an extended pool sustains active transport.

In addition, in the present study, CD151 antigen-like, CD63 antigen-like, engulfment, and cell motility protein 1-like isoform X2, were also up-regulated in G (Supplementary Table 1). Mucin-17-like, mucin-5AC-like, histone H2B 1/2-like, lysosome-associated membrane glycoprotein 5-like, and peroxiredoxin-2-like, were down-regulated in G (Supplementary Table 2). The result suggested that two sub-population haemocytes may reflect their different roles in regulating cellular immune response in *B. areolata*.

5. Conclusions

In present study, hyalinocytes and granulocytes were successfully separated in high purity by density gradient centrifugation with Percoll. The differentially expressed proteins were determined between granulocytes and hyalinocytes using proteomic analysis. It is to report firstly the proteome of haemocyte sub-populations: hyalinocytes and granulocytes in the ivory shell, *B. areolate*. The results showed cathepsin, p38 MAPK, toll-interacting protein-like and beta-adrenergic receptor kinase 2-like were up-regulated in G; alpha-2-macroglobulin-like protein, C-type lectin, galectin-2-1, galectin-3, β-1,3-glucan-binding protein, ferritin, mega-hemocyanin, and catalytic subunit of protein kinase A were down-regulated in G. The number of down-regulation proteins in G was more than that of up-regulation proteins in phagosome and endocytosis pathway, the contrary is the case in lysosome pathway. The results may lead to better understanding of the functions of haemocyte sub-populations. However, further studies are needed to determine functions of the described proteins and their specific roles in immune response of hyalinocytes and granulocytes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.036.

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