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Innate immune system response against environmental temperature changes as a dangerous abiotic factor

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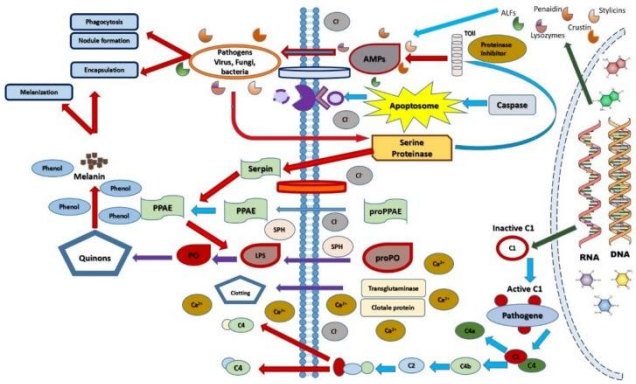
Graphical abstract



Abstract

Innate immune system is the first line response against environmental changes in invertebrate. It protects the animal from environmental changes such as temperature change, PH and salinity as well as pathogens such as bacteria and viruses through different biochemical pathways. In fact, the innate immune system relies on different biochemical reactions which are protecting the animal under adverse environmental circumstances. Among all of the environmental factors, temperature is a dangerous abiotic factor which affects organisms on its ecological level through infiltrating it's the molecular and cellular levels. Invertebrate could survive from a wide range of environmental effects and possesses innate immunity as its defense systems. This review paper aimed at presenting the main innate immune pathways that are activated against the most abiotic environmental changes. We reviewed fundamental aspects of invertebrates' defense process by focusing on the important innate immune pathways including: Pattern recognition receptors (PRRs), Antimicrobial peptide (AMP), Pro-PO activating system, Melanization Pathway, Lectin Pathway, Apoptosis Pathway, Plasma clotting protein.

Keywords: Innate immune system, invertebrates, environmental changes, temperature.

1. Introduction

Invertebrates and other arthropods share similar defense responses without the presence of immunoglobulins. Despite the absence of antigen-antibody specificity, the innate system could possess good identification and respond swiftly to incapacitate and eradicate pathogens. Primarily, the exoskeleton that shields the body structure is a natural physical barrier towards any types of pathogenic microorganisms (Pillai *et al.*, 2010; Zulkapli *et al.*, 2018). The existence of cuticle serves as a lining for foregut, hindgut of a prawn and its body surface. Moreover, gills could allow any exchange due to lack of epicuticle; whereas a gut present without outer lipid layer could allow the permeability. Crustaceans are enclosed with an open circulatory system that transports oxygen, hormones, nutrients, and cells via the hemolymph (Hosseini and Sharifi, 2019; Lorena Vazquez *et al.*, 2009). Association of humoral and cellular defense mechanisms is observed during the hemocyte flow and helps to integrate with plasma cells. Humoral defense system involves the production and release of lectins, antimicrobial peptides (AMP) and prophenoloxidases (proPO) while cellular defense system includes hemocyte mediated responses for encapsulation and phagocytosis. The circulating fluids are known as crustacean hemolymph or blood comprise hemocytes that include fluids or plasma. The defense mechanism is stimulated based on the diverse arrangements of pathogen characteristics which are pointed at the surface level and involved in various immune responses. Upon intrusion of pathogens into the tissue, proteolytic pathways will be activated immediately in order to exterminate the pathogens (Ratcliffe *et al.*, 1985). Firstly, it will be recognized and various effector cascades will be triggered such as antimicrobial proteins (Islam *et al.*, 2020; Underhill and Ozinsky, 2002), clotting protein (Iwanaga and Morita, 1978; Iwanaga *et al.*, 1978), lectin (Fujita, 2002), proPO system (Lorena Vazquez *et al.*, 2009), encapsulation and phagocytosis (Bogdan *et al.*, 2000). The proPO cascade could help activate other immune cascades such as the assembly of melanization, phagocytosis, encapsulation and nodule formation that are mediated

through pathogen-associated molecular patterns (PAMPs) by crustacean proteins. The main question is that how, when and why a specific immune defense mechanism would be chosen in crustaceans among Pattern recognition receptors (PRRs), or pattern recognition proteins (PRPs), Apoptosis pathway, Melanization, Pro-PO activating system, Plasma Clotting protein, and Lectin Pathway (Akira *et al.*, 2006). In crustaceans, the physiological barricade is the first hindrance to keep back any invading particles or pathogenic micro-organisms. When any damage, particles or pathogenic micro-organism is recognized, proteolytic pathways become active immediately (Haroon *et al.*, 2018; Roth and Kurtz, 2009). This instantly activated pathway will eliminate the invaded particle. In second step hindrance, the coagulation cascade would be activated to avoid the loose of hemolymph. Then the proPO system will join the immune picture by stimulation of oxidative metabolites and melanin production. Prophenoloxidase is the most effective protein (Labbe and Little, 2009; Ogunyele *et al.*, 2018). Its activation will stimulate other important procedures in the immune response. Phagocytosis and encapsulation and nodule formation will be activated by prophenoloxidase incitement. All of these pathways are interacting in a complex biochemical reaction that still has a lot of unveiled points. The complexity of the innate immune system reactions has been demonstrated in the Figure 1.

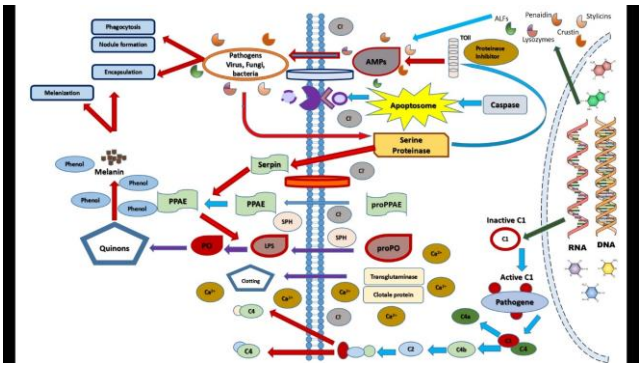


Figure 1. The figure is demonstrating the signal transduction and gene regulation of the innate immune system of crustacean. It shows the consequence of different pathways during the innate immune system activation under the adverse circumstances

2. The effect of the temperature on the innate immune system molecular response

The environmental temperature might have many fluctuations for many creatures throughout their lives. This effect may occur yearly, seasonally, or daily and usually does not remain constant. There is a question to ponder on, and that is that how an organism struggles with long-term or severe temperature changes (Asadullah *et al.*, 2018; Hochachka and Somero, 2002; Youdeowei *et al.*, 2019). Initial heat shock research investigates the mechanisms behind the response to critical heat stress by observing the heat shock in Drosophila. In this study, puffs characteristics had been studied in the salivary gland chromosomes of Drosophila (Ritossa, 1962). Later, it was understood that these studied chromosomal puffs were associated with RNA synthesis and heat shock protein expression. Since 1974 up till now, it is well known that heat shock protein family including (e.g, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock protein families) are regulated under “heat shock response” mechanisms in almost all organisms (Lindquist, 1986). The heat shock proteins are grouped by size or categorized by function. They are also famed as molecular chaperones because they are involved in protein folding. It has also been reported that they are preventing the formation of protein accumulation inside the cell (Parsell and Lindquist, 1993). Additionally, it have been reported that they have functions in ATP-independent’ (small heat shock proteins) ATP-dependent (Hsp60, Hsp70) mechanisms (Fink, 1999). Initial studies revealed that the binding of the heat shock factor (HSF) to cis-regulatory heat shock element (HSE) regions induced heat shock regulatory networks. Furthermore, interaction between Hsp70 with HSF and can cause heat shock auto regulation or block it (Amina and Kamel, 2019; Morimoto and Santoro, 1998).

Researchers have approved that the heat stress response is a complicated mechanism. Research has also confirmed that the heat shock response has a variation in the initiation or termination timing or in the stress intensity. It furthermore depends on different types of Hsps in different organisms. It also may comprise from the induction of many other non Hsps genes (Brokordt *et al.*, 2015; Junprung *et al.*, 2017; Lindquist, 1986; Madeira, 2014; Ravaux *et al.*, 2016).

The comparison between Drosophila and mussel *Mytilus trossulus* showed that heat in the Drosophila shock protein is synthesized during heat stress, but it will only be expressed after stress in mussel *Mytilus trossulus*. A range of HSPs proteins involved in response to heat were detected by faint SDS-PAGE bands in marine snails, yeast, and mussels. Hsps protein also maintains a fundamental influence for thermos-tolerance in yeast (Hoffmann, 2003).

Moreover, recent work on Drosophila and yeast has exposed no limitation in the binding target of the HSF to heat shock proteins. This confirmed that almost 3% of genomic loci are available as binding target (Hahn *et al.*, 2004). The recent interest in gene and mechanisms characterizations has risen by the development of genomic tools. The accessibility to the heat shock response studies by gene expression monitoring on a wide-ranging scale by high throughput technology and quantitative PCR has increased (Birch-Machin *et al.*, 2005). Post research has shown that the level of change in gene and protein expression are not essentially correlated. Recent researches have confirmed that by some protein regulations. For instance (e.g., elongation factors) showed that these proteins are expressed at the translational level, but not in protein level or vice versa. This means that an increased in this gene expression generally does correlate with an increase in their protein expression (Suzuki *et al.*, 2006). Moreover, additional research on Drosophila confirmed a parallel linkage between genomic and metabolomic profiles resulting in heat stress.

Previously, most studies had focused on simple modeled organisms such as prokaryotes, Drosophila, and yeast. Recent studies; however, focused on the heat shock response in higher organisms (Hoffmann, 2003). Heat stress can cause many types of changes, fluctuations or variations in higher organisms or animals?

Higher aquatic organisms that are living in an aquatic habitat are widely exposed to a range of biophysical challenges. They are widely exposed to temperature fluctuations. These creatures may counteract these challenges by increasing or decreasing their body temperatures (Hofmann *et al.*, 2005).

A noteworthy example is the *T. funerals* which was monitored in a study for 26 days from March-April. Nearly after half of the 26 days, the water temperature was midday low, but the body temperature was 27°C. Its body temperature was high enough to induce Hsp70 and Hsp90 gene expressions (Tomanek and Somero, 1999).

Fascinatingly, in the similar heat stress condition, before midday (2.5h at 30°C), Hsp70, Hsp38, Hsp90, and Hsp77 expression was increased and after 6h, 30 min., 6h, and 14h it dropped to normal levels, this showed that *T.funebrails* can tolerate the stress response during the two low-temperature waves (Tomanek and Somero, 1999). In addition, the up-regulated HSPs levels that contribute to survival with reversible protein denaturation also exist following heat stress in the studied area. Reversible protein denaturation is the mechanism that handle the reversible denaturation through protein degradation (Hofmann *et al.*, 2005).

Species-distributions borders are expected to change in response-to global warming. For example, in Monterey, California, southern species in the intertidal zone shifted northwards in the time span of 60 years (Sagarin *et al.*, 1999). Environmental temperature is swiftly increasing both in domestic or aquatic habitats therefore understanding the aspects-of thermal tolerance physiology is essential. In the order to describe the impact that global warming and climate change may have on an organism (Stillman, 2003). It has been known that the upper and lower thermal tolerance limit (CTmax and CTmin) of an organism can be modified with acclimatizing to its surrounding temperature (Cuculescu *et al.*, 1998). Thermal tolerance often depends on the thermal scale availability related to the conditions of the natural habitat. Stillman in 2003 used comparative approaches which demonstrated that during in created temperature, warm adapted species are less able to select their CTmax intolerance while cold-adapted species are the opposite (Stillman, 2003). The geographical and latitudinal study may also be helpful for assessing the ecological consequences of climate change. It might have proper evidence in explaining the bases of an organism's distribution borders. A critical aspect of this study is the impact of global warming effects on species population in a latitude dependent approach. This aspect is assessed by thermal stress levels and the organisms, response in their natural habitat. Seasonal temperature conditions influence a thermal phenotype (Hopkin *et al.*, 2006). It would be insightful to know the level of gradual acclimatization between seasons in natural habitats. There is evidence which shows that the thermal phenotype varies at both molecular and physiological levels (Dietz, 1994). For instance, in crab *C. maenaas* and *C. pagurus*, the maximum thermal tolerance was significantly lower in winter than it was in summer (Cuculescu *et al.*, 1998).

There are some studies on the recovery from temperature fluctuation of intertidal specious such as mussel *M. trossulus* and crab, *Petrolisthes cinctipes*. Studies on the heat shock response in these species have confirmed the immersion of Hsp70 and Hsp90 in reversible protein denaturation (Stillman, 2003). In this stage, a question arises that, what other mechanisms or genes either than Hsps family genes might be up or down-regulated in response to the thermal stress? And how do these changes may vary over time? And what is the immune system response?

3. Pattern recognition receptors (PRRs)

In this innate immune system mechanism, the invading microorganism would be recognized by germline-encoded RRs (Akira *et al.*, 2006). The pattern recognition receptors will bind to the first available pathogen-associated molecular patterns (PAMPs) on the surface of the microorganism. Each PRRs specifically recognizes specific AMPs and activates signaling pathways of the immune responses. It will be active phagocytosis, encapsulation, nodule formation depending on the PAMPs or may be active AMPs synthesis (Medzhitov R and C., 2000).

4. Antimicrobial peptide (AMP)

AMPs could normalize and/or destroy various types of invading pathogens including Gram-positive, Gram-negative, fungal, viral and protozoans. It is reported in numerous species ranging from plants, invertebrate to vertebrates. It is present as a small cationic protein which has features of antimicrobial response and enhanced immunity by functioning as an immunomodulator. It was also best described as a candidate for development of novel antibiotics and also in the development of therapeutic agents. In shrimp, numerous types of AMPs have been characterized such as crustins, anti-lipopolysaccharides factor, penaeidins, hemocyanin, histones and many more. The two well-known AMP is well described in *M. rosenbergii* which is known as cousin and anti-lipopolysaccharides factor (Rosa *et al.*, 2010).

Crustin is one of the antimicrobial peptides that is classified as a cationic cysteine-rich antibacterial polypeptide in crustaceans. Crustin which is the homolog of 'caring' was first identified inshore crab, *Carcinus maenas* as an 11.5kDa peptide (Relf *et al.*, 1999). Crustins were isolated from a broad range of crustaceans which include *Litopenaeus vannamei*, *Litopenaues setiferus* (Bartlett *et al.*, 2002), *Panulirus argu* (Stoss *et al.*, 2004), *Penaues monodon* (Supungul *et al.*, 2004), *Marsupenaeus japonicas* (Rattanachai *et al.*, 2004), *Homarus gammarus* (Hauton *et al.*, 2006), *Fenneropenaeus chinensis* (Zhang *et al.*, 2007), *Carcinus maenas* (Brockton *et al.*, 2007), *Pacifastacus leniusculus* (Jiravanichpaisal *et al.*, 2007), *Litopenaeus*

schmitti, *Farfantepenaeus brasiliensis*, *Farfantepenaeus paulensis* (Rosa *et al.*, 2007), *Farfantepenaeus subtilis* (Rosa *et al.*, 2007), *Fenneropenaeus indicus* (Antony *et al.*, 2011), *M. rosenbergii* (Arockiaraj *et al.*, 2013).

The term crustin was first coined to define the mRNA transcript found in *L. vannamei* and *L. setiferus* (Bartlett and Cuthbertson, 2002). Crustins are cationic, cysteine-rich AMPs with an isoelectric point ranging from 7.0 to 8.7 with a molecular weight ranging between 7 and 14 kDa with one whey acidic protein (WAP) domain at the carboxyl terminus (Bartlett *et al.*, 2002). This domain consists of tightly packed 8 cysteine residues in a conserved architecture which is known as a four-disulfide core (4 DSC). The WAP domain containing proteins are widely distributed from invertebrates to vertebrates. The term 'WAP' is given to a family of proteins that are derived from whey fraction of mammalian milk, and it comprises of two WAP domains with 50 amino acids (Ranganathan *et al.*, 1999). The WAP domain in mammals includes trapping, elafin, and antileukoproteinase. It is one of the conserved points among many species and few crustins mainly in shrimp (Bartlett *et al.*, 2002).

The molecular assemblies between the signal sequence and WAP domain vary in crustin, yet it is substantiated by its unique molecular architecture. The cysteine rich region lies in between the signal sequence (N-terminus) and the WAP domain (C-terminus) for Type I crustins which are present in crabs (Brockton *et al.*, 2007; Imjongjirak *et al.*, 2009) crayfish (Jiravanichpaisal *et al.*, 2007), shrimp (Sun *et al.*, 2010), freshwater prawn (Dai *et al.*, 2009) and lobsters (Christie *et al.*, 2007) and other crustins in the Pleocyemata family. This type of crustin was categorized under Type I since it resembles similar domain organizations as a carcinogen (Relf *et al.*, 1999). Likewise, Type II crustin exists with both long glycine-rich and cysteine regions within the signal sequence (N-terminus) and a WAP domain (C-terminus) which are present mainly in crayfish (Jiravanichpaisal *et al.*, 2007) and shrimp (Rattanachai *et al.*, 2004). The Type III crustin is also known as a single whey domain (SWD), chelonianin-like protein or antileukoproteinase-like proteins. It is a peptide that lacks both cysteine and glycine-rich regions, but it is substituted with proline or arginine region between the signal sequence and WAP domain. So far only few Type III crustins were identified in crayfish and shrimp (Amparyup *et al.*, 2008).

M. rosenbergii crustin carries a signal sequence and WAP domain along with proline and arginine-rich regions. It was also classified as a type III crustin (Arockiaraj *et al.*, 2013). It also has 12 conserved cysteine residues that were previously observed in many other crustins as well as antimicrobial peptides like a carcinogen. The conserved residues were predicted to form six disulphide bonds in the tertiary structure. This WAP domain shows a wide range of functions including antiproteinase and antimicrobial activities. Moreover, the amino acid residue of protease inhibitors, methionine, which is located near to the second cysteine in WAP domain is replaced by cationic and hydrophobic amino acids in crustin WAP. This alteration turns the protein to be amphipathic and permits insertion of the protein into the outer layer of microbes.

Crustin as an AMP effector was reported to carry antibacterial activities especially in Gram-positive bacteria (Arockiaraj *et al.*, 2013). *Mr* crustin could exhibit its response towards *infectious hypodermal and hematopoietic necrosis virus* (IHHNV) and *white spot syndrome virus* (WSSV) and bacteria *Aeromonas hydrophila* (Gram-negative) and *Enterococcus faecium* (Gram-positive). Besides, the recombinant crustin also shows good response towards both Gram positive and Gram negative bacteria by distinguishing the pathogens (Arockiaraj *et al.*, 2013). The first identified crustin from *C. maenas* has the ability to encounter the growth of Gram-positive bacteria that include *Aerococcus viridans var tomato*, two strains of marine *Planococcus* spp. and a salt-tolerant strain of *Micrococcus luteus* (Relf *et al.*, 1999). The recombinant crustin of *F. chinesis* also aims at Gram-positive bacteria that include *Staphylococcus aureus*, *M. luteus* and 3 other bacilli (Zhang *et al.*, 2007). One of the recombinant crustins of *P. monodon* highly inhibits the activity against *Streptococcus iniae* and *S. aureus* but not towards *A. viridans var homari* and *M. luteus* (Supungul *et al.*, 2008). Nevertheless, Type II crustin identified from *P. monodon* indicates robust antibacterial activity against both Gram-positive and Gram-negative bacteria that include *A. viridans var homari*, *Escherichia coli 363* and *Vibrio harveyi* (Amparyup *et al.*, 2008).

On the other hand, anti-lipopolysaccharides factor (ALF) is one of the important conserved AMP found in crustaceans. Initially, ALF was known as anti-lipopolysaccharide LPS factor as it was recognized as an inhibitor factor of LPS-mediated hemolymph coagulation in *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka *et al.*, 1982). ALF was further identified and cloned in many other crustacean species such as *Litopenaues setiferus* (Gross *et al.*, 2001; Li *et al.*, 2014), *Penaeus monodon* (Supungul *et al.*, 2002), *Macrobrachium olfersi*, *Farfantepenaeus paulensis* and *Litopenaeus schmitti* (Rosa *et al.*, 2007), *Macrobrachium rosenbergii* (Jesu *et al.*, 2012; Liu *et al.*, 2014; Ren *et al.*, 2012), *Macrobrachium nipponense* (Wang *et al.*, 2015), *Fenneropenaeus chinesis* (Liu *et al.*, 2005, Tang *et al.*, 2014), *Litopenaeus vannamei* (Vega *et al.*, 2008), *Marsupenaeus japonicas* (Hiroki Nagoshi et al., 2006; Mekata *et al.*, 2005), *Pacifastacus leniusculus* (Liu *et al.*, 2006), and *Scylla paramamosain* (Imjongjirak *et al.*, 2009), *Eriocheir sinensis* (Zhao *et al.*, 2009), *Scylla serrata* (Ko *et al.*, 2007), *Procambarus clarkii* (Li *et al.*, 2009) and *Portunus trituberculatus* (Yue *et al.*, 2010).

Moreover, multiple isoforms also have been found in the many types of other crustacean's species. To date, seven forms of ALF genes were identified from *M. rosenbergii* (Arockiaraja *et al.*, 2011). Five forms of ALF genes were reported from the two genomic loci from *P. monodon* that are actively involved in defense mechanisms against various types of pathogen infections (Antony *et al.*, 2011). *Portunus trituberculatus* has four types of ALF genes (Yue *et al.*, 2010).

ALF is classified as a single domain AMP that has an LPS domain with a signal peptide. The LPS has two conserved cysteine residues that form the disulfide bonds. The deduced ALF from *M. rosenbergii* shows the conserved structure of the signal peptide, LPS binding domain and two cysteine residues (Arockiaraj *et al.*, 2014). According to Yang *et al.* (1992), the second and third sheet of β-sheet is enveloped by the LPS domain along with the four antiparallel β-sheets. The structure of *Macrobrachium rosenbergii* showed 5 α-helices and 4 β-sheet along with two conserved cysteine residues in β-sheet that forms disulfide bonds in a hairpin loop (Jesu Costa Ferreira *et al.*, 2012) This similar pattern was also observed in ALF from *E. sinensis* (Wang *et al.*, 2011). The pI value of ALF and LPS binding domain of *M. rosenbergii* falls in a range of 5.00 to 10.00 among crustaceans (Ren *et al.*, 2012). Basically, ALF is a basic protein that is responsible for binding and neutralizing LPS. The basic ALF shows antibacterial activity, while the acidic ALF may show antiviral or antifungal activity (Ren *et al.*, 2012). Similar results were obtained for *E. chinesis*, *M. japonicas*, *Litopenaeus stylirostris* (Zhang *et al.*, 2010).

ALFs were reported to be involved in immune defense mechanisms in a wide range of pathogenic infections including bacteria, virus, fungi or yeast. Generally, ALF can bind to the liposaccharides (LPS) on the pathogens (Imjongjirak *et al.*, 2007). *Mr*ALF shows it response against white spot syndrome baculovirus (WSBV), white spot syndrome virus (WSSV), *Aeromonas hydrophila*, *Escherichia coli*, *Vibrio anguillarum*, and *Staphylococcus aureus*. It is reported that cationic and hydrophobic properties of AMPs allow the interaction and insertion into the anionic cell walls and phospholipid membranes of the pathogens towards antimicrobial action (Morita *et al.*, 1985; Oren *et al.*, 1998). The ALF from *Portunus trituberculatus* increases upon *Vibrio alginolyticus* challenge. In *Scylla paramamosain* an ALF had demonstrated antimicrobial activity against *Vibrio* (Imjongjirak *et al.*, 2011). While, the ALF in *Pacifastacus leniusculus* shows antiviral activity upon challenge with the white spot syndrome virus (WSSV) (Lu *et al.*, 2006). The ALF from *Penaues monodon* was shown to be up-regulated upon *Vibrio harveyi* challenge (Tharntada *et al.*, 2008).

Besides that, the functional property was indicated by recombinant ALF with LPS domain through various types of Gram-negative and Gram-positive bacteria (Liu *et al.*, 2014). The recombinant ALF from *M. rosenbergii* could show antibacterial activity towards *Aeromonas hydrophila*, *Vibrio cholera,* and *Escherichia coli*. Moreover, it could also bind to both bacterial types that were studied including LPS of *E. coli*, and lipoteichoic acids of *B. subtilis* and *S. faecalis*. Meanwhile, (Arockiaraj *et al.*, 2014) reported that *Mr*ALF shows a high antimicrobial response to almost more than five types of bacteria which were tested. Moreover, the bactericidal test after 24 hours also indicates that *Mr*ALF could kill all the bacteria. A similar type of activity was also shown by recombinant ALF protein from *Scylla paramamosain*, *Penaeus monodon* and *Pacifastacus leniusculus* (Somboonwiwat *et al.*, 2008). The recombinant ALF from *Penaeus monodon* has successfully decreased the replication of WSSV (Tharntada *et al.*, 2009). This indicates the participation of *Mr*ALF as a part of defense mechanism during pathogen attack.

5. Prophenoloxidase pathway

The prophenoloxidase protein is the active form of pro-Po system and is the main domain protein of Pro-PO activating system. Pro-PO is synthesized and localized in crustacean's granules hemocyte. Then it triggers the pattern recognition protein and migrates to plasma by exocytosis process (Amparyup *et al.*, 2013). The pro-Po system contains several protein complexes that will participate in melanin formation, cytotoxic reaction, cell adhesion, encapsulation, Prophenoloxidase, and phagocytosis (Söderhäll and Cerenius, 1998). Each crustacean species has a specific composition of the zymogens of either protease cascade, pattern recognition proteins or serine protease and pro-PO. The complex of pro-PO and LPS will bind to the microbial cell wall components and induce activation of serine protease zymogens in the pro-PO system (Amparyup *et al.*, 2012). The most triggering molecules to pro-Po are LPS binding protein which is found in crayfish pro-PO system. In an in vitro study, the cleavage of pro-PO activating serine proteases showed antimicrobial activities which suggest that pro-PO activation with serine protease may have a dual function (Gupta *et al.*, 2005).

A humoral response that plays a key role in antigen recognition and neutralization in a wide range of host models is known as prophenoloxidase (proPO) (Cerenius *et al.*, 2008; Söderhäll and Cerenius, 1998). The proPO system was also identified as a center of the defense mechanism characterized in many organisms including arthropods, ascidians, cephalochordate, molluscs, and annelids (Theopold *et al.*, 2004). A segment of complex enzyme cascade could activate PO along with Ca^{2+} ions that oxidizes phenols into quinone and further catalyzes it into melanin cascade for encapsulation, wound healing and for antimicrobial activity (Arockiaraja *et al.*, 2011). Various types of microorganisms or parasites may activate the proPO cascade, especially the large size pathogen. This type of pathogen encapsulation and further melanization appears on the host system by the action of phenoloxidase (Arockiaraj *et al.*, 2012; Söderhall *et al.*, 2003). The proPO involves mainly in defense reactions, wound healing and cuticular hardening processes (Arockiaraj *et al.*, 2012). The activation system of proPO can also be stimulated by very low quantities of microbial cell components such as β-1,3-glucans, lipopolysaccharide (LPS), and peptidoglycan (PG) (Arockiaraj *et al.*, 2012). These intruders could indirectly or directly induce the granulocytes via enzyme activating cascades to produce phenoloxidase (PO) (Arockiaraj *et al.*, 2013).

The first cloned and purified proPO was from *Pacifastacus leniusculus*, a freshwater signal crayfish (AsPAN *et al.*, 1995). Following which many other proPO genes were isolated and characterized from a wide range of species including *Drosophila melanogaster* (Fujimoto *et al.*, 1995), *Manduca sexta* (Hall *et al.*, 1995), *Anopheles gambiae*

(Christophides *et al.*, 2002), *Scylla serrata* (Ko *et al.*, 2007), *Anopheles stephensi* (Cui *et al.*, 2000), *Aedes aegypti* (Taft *et al.*, 2001), *Bombyx mori* (Asano *et al.*, 2001), *Armigeres subalbatus* (Huang *et al.*, 2001), *Litopenaeus vannamei* (Liu *et al.*, 2004), *Penaeus monodon* (Nayak *et al.*, 2010), *Homarus gammarus* (Hauton *et al.*, 2005), *Macrobrachium rosenbergii* (Arockiaraj *et al.*, 2012). Upon infection, one of the major enzymes that are stimulated is PO via proPO activation system. This takes place upon stimulation by components of the pathogen-associated molecular pattern (PAMP). The activation of proPO cascade may involve serine protease breaks, to trigger the zymogen into active PO. The PO is a copper-dependent enzyme that acts as a terminal enzyme with two functions. PO can catalyze monophenols into o-hydroxylation (cresolase activity) and oxidation of phenols into quinones (Sugumaran and Nellaiappan, 1996). Therefore, PO can convert tyrosine to dihydroxyphenylalanine (DOPA) and also DOPA to DOPA-quinone along with non-specific pathways between neighboring molecules for melanin formation. Moreover, the PO is also able to regulate the negative stimulus to the host through reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Cerenius and Soderhall, 2004; Ko *et al.*, 2007).

The first *Mr*proPO (*Mr*proPO1) was cloned by (Lu *et al.*, 2006) with six histidine residues and a thiol ester like motif. This peptide has similar structural formation as *Penaues monodon*, *P. leniusculus,* and *H. gammarus*. Furthermore, (Lu *et al.*, 2006) also had reported proPO (MrproPO2) from *M. rosenbergii* with six histidine residues within two copper binding sites, thiol esters like motif, a proteolytic activation site, and a conserved c-terminal region. The proPO activating enzyme III (*Mr*proPO3) is a conserved copper-containing enzyme that was isolated and characterized in *M. rosenbergii* (Arockiaraj *et al.*, 2012). The amino acid sequence has a characteristic feature of the clip-serine protease (clip-SP) that comprises of histidine active sites at N- and C- terminal, signal peptide and a domain from serine proteases trypsin family. This clip also shows conserved catalytic amino acid that is important for the stabilization of the catalytic site in the three-dimensional structure. The clip-SP is an important molecule that plays important roles in immune regulation and embryonic development. This sequence characteristic was also found in another proPO activating enzyme such as *C. sapidus* and *P. monodon*. In addition, another *Mr*proPO (*Mr*proPO4) was also found by (Arockiaraja *et al.*, 2011) with two copper binding sites (CuA and CuB) that have hemocyanin features, a signal sequence that is important in secretion at endoplasmic reticulum site and thiol-ester like motif. Hemocyanin has a wide range of physiological processes such as osmoregulation, molt cycle, exoskeleton arrangement and protein storage. The similar structural study was also reported in *Manduca sexta* (Li *et al.*, 2009). Besides that, the tertiary structure also indicates tyrosine signature that is postulated to bind to proPO and activate the melanin synthesis (Li *et al.*, 2009). In addition, the copper binding sites with conserved histidine residues were found in most insects and crustaceans that are responsible for melanization, encapsulation and sclerotization mechanism (Hughes, 1999).

*Mr*proPO1 gene expression and PO activity were measured using CpG oligodeoxynucleotide. This has clearly shown that the proPO system can be activated through CpG oligodeoxynucleotide via protein kinase C signaling pathway. This supports their previous study as the challenge was carried out with *Aeromonas veronii* and *Lactococcus garvieae*. As a whole, the proPO system can be triggered by the stimulation of bacterial challenge, bacterin or oligodeoxynucleotide stimulation (Lu *et al.*, 2006). Meanwhile, the expression of *Mr*proPO2 was studied based on five different molting stages of *Mr* (Liu *et al.*, 2006). The gene expression of proPO in the postmoult stage showed a gradual increase and further reached the higher expression levels during the later stage of the postmoult stage. The proPO expression declined sharply in the intermoult stage, and the lowest level was observed in the premoult stage. Therefore, it was concluded to be an immunomodulatory system (Liu *et al.*, 2006). *Mr*proPO3 shows its responses against IHHNV (Arockiaraja *et al.*, 2012) while *Mr*proPO4 shows responses towards a wide range of pathogens including WSBV, *Mr*NV, *A. hydrophila* and *V. Harvey (J et al., 2013)i* (Arockiaraj *et al.*, 2013). This indicates that *Mr*proPO is actively involved in the defense mechanism of innate immunity in an invertebrate. The PO activity of *Mr*proPO3 is not similar to gene expression study that might be because of its complex activity at different phases of the host-pathogen interaction. *Mr*proPO4 shows a robust association between gene expression and enzymatic activity that might be because of its involvement of various phenoloxidases. A similar mechanism was also suggested by (Adachi *et al.*, 2003).

6. Melanization pathway

Melanin is synthesized by the proPO-activating system and is an enzymatic cascade which is involved in several enzymes (Gorman MJ *et al.*, 2008). This protein is the main substance for melanization which an important immune defense pathway in crustaceans is. The melanization is a prominent immune response which involves the synthesis of melanin to encapsulate pathogens. The process of melanization is a complex process which starts from proPO-activating system binding to microbial cell wall components. This complex will activate the stimulation of the SP cascade which promotes proteolysis to convert proPO into active PO. The active PO oxidase phenols into quinines which can non-specifically crosslink nearest molecules to form melanin. To date, several genes associated with the shrimp pro-PO activating system are identified and characterized (Jang *et al.*, 2011).

7. Lectin pathway

Invertebrates have a well-developed defense mechanism to detect and exterminate any opportunistic microorganism or potential pathogens. Anciently diverse conserved molecules establish their non-self- recognition system through pathogen-associated molecular patterns (PAMPs). In response to this architecture, a family of proteins that were termed as pattern recognition proteins

(PRPs) will be exerted by the immune system to initiate the downstream mechanisms. A well-studied protein, the lectin is a functional precursor of antibodies which recognizes the signature molecules of non-self-particles via PAMP (Kilpatrick, 2002). Lectins are organized in a broad spectrum of protein units, and every architecture shows different molecular mass depending on the species and the molecular assemblies. Lectins are proteins or glycoproteins. They normally do not have catalytic activity. They are mostly recognized while they don't have any covalent bind to sugar moities. Thus they agglutinate cell by binding to glycoproteins or glycoconjugates to the cell surface (Agundis *et al.*, 2000) . Therefore, they are considered as significant pattern recognition proteins in crustacean innate immunity. They also play important roles in non-self-recognition and clearance of invading microorganisms. They are existing in circulating fluids, either as cell surface receptors or soluble proteins (Marques and Barracco, 2000). Different types of lectins have been diagnosed in a crustacean. Lectin1, lectin2, lectin3, l3ctin4 and C-type lectins are the most recognized lectins in crustaceans. C-type lectins are the largest group of immune function that is mostly found in crustacean hepatopancreas (Zhang *et al.*, 2007). A large number of lectins have been purified and characterized from crustacean hemolymph (Pereyra *et al.*, 2004). In crustaceans, lectins have been reported to contribute to innate immune responses, including prophenoloxidase activation, encapsulation1.nodule formation of hemocyte, opsonin formation, antibacterial activity, and antifungal activity and may also be contributing to injury healing (Smith *et al.*, 2003).

It is reported that lectin serves a decisive role for both physiological and pathological processes with exclusive interactions between intricate carbohydrates that include glycoproteins, glycolipids, polysaccharides or proteoglycans (Vázquez-Mendoza *et al.*, 2013). Likewise, some lectins respond only to certain types of carbohydrate forms that encompass the whole sugar molecule, a portion of the molecule or the glycosidic linkages (Sharon and Lis, 1998). Generally, lectins are described to be activated based on the conserved sugar binding activity towards the N- or O-acetylated sugar residues, such as N-acetyl-glucosamine (GlcNAc), N-acetyl- galactosamine (GalNAc), N-acetyl-neuraminic acid, or O-acetylated sialic acid in decapod (Lorena Vazquez *et al.*, 2009) Several activities take place during these multifaceted interactions such as the embryonic development, intracellular-trafficking, cell-cell and cell-matrix recognition, cell homing, protein synthesis and transport, signal transduction, endocytosis, phagocytosis and inflammation (McGreal *et al.*, 2004).

To date, many lectins are recognized and studied in their molecular configuration. As non-self- recognition molecules, lectins show a high abundance of activity in domain assemblies, sugar substrates, and tissue distribution and even expression upon pathogen challenge to describe the potential functional roles in immunological assays. Lectins are highly heterogeneous molecules that are categorized based on their structures, motifs, and functions. The motifs are known as carbohydrate recognition domains (CRD) that show the disparities between the structures. The Animal Lectins Homepage (http://www.imperial.ac.uk/ research/animal lectins/default) declared that animal lectins are termed into 13 different groups namely the C-type, F-type, M-type, L-type, P-type, I-type, R-type, F-box lectins, chitinase-like lectins, ficolins, calnexin, galectins, and intelectins. The intracellular lectins were reported in protein sorting and trafficking whereas extracellular lectins mainly were shown to function in cell signaling and pathogen recognition (Kilpatrick, 2002). Even though repertoires of lectin show a large divergence, almost 7 types of lectins were identified in the GenBank that are known as C-type, M-type, L-type, P-type, fibrinogen-like domain lectins, galectins and calnexin/calreticulin. Sequence distinction in CRD indicates diverse interactions between sugars that are known as mannose-binding lectins, fucose binding lectins, rhamnose binding lectins, galactose-binding lectins, GlcNAc-specific lectin, and GalNAc-specific lectins. Among, this C-type and mannose-binding lectins were discovered in *M. rosenbergii*.

C-type lectin is one of the proteins that is well studied in many invertebrates and constitutes common structural features such as a carbohydrate recognition domain (CRD), disulfide bond sites and calcium binding sites. The CTL reported in *M. rosenbergii* has a single or double CRD, a signal peptide that may act like a secretory protein that participates in transporting mature proteins outside the cell, a cysteine residue that forms disulfide bridges, and calcium binding sites. The CRD structure is composed of a double loop composed of four conserved cysteine residues and stabilized by two disulfide bridges. However, some CTL is present without signal peptides that may act as receptors or calcium-independent binding sites. The position of the hydrogen bond from donor and acceptors site on the Ca2+ binding site regulates the binding of carbohydrate. The CRD fold is formed by a double loop structure that is stabilized by two conserved disulfide bridges along with hydrophobic and polar interactions. CRD that has a Glu-Pro-Asn (EPN) motif binds to mannose whereas CRD that bears at Gln-Pro-Asp (QPD) motif binds to galactose. The Trp-Asn-Asp (WND) motif shows a connection with monosaccharide residues in the presence of the Ca2+ binding site. This variation shows CRD binding capability towards different sugar moieties on invading pathogens (Zelensky and Gready, 2005).

Though of CTL found in higher levels in invertebrates with diverse functionality, it is highly conserved in vertebrates. Many types of CTL's were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, also in *Manduca sexta* that help in activation of other immune-related genes likely prophenoloxidase, encapsulation, and melanization. CTL is also found in many different penaeid shrimps that carry antimicrobial activity with single CRD, whereas the double CRD may act as PRR via binding to bacteria for further neutralization processes. The *M. rosenbergii* shows its possible anti-viral and antimicrobial roles upon *V. anguillarum*, *V. parahaemolyticus*, WSSV and spiroplasma challenge. Similarly, *P. monodon*, *M. japonicas,* and *L.*

vannamei also show anti-viral and antimicrobial activity (Luo *et al.*, 2003). *M. rosenbergii* CTL knockdown shows its association with activation of other types of innate immune systems such as anti-lipopolysaccharide factor (ALF) and lysosomes. It is also shown that CTL could initiate multiple signal transduction cascades upon pathogen challenge with the stimulation of other immune molecules.

CTL has even shown its robust activity to many types of pathogens like bacteria and virus. *M. rosenbergii* shows agglutination activity towards both Gram positive and Gram-negative bacteria which shows the possible association of lectin and carbohydrate component on the surface of pathogens. This similar activity was also observed in *F. chinesis*, *P. monodon*, *P. japonicus*, and *L. vannamei*. The recombinant CTL also shows its broad binding activity towards both Gram-positive bacteria like *S. aureus*, *M. luteus*, *B. subtilis*, *B. cereus* and *B. megaterium* and Gram-negative bacteria such as *A. hydrophila*, *V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus* and *E. coli*. This indicates its wide range response in defense mechanism against a range of pathogens.

Meanwhile, a mannose-binding lectin that was categorized as an AMP was also found in *M. rosenbergii*. Many mannose-binding lectins were identified and characterized, ranging from plants to vertebrates, invertebrates especially in insects and shrimps which indicates the evolution and diversity of mannose-binding lectin gene. *Mr.* MBL is also predicted to have a signal peptide region,a CRD domain, a cysteine region and a calcium binding site similar to CTL. These cysteine residues are highly conserved and important in forming disulfide bonds. It even acts as a calcium-dependent lectin with four calcium binding sites and is composed of highly conserved 'specific carbohydrate recognition motifs' at the Tyr-Ser-Asn (YSN) and Gly-Asp-Leu (GDL) sites. The tertiary structure of this molecule is composed of α-helix, coil regions, and β-sheets (Antony *et al.*, 2016).

Antimicrobial activities of MBL were also observed in response to different bacteria such as A. hydrophila, E. coli, V. parahaemolyticus, V. alginolyticus, V. harveyi, B. subtilis, B. licheniformis, B. coagulans, S. pyogenes, M. luteus and L. monocytogenes. Among which, the Gram-negative bacteria show a better binding activity compared to Gram-positive bacteria which may be due to the binding capacity of mannose and the carbohydrate from both bacteria. A similar result was observed by since LPS is the main pathogen associated with molecular patterns in Gram-negative bacteria. The gene expression studies show that mannose-binding lectin can be regulated upon WSBV, MrNV, A. hydrophila and V. harveyi infection. Previous reports show similar results for lectin response to Vibrio challenge in M. rosenbergii and Fenneropenaues chinesis (Zhang et al., 2009; Xu et al., 2010). Moreover, the bactericidal efficiency test shows the possibility of killing viral and bacterial colonies efficiently. This shows that MrMBL also takes part in defense mechanism towards pathogen eradication.

8. Apoptosis pathway

Apoptosis is genetically controlled by the cell death program. This pathway will eliminate harmful cells (such as a viral cell) and damaged cells. This pathway is very important in some of the normal processes such as the development of the embryo, metamorphosis and more importantly in immune defense (Wen *et al.*, 2012). Apoptosis pathway plays an important key role in crustacean immune defense against viral attacks. There are many viruses with different strategies to inhibit apoptosis in host cells during viral infections. The virus will try to delay the host cell capability till sufficient offspring viruses have been produced (Wang *et al.*, 2008). Even though the viruses always try to inhibit apoptosis, there is an exception in some viruses that they try to use apoptosis pathway to spread their progeny virus to other cells. The most important molecules which mediate the apoptosis pathways are caspase (Elmore, 2007). Caspase overexpression was diagnosed in *M,japonicas* under WSSV infection (Jiann and Lo, 2011).

9. Plasma clotting protein

Blood loose prevention is an essential immune process in case of injury. This mechanism is different in different crustacean species. Lipoprotein is another clotting protein which is found in freshwater crayfish (Hall *et al.*, 1999). Each subunit of lipoprotein has lysine and glutamine side chain. Then the injured tissue or hemocyte will release transglutaminase which will cause the chains to cross-link to each other covalently. All these reactions will happen in the presence of Ca2+ (Laszlo and Graham, 2003) as shown in Figure 1.

In some species such as crayfish, the hemolymph clotting is based on the direct transglutaminase-mediated cross-linkage of specific plasma protein. This protein is a homologous protein to the female-specific vitellogenins (Hall *et al.*, 1999). An interesting fact about clotting protein is the similarity sequence with female-specific protein, vitellogenin, in both sexes which constitute a separate group of clotting factors. Therefore, the crustacean clotting proteins are evolutionarily related to vitellogenins, but they have completely-different functions-and are expressed in both sexes. So, they should not be considered as true vitellogenins (Lee *et al.*, 2008).

10. Conclusion

Temperature is a-critical-abiotic factor that affects organisms thoroughly from the-ecological macro-environment to the fundamental cellular and internal molecular environments. Generally, all microorganisms experience temperature fluctuations during day and night and also by seasonal changes in their natural habitat. - There is a strong monitoring evidence that global temperature has been increasing in the past decades. The evidence also confirms that it will continue to increase in the future as well (Hornbach *et al.*, 2016). The question of how organisms can be affected by global warming and climate changes has been argued since 1999, and it is still heavily concerning to humankind (Hughes *et al.*, 2003; Sagarin *et al.*, 1999; Stillman, 2003).

Providing the molecular response-repertoire derived from environmental changes is essential to develop-strategies to prevent mortalities-related to climate parameter in the natural environment and in aquaculture farms. In this study, through the essential pathway of gene expression analysis, we tried to identify the resistant-related gene expression signatures in invertebrate under temperature changes. To understand the-mechanisms that underlie resistance in invertebrates, some studies have been conducted to identify gene regulations by environmental changes.

In conclusion, the innate immune system is the primordial immune system which has been evolving until today in every living system. According to a recent study, invertebrates were classified as ancestors to insects in the group of invertebrates since they could survive from a wide range of environmental effects. Hence, *M. rosenbergii* which possesses innate immunity as one of its defense systems was discussed in this review. To get a virtuous view of an important defense mechanism of *M. rosenbergii* from the context of a humoral peptide, this review focused mainly on important antimicrobial peptides, lectin, and prophenoloxidase that are being studied to date and their reactions under adverse environmental condition. The discovery of these genes in different invertebrates could help further research in applications to develop natural antibiotics or therapeutic agents which could further ensure the healthy invertebrate cultivation and maintain food security for human consumption.

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