Hemocyte marker proteins and regulation of the proPO system in a crustacean, *Pacifastacus leniusculus*

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ABSTRACT

In crustaceans and other invertebrates, hemocytes are essential in immunity. The development of new hemocytes is important in the defence mechanisms in invertebrates. In crustaceans, new hemocytes which are synthesized in a specific organ called the hematopoietic tissue (Hpt). The hemocytes are produced and partly differentiated in the Hpt before they are released into the hemolymph and become freely circulating peripheral hemocytes. The final step of differentiation to mature hemocytes containing prophenoloxidase (proPO) takes place in the hemolymph.

This paper presents the studies to identify proteins associated with development of different hemocyte types and a negative regulator of phenoloxidase-induced melanization in the freshwater crayfish *Pacifastacus leniusculus.*

To obtain tools for more detailed investigations about the connection between semigranular cells, granular cells and precursor cells in Hpt of freshwater crayfish, *Pacifastacus leniusculus* and possibly also in other crustaceans, we have used two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to identify specific proteins expressed in different hemocyte types differentially. In this study we report the specific expression of some genes in different hemocyte lineages and their transcript levels in Hpt cells in normal or previously laminarin or LPS treated animals analyzed by RT-PCR. Moreover, RNA inference experiments were also included to study the differentiation of Hpt cells.

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Melanin formation is essential for host defence in crustaceans and insects, and this process needs to be tightly regulated since unwanted production of quinone intermediates or melanization is also dangerous to the animal when they are not synthesized appropriately. We also report the presence in the crayfish *Pacifastacus leniusculus* of a plasma protein with a distinctly similar function as mealworm *Tenebrio molitor* melanization inhibiting protein (MIP). Crayfish MIP as well as *Tenebrio* MIP interfere with the melanization reaction from quinine compounds to melanin, but do not affect phenoloxidase activity by themselves instead. Interestingly, this protein has a completely different structure from *Tenebrio* MIP and is similar to vertebrate ficolin and horseshoe crab Tachylectin 5. Moreover, an Asp-rich region similar to that found in ficolins that is likely to be involved in Ca^{2+}-binding is present in crayfish MIP. However, crayfish MIP did not show any hemagglutinating activity which is common for the vertebrate ficolins. The crayfish MIP is very efficient in inhibiting activation of the proPO-system and thus functions as an important regulatory molecule to prevent unwanted proPO activation in the crayfish.

Key words: *Pacifastacus leniusculus*, hemocyte marker protein, proPO system

INTRODUCTION

Background

Crustacean aquaculture production is an important income for many developing or low-income countries (Rosenberry, 1998). The increase in aquaculture also has many negative effects, for instance, it causes environmental destruction and the farming often faces severe disease problems. To optimize aquaculture condition and avoid disease outbreaks, the development of tools for rapid recognition and control of pathogens are imminently needed (Bachère, 2000).

Crayfish are immunologically related to other more economically important crustaceans, so insight into crayfish immunity and their defence system is valuable for aquaculture development and good for optimizing farming conditions.

Vertebrate immunity is composed of innate and adaptive response, but invertebrates, including arthropods, only rely on very sensitive innate immune system since they lack true antibodies and adaptive immunity. The innate immune system seems to be enough to protect them against infections or intruders, and it includes cellular and humoral mechanisms, both of which are activated upon immune challenge. The cellular response mediated by hemocytes in hemolymph involves nodule formation, phagocytosis, encapsularion of pathogens and coagulation (Ratcliff *et al.,* 1985, Johansson and Söderhäll, 1989; Theopold *et al*., 2002;). Moreover, hemocyte**s** are also involved in another cellular response, melanization, which is activated immediately upon injury/infection and normally is localized to the place of injury or the surfaces of invading microorganisms, so that toxic phenol intermediates or melanin

are released and the intruders are immobilized and killed Söderhäll and Cerenius, 1998). Humoral defense is characterized by synthesis and secretion of immune components after challenge, for example: antimicrobial peptides (AMPs) can accumulate in hemolymph to defend against invading microorganisms (Lemaitre and Hoffmann, 2007). But it is important to keep in mind that cellular and humoral responses are connected to each other, and cannot be separated completely in the immune response.

There are various strategies for the invertebrates to combat different invading pathogens, but most of them are evolutionarily conserved such as: activation of phagocytic cells, production of AMPs and generation of toxic or reactive oxygen species (ROS).

In decades, progress has been made in different aspects of crustacean immune system, especially the understanding at molecular and biochemical level of some highly conserved immune response pathways (eg. Melanization, production of AMPs and clotting) (Bachère *et al*., 2004; Cerenius and Söderhäll 2004, Sritunyalucksana and Söderhäll, 2000). However, the research of these animals is being hampered by a lack of access to knowledge of genetic data and of well-developed cell lines. Therefore, the hematopoietic tissue (Hpt) cell cultures technique developed in the freshwater crayfish *Pacifastacus leniusculus*, provide a useful tool for gene functional studies in crustaceans (Söderhäll *et al*., 2005), since an efficient method for RNA silencing is at hand for these cells (Liu and Söderhäll, 2007).

Hematopoiesis

Hematopoiesis is the formation and development of new hemocytes and/or blood cells. This is the process whereby undifferentiated hematopoietic stem cells develop into mature cells involving proliferation, commitment and differentiation (Barreda and Belosevic 2001; Medvinsky and Dzierzak 1999). In crustaceans' three morphologically different classes of hemocytes, hyaline, semigranular cells (SGCs), and granular cells (GCs) are observed within the hemolymph, and all of them are important in immobilizing or destroying invasive pathogens (Johansson *et al*., 2000; Cerenius *et al*., 2008). Hyaline and SGCs are involved in phagocytosis (Söderhäll, 1986), whereas the GCs are responsible for storage and release of the proPO system and also antimicrobial peptides (Sricharoen *et al*., 2005). SGCs and GCs can be cytotoxic and lyse foreign eukaryotic cells (Söderhäll, 1985). In crustaceans, the number of free hemocytes can vary a lot in different individuals and also vary in response to environmental stress, hypoxia, endocrine activity during the moulting cycle and infection (Jiravanichpaisal *et al.,* 2006; Johansson *et al.,* 2000; Le Moullac *et al.*, 1998; Söderhäll *et al*., 2003). In addition, the hemocytes number drops dramatically or nearly disappears when the crayfish is unhealthy, dying or infected with virus such as white spot syndrome virus (WSSV) (personal observation). Generally, hemocytes do not divide in the circulatory system of most crustaceans (Söderhäll and Cerenius, 1992; Jiravanichpaisal *et al*., 2006). Thus, new hemocytes need to be continuously and proportionally produced from a separate organ called as hematopoietic tissue. However, there are not many studies published elucidating the mechanisms by which blood cells are released into the circulation in crustaceans. In the crayfish *P. leniusculus*, mature hemocytes come from the sheet-like Hpt which is surrounded by connective tissue and situated on dorsal sides of the stomach (Chaga *et al*., 1995).

In *P. leniusculus* the Hpt cells were studied by light and electron microscopy and the cells were subdivided into five morphologically different cell types that might correspond to different developmental stages of SGCs and GCs (Chaga *et al*., 1995; Johansson *et al*., 2000). The connection between the circulating hemocytes and the hemocyte precursors in the Hpt is still unclear, and the proposed hemocyte lineages have mainly been based on morphological characters. For instance, morphological studies of the hematopoietic tissue have been carried out in blue crab *Carcinus sapidus* (Johnson 1987), shrimp *Sicyonia ingentis* (Hose *et al*., 1992), the lobster *Homarus americanus* (Martin *et al*., 1993), and the black tiger shrimp *P. monodon* (van de Braak *et al*., 2002), but these studies did not reveal any details about maturation and release of the hemocyte in these crustaceans. Data also did not show that the new synthesized hemocytes were released directly from the Hpt or similar tissue, or stored somewhere and released upon activation when they were needed.

Hematopoiesis is the lifelong production of blood cells and is tightly regulated by the various transcription factors that promote or limit cell diversification (Orkin, 1998; Orkin 2000; Sieweke and Graf, 1998). Several hematopoietic transcription factors have been characterized and those are conserved across taxonomic groups including both protostomian and deuterostomian organisms, ranging from flies to humans (Fossett *et al*., 2001b). The crystal cells and the plasmatocytes are two primary blood cell lineages in *D. melanogaster* embryo, and they develop from a common hemocyte progenitor expressing GATA protein Serpent (Srp) (Lebestky *et al*., 2000). The other genes encoding transcriptions factors are: glial cell missing (Gcm), U-shaped (Ush), Lozenge (Lz) also have been identified to be involved in the hematopoietic lineage commitment in *D. melanogaster*. Gcm promotes plasmatocyte development; Ush limits crystal cell development, while Lz promotes crystal cell development; Srp acts upstream of the other factors and is required for late plasmatocyte differentiation. (Fossett and Schulz, 2001a; Lebestky *et al*., 2000; Rehorn *et al*., 1996). In *P. leniusculus*, also some transcription factor proteins have been found. The Hpt cells were shown to be actively proliferating and the Lz-homologue *Pl*Runt was shown to be important in crayfish hematopoiesis (Söderhäll *et al*., 2003). The number of hemocytes dramatically decreased when the crayfishes were injected with a β-1, 3-glucan, resulting in an accelerated maturation of hemocyte precursors in the Hpt followed by release into the circulation of new hemocytes that later develop into functional SGCs and GCs expressing the proPO transcript (Söderhäll *et al*., 2003). In crayfish *P. leniusculus*, all mature hemocytes are expressing proPO while less than 3% of the Hpt cells were found to express this transcript, so we suggested the presence of the proPO transcript can be used as a putative marker for final hemocyte maturation (Söderhäll *et al*., 2003). Moreover, an endogenous cytokine named as astakine from crayfish *P. leniusculus*, containing a prokineticin domain and is critical in the differentiation and growth of hematopoietic stem cells *in vitro* and *in vivo* (Söderhäll *et al*., 2005; Lin *et al.*, 2008) (Fig. 1).

In the five different morphological type cells as suggested by Chaga *et al.* (1995), proPO mRNA is restricted to type 4 cells, and expression of *Pl*Runt in Hpt in this stage is low in all cell types. A short period prior to the release of the cells into circulation, the *Pl*Runt transcript was induced significantly whereas proPO expression was delayed until

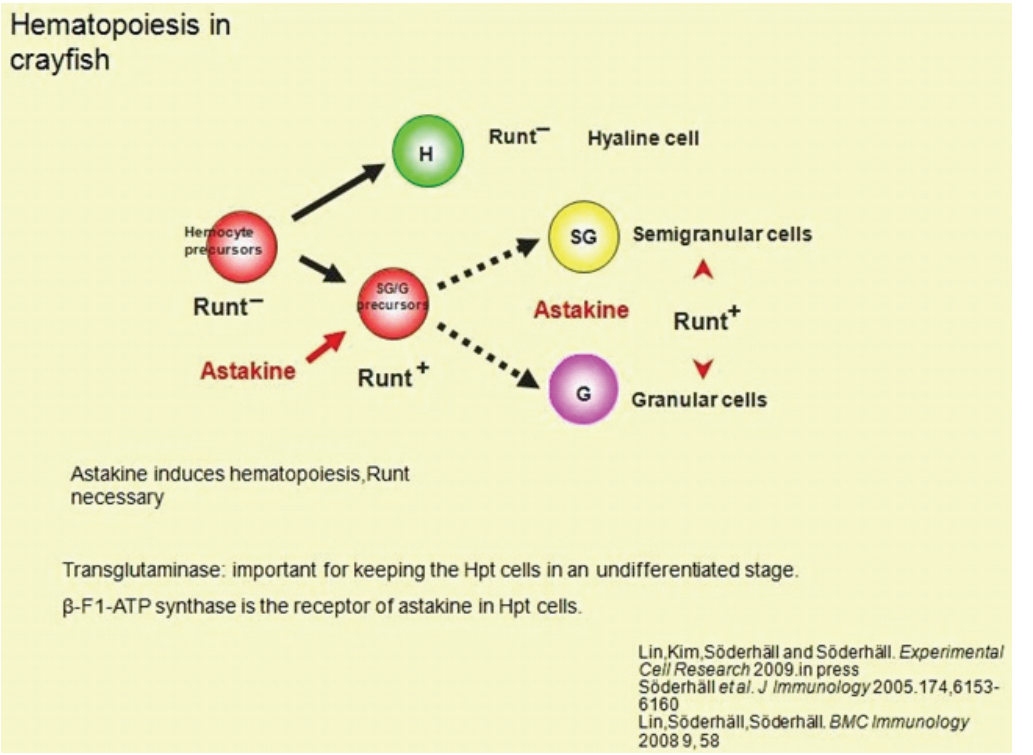

Figure 1 A model for hematopoiesis in crayfish. Astakine 1 is inducing the lineage to SG cells and astakine 2 to the G cells. (For more details see Lin and Söderhäll. 2011. Blood 117:6417-6424).

the hemocytes matured and reached the circulation (Söderhäll *et al*., 2003), showing that the final stage of development into functional semigranular or granular hemocytes happen after their release from the hematopoietic tissue. Morphological observation also showed the presence of these newly released hemocytes with an appearance more similar to type 4 cells (Söderhäll *et al*., 2003).

Absence of proPO transcript in the majority of Hpt cells indicates that this protein is not required until the cells are released into the circulation, which is of considerable interest since this protein is the very important protein in innate immune reactions as well as during sclerotization of the cuticle (Söderhäll *et al*., 2003; Söderhäll and Cerenius, 1998).

In crustaceans and other invertebrates the circulating hemocyte**s** play the most important role in the protection of the animal against invading microorganisms. Therefore, hematopoiesis is essential to produce new hemocytes which can particpate in the cellaular defence. Recently we have shown that crayfish hemocytes have different marker proteins which can be used to understand the differentiation of hemocytes from Hpt cells. Thus we found that proliferating cell nuclear antigen (PCNA) is specific for Hpt cells, a Kazal

proteinase inhibitor for SG cells and Superoxid dismutase for G cells (Wu et. al. 2008) and also that transglutaminase is essential for the maintenance of the Hpt cells in their stem cell form. Further we recently also isolated and characterised the membrane receptor for the cytokine, astakine which is invoved in hematopoiesis (Lin *et al*., 2007).

Prophenoloxidase activating system (proPO-system) and melanization

The melanization reaction is an important immune reaction in arthropods (Cerenius *et al*., 2008) (Figure 2). It is a rapid immune response important in encapsulation and killing of microbial pathogens in invertebrates and melanin depositions are often observed on the surface of invading parasites in the hemocoel or at the site of cuticular injury. Melanin synthesis is achieved by the proPO activation system, a proteolytic cascade similar to vertebrate complement (Cerenius *et al*., 2008). In invertebrates, the proPO cascade (Figure 3) is a very efficient non-self-recognition system, which can be triggered by minute amount of microbial components, e.g. β-1, 3-glucans from fungi and lipopolysaccharides (LPSs)/ peptidoglycans (PGNs) from bacteria, and includes several immune defense proteins resulting in melanization, cell adhesion encapsulation, and phagocytosis. In crustaceans, the proPO-system including several pattern recognition proteins (PRPs), such as β-1, 3-glucans binding protein (βGBP) and lipopolysaccharide and β-1, 3-glucans binding protein (LGBP), and several serine protease zymogen involved in the proteinase cascade have been well studied (Cerenius and Söderhäll, 2004). In the crayfish *P. leniusculus*, proPO is mainly synthesized in granular cells (Gcs) and stored in the secretory granules, similar to the clotting system of horseshoe crabs (Theopold *et al*., 2004), and then released into hemocoel by exocytosis triggered by PRPs. After binding of the PRP ligand with microbial components, serine proteinases zymogens was activated in the proPO-system, and the final step in this process is the conversion of proPO into active phenoloxidase (PO) by the prophenoloxidase activating enzyme (ppA) (Aspán *et al*., 1995). The first primary structure of proPO cDNA was reported from crayfish (Aspán *et al.,* 1995), and after that more than 40 proPO sequences have been reported from different invertebrates, such as insects, echinoderms, ascidians, bivalves, mollusks, millipedes, and brachiopods (Cerenius and Söderhäll, 2004; Cerenius *et al.,* 2008). PO is a copper-containing proteins, customarily is classified as tyrosinase, but actually arthropod POs are much more similar to arthropod hemocyanins (Burmester, 2001; Terwilliger and Ryan, 2006). Hemocyanin is a respiratory protein localized in the plasma and normally produced by the hepatopancreas in many invertebrates, and under certain conditions it exhibits PO activity and may also play important roles in immune defense against invading microorganisms (Baird *et al*., 2007; Decker and Rimke, 1998; Jaenicke and Decker, 2008; Jiang *et al*., 2007; Lee *et al*., 2004; Nagai *et al*., 2001). For instance, crayfish hemocyanin subunit 2 exhibits phenoloxidase activity after the cleavage of at the N-terminal part with trypsin, although its PO activity is relatively lower (Lee *et al.,* 2004). In the process of melanin formation, PO catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity), followed by several intermediate steps which result in the formation of melanin, a dark brown pigment.

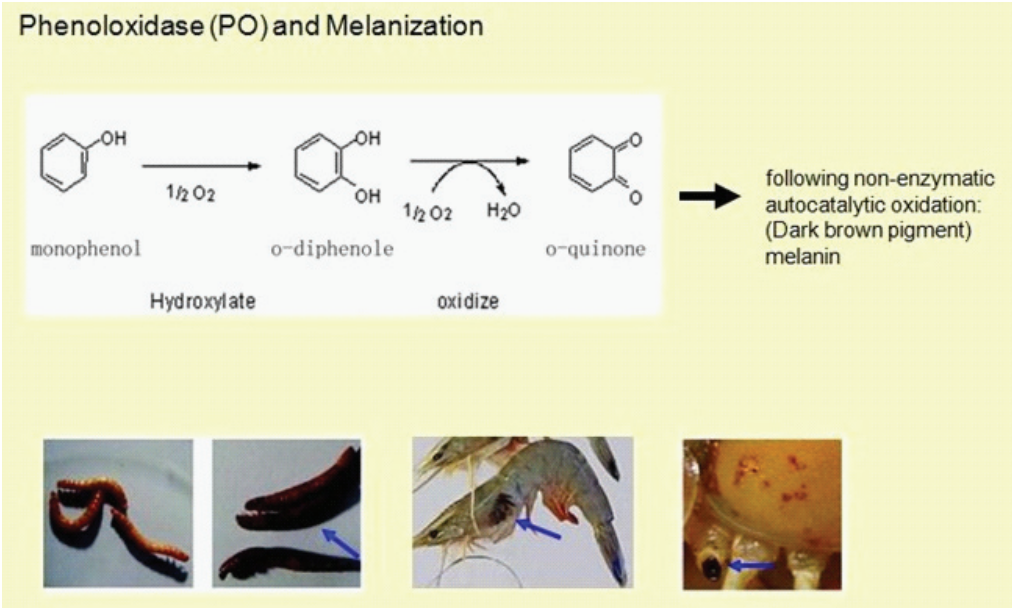

Figure 2 The reaction carried out by the active enzyme phenoloxidase. Photos below show some different animals with melanised spots caused by phenoloxidase.

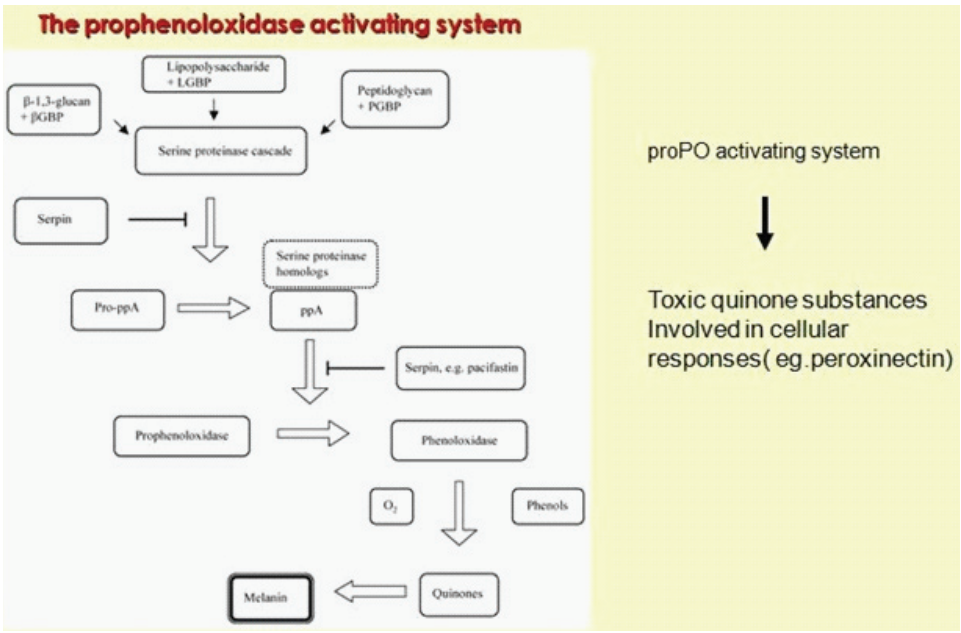

Figure 3. The melanization reaction caused by activation of the prophenoloxidase activating system. (For more details see (1) Cerenius, Lee and Söderhäll. 2008. Trends in Immunology 29:263-27; (2) Cerenius et al. 2010. Trends in Biochemical Science 35:575-583).

Crayfish ppA has similar amino acid sequence as horseshoe crab defensin (Saito *et al*., 1995). The recombinant clip domain of the endogenous trypsin-like serine proteinase ppA from crayfish accordingly shows antibacterial activity *in vitro* against Gram-positive bacteria suggesting a multiple function of crayfish ppA, which is possible for other ppAs (Wang *et al*., 2001). Several ppAs and cofactors have been identified from insects: *Manduca sexta*, *Holotrichia diomphalia*, and *Bombyx mori*, (Cerenius and Söderhäll, 2004). Crayfish and *Bombyx mori* ppAs alone can produce an active PO (Wang *et al*., 2001; Satoch *et al*., 1999), but *Manduca sexta* or *Holotrichia diomphalia* ppA need a cofactor to generate active PO (Jiang *et al*., 1998, 2003). The primary structures of these proteins show that they all exist as zymogens of typical serine proteinases, which are similar to *Drosophila* serine proteinases involved in the organization of the developing embryo (Jiang and Kanost, 2000). Masquerade-like proteins and serine proteinase homologues, other proPO activating factors, have an essential role in activation of the proPO-system in invertebrates. These proteins have no proteinase activity since the primary structures of them show a serine proteinase domain lacking the catalytic triad residues which is necessary for serine proteinase activity. This kind of proteins have been characterized from the coleopteran insects *H. diomphalia* and *Tenebrio molitor* (Kwon *et al*., 2000; Lee *et al*., 2002; Piao *et al*., 2005), the crayfish *P. leniusculus* (masquerade-like protein *Pl*MasI) (Lee and Söderhäll, 2001), the *D. melanogaster* (masquerade protein) (Murugasu-Oei *et al*., 1995), horseshoe crab (factor D) (Kawabata *et al*., 1996) and mosquito *Anopheles gambiae* (infection-responsive serine proteinase-like protein (ispl5)) (Dimopoulos *et al*., 1997). Recently, many details have continued to be collected on the activation and the regulation of the proPO activating proteinase cascade about PRPs, serine proteinase zymogens and serine proteinase homologues. For example, hemolymph serine proteinase, HP21 was proved to act as an activator of proPO-activating proteinase 3 (PAP-3) in *Manduca sexta* (Gorman *et al.,* 2007); One synthetic Lys-PGN derivative fragment was identified as an inhibitor of the melanization cascade, and helped the understanding of how the melanization is regulated and controlled in *Tenebrio molitor* (Park *et al*., 2006); Clustering of PGRP-SA is required to activate both Toll and proPO pathways in *Tenebrio molitor* (Park *et al*., 2007). In *Holotrichia diomphalia,* the activating mechanism of proPO-activating enzyme I (PPAF I) and PPAF II was enlightened by analyzing the crystal structure (Piao *et al*., 2007; Piao *et al*., 2005). Melanin, with a strong efficiently bactericidal effect, was produced on the surface of bacteria by an active melanization complex formed with *Tenebrio* proPO and *Tenebrio* clip-domain SPH1 zymogen after both of them were converted to active forms by *Tenebrio* Spätzle processing enzyme (Kan *et al*., 2008). Zou *et al.* (2008) also found that RUNT-related transcription factor 4 regulated proPO gene expression by binding to the promoter of mosquito proPO under the control of Toll pathway in *Aedes aegypti*. All these results indicate that the two major innate immune responses, Toll pathway and the melanization reaction, share a common serine protease for their regulation (Kan *et al*., 2008).

Evidence (Liu *et al*., 2007; Johansson and Söderhäll, 1995) shows that the activation of the proPO-system can also trigger cellular responses including blood-cell adhesion, degranulation, phagocytosis, nodule formation, and encapsulation. In *Tenebrio molitor,* PO participated in cell adhesion reaction and/or clumping after binding to the hemocytes

membrane (Lee *et al*., 1999). There are reports showing that proPO binds to the surface of some hemocytes resulting in a strict spatial localization of the melanization response in *Manduca sexta* (Ling and Yu, 2005; Mavrouli *et al*., 2005). Studies from *Anopheles gambiae* indicate that PO activity is required for coagulation by causing lipophorin particles to coalesce into the sheet structures (Agianian *et al*., 2007), which increase the efficiency of hemolymph coagulation and cellular defense reactions. Some studies fully imply that the components of proPO activating system provides factors (also include the factors associated with the proPO activating system) to stimulate cellular defense through increasing phagocytosis (Cerenius *et al*., 2008). Other molecules can also gain their function when they are generated concomitant with the activation of proPO-system, for instance, peroxinectin, a cell adhesion protein from crayfish and shrimp is stored in the secretory granules of the SGCs and the GCs, which can be released during exocytosis and proPO-system activation to mediate cell attachment and spreading. (Johansson, 1999; Lin *et al*., 2007; Johansson and Söderhäll, 1988; Sritunyalucksana *et al*., 2001). By RNA interference (RNAi) of pacifastin, an efficient inhibitor of the crayfish proPO activation cascade, higher PO activity was determined, which led to lower bacterial growth, increased phagocytosis, increased nodule formation, and delayed mortality. On the contrary, silencing of crayfish proPO resulted in increased bacterial growth, lower phagocytosis, lower PO activity, lower nodule formation, and higher mortality when infected with this bacterium (Liu *et al*., 2007). The crayfish PO studies suggest that PO play a critical role in crayfish *P. leniusculus* defense when it was infected with a highly pathogenic bacterium, *Aeromonas hydrophila*.

Although melanin formation is essential for host defence in crustaceans and insects, the initiation of proPO system needs to be tightly regulated due to the danger to the animal of unwanted production of quinone intermediates and melanization in places where it is not appropriate (Ashida and Brey, 1998; Gillespie *et al*., 1997). Pacifastin is highly efficient in inhibiting the crayfish ppA, and forms a new family of proteinase inhibitors, which was named as pacifastin-like serine proteinase inhibitors, and it can inhibit the proPO system in many insects (Simonet *et al*., 2002; Vanden Broeck *et al*., 1998). Pacifastin is a high molecular weight and heterodimeric inhibitor composing of one light chain containing nine proteinase inhibitor subunits and a heavy chain that contains three transferrin lobes (Liang *et al*., 1997). Challenging crayfish with *Aeromonas hydrophila* always resulted in 100% mortality when pacifastin was silenced, indicating that endogenous proteinase inhibitors might participated into the protection of host cells or tissues by inhibiting the production of inappropriate highly toxic productions (Liu *et al*., 2007). Injection of low amount of this bacterium killed crayfish anyway in the end, which means that the *A. hydrophila* or other bacteria adopt some strategies to overcome host defenses (Vallet-Gely *et al*., 2008). By using the genetic studies, several genes expressing serine proteinase inhibitor (serpin) were shown to be involved in the melanization response toward different pathogens in *Anopheles* mosquitoes (Michel *et al*., 2005) and *Drosophila* (Ligoxygakis *et al*., 2002). It was shown that *Drosophila* Serpin 27A can inhibit its proPO activating enzyme, PPAE and prevented the melanin synthesis induced by the proPO-system (De Gregorio *et al*., 2002; Ligoxygakis *et al*., 2002). Mutations in Serpin 27A led to severe melanization and increased lethality (De Gregorio *et al.,* 2002). Injections of Serpin 27A into serpin mutant flies blocked the

melanization response on cuticle, when it is experimental injury. The survival rate of parasitoid wasp *Leptolinina boulardi* in these flies increased by the injections of Serpin27A into *Drosophila* larvae (Nappi *et al*., 2005). RNAi and loss-of-function mutation studies show Serpin 28D, one of 29 serpins in *Drosophila,* is the most strongly induced upon injury, and it regulates hemolymph PO activity in both larvae and adults. The study claims that Spn27A confines the melanin produced at the wound site, while Spn28D rather limits PO availability by controlling its initial release (Scherfer *et al*., 2008). The expression of SRPN6, another member of the serpin family, is induced by *Escherichia coli* and both rodent and human malaria parasites, and depletion of this inhibitor by RNAi delays the lysis of parasite without changing the number of developing parasites in *An. Gambiae,* indicating that SRPN6 acts on parasite clearance by inhibiting melanization and promoting parasite lysis (Abraham *et al*., 2005). *Microplitis demolitor* bracovirus carried by the wasp *M. demolitor* encodes a protein Egf1.0, which was recently shown to disable melanization reaction in *Manduca sexta* by inhibiting both the activity of PAP3 and also prevented processing of pro-phenoloxidase serine proteinase homolog (SPH) 1, and SPH2 (Beck and Strand, 2007; Lu *et al*., 2008). In addition to inhibitors of the proPO system activation, some PO inhibitor directly inhibiting the activity of PO have been studied from different arthropods (Daquinag *et al*., 1995; Daquinag *et al*., 1999; Sugumaran and Nellaiappan, 2000), and some specific pathogens have also developed strategies to prevent proPO activation: many endogenous factors function as competitive inhibitors of PO activity; several microbial derived factors also have been identified with the capacities to interfere with active PO (Daquinag *et al*., 1995; Eleftherianos *et al*., 2007; Zhao *et al*., 2005; Cerenius *et al*., 2008). For instance the phenoloxidase inhibitors (POIs), basic lysine rich peptides, have been found in *Musca domestica* and homologous structures were also detected in *Anopheles gambiae* and *Manduca sexta* (Shi *et al*., 2006; Lu and Jiang, 2007). Moreover, Volz *et al.* (2005, 2006) showed that several clip-domain serine proteases are involved in limiting parasite numbers and/or affecting the regulation of melanization. Interestingly, the PO-induced melanization reaction was found to be strictly regulated by *Tenebrio* proPO, which functions as a competitive inhibitor for the formation of melanization complex (Kan *et al*., 2008).

Melanization is vital for wound healing as well as an immune defense. Since the toxic quinone substances and by-products (intermediates) are also harmful to the host, it is necessary to tightly control the melanization reaction.

Recently, Zhao *et al*. (2005) have discovered a novel 43 kDa protein from the hemolymph of the beetle *T. molitor* (*Tenebrio* MIP) which acts as a negative regulator of melanin synthesis. This protein, the target of which is still presently unknown, is consumed during melanization, and no similarity was found between *Tenebrio* MIP and any other known protein (Zhao *et al.,* 2005). Interestingly, the *Tenebrio* MIP-antibody recognizes a protein in crayfish plasma. Since this crayfish 43 kDa protein also is involved in regulating the proPO-system and melanization, we named it *Pacifastacus leniusculus* melanization inhibiting protein (*Pl*-MIP). The sequence analysis shows that *Pl*-MIP is totally different from the *Tm*-MIP, and instead it contains of a C-terminal domain similar to vertebrate fibrinogens, but the collagenous domain found in ficolins is missing in the *Pl*-MIP corresponding region. Importantly, fibrinogen-like domain is the substrate-recognition domain of vertebrate

L-ficolins known as activators of vertebrate complement. However, crayfish MIP did not show any hemagglutinating activity as is common for the vertebrate ficolins. The structural similarities of *Pl*-MIP with ficolins indicates interesting parallels in the regulation between proteolytic cascades involved in defence in vertebrates and invertebrates. We also analysed the expression pattern in different tissue by RT-PCR. The result shows that hemocytes and Hpt did not express *Pl*-MIP. This transcript was detected at fairly low level in hepatopancreas and eyestalk, whereas high expression occurred in nerve-tissue, heart and intestine. When recombinant *Pl*-MIP (*Pl*-rMIP) was added to HLS containing an inactive proPO systeim, *Pl*-rMIP could inhibit LPS-PGN or laminarin induced PO-activity in a dose dependent manner assayed with L-Dopa as substrates, whereas no such inhibition was achieved when the proPO was activated prior to incubation with the *Pl*-rMIP. Therefore *Pl*-MIP has two functions. One is to inhibit proPO activation and the other is to block or delay melanin formation, once PO is activated. Surprisingly, an Asp-rich region similar to that found in ficolins that is likely to be involved in Ca^{2+}-binding is present in crayfish MIP. The antigenic Asp-rich region is important for the function of *Pl*-MIP, since site-directed mutagenesis was perfomed by deleting the four-Asp amino acids in the recombinant protein and this mutant form of MIP acids lost its original function, which implicates that MIP is involved in regulating the PO activating cascade.

The sequence similarity between *Pl*-MIP and human L-ficolin was used to build a homology model of *Pl*-MIP 3-D structure. The model suggests that *Pl*-MIP is an alpha/beta protein stabilized by two cysteine bridges. The binding site for Ca^{2+} ions as described in the L-ficolin structure and similar to tachylectin 5A is well conserved in *Pl*-MIP.

CONCLUSION

Altogether, we have identified three different proteins which can be used as markers for Hpt cells, SGCs and GCs of *P. leniusculus* respectively, and the newly described hemocyte lineage marker protein genes from crayfish *P. leniusculus* provide more information about the differentiation of different stages of the crayfish hemocytes. These data taken together may be helpful for future studies to reveal the connection between SGCs, GCs, and precursor cells in Hpt and also the role of the growth factors (e.g. astakine) as regulators of hemocyte maturation and development in crustaceans.

In the second study, we have identified a novel protein that acts as a negative regulator of proPO activation and melanization. Our data shows that the *Pl*-MIP is most likely an important regulator of the proPO-system and will keep the proPO-system in a non active form until certain inducers such as pathogen-associated molecular patterns (PAMPs) or microorganisms are present, then *Pl*-MIP as well as *Tenebrio* MIP (Zhao *et al*., 2005) are specifically degraded which then will allow activation of the proPO-sytem and melanization. Altogether, both crayfish MIP as well as *Tenebrio* MIP do not affect phenoloxidase activity in itself, but instead interfere with the melanization reaction from quinone compounds to melanin, which is different from Pacifastin, a highly efficient inhibitor of the crayfish ppA.

This study suggests that there may be more unknown factors involved in regulating the proPO system, and the mechanism of *Pl* MIP or *Tenebrio* MIP degradation in the proPO activation need further studies.

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