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Initiation of Innate Immune  
Responses in the Freshwater Crayfish  
*Pacifastacus leniusculus*

BY

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### **Abstract**

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Prophenoloxidase (proPO) is a key enzyme for generation of melanin and is activated by the proPO activating enzyme (ppA) to its active form, PO. The active ppA was purified and cloned from crayfish hemocytes and it is a typical serine proteinase containing a clip, a proline-rich, and a glycine-rich domain. A recombinant protein containing the clip-domain, with homology to horseshoe crab big defensin and mammalian  $\beta$ -defensin, had antibacterial activity *in vitro* against gram-positive bacteria.

The proPO activating system (proPO system) is triggered by lipopolysaccharides (LPS) or  $\beta$ -1,3-glucans. An LPS and  $\beta$ -1,3-glucan binding protein (LGBP) was characterized from crayfish hemocytes. The results of an LGBP antibody inhibition assay suggest that LGBP is directly involved in the proPO system.

The primary structure of a crayfish masquerade-like (mas) protein has homology to serine proteinases except for a substitution within the catalytic triad, which renders it without proteinase activity. The crayfish mas-like protein has also binding activity to various gram-negative bacteria and yeast. When the mas-like protein binds to microorganisms, it is processed by a proteolytic enzyme. The mas-like protein exhibited cell adhesion and opsonic activities suggesting that it plays a role in defense against parasites.

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## **PREFACE**

This thesis is based on three original papers and one manuscript.

The papers will be referred to by their Roman numerals.

- I. Wang, R., Lee, S. Y., Cerenius, L. and Söderhäll, K. (2001) Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *Eur. J. Biochem.* **268**, 895-902.
  
- II. Lee, S. Y., Wang, R. and Söderhäll, K. (2000) A lipopolysaccharide- and  $\beta$ -1,3-glucan binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization, and cDNA cloning. *J. Biol. Chem.* **275**, 1337-1343.
  
- III. Huang, T., Wang, H., Lee, S. Y., Johansson, M. W., Söderhäll, K. and Cerenius, L. (2000) A cell adhesion protein from the crayfish *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade. *J. Biol. Chem.* **275**, 9996-10001.
  
- IV. Lee, S. Y. and Söderhäll, K. (2001) Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus* (submitted)

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## **ABBREVIATIONS**

$\beta$ G :  $\beta$ -1,3-glucan

$\beta$ GBP :  $\beta$ -1,3-glucan binding protein

CCF-1 : coelomic cytolytic factor-1

D-GalNAc : N-acetyl-D-galactosamine

D-GlcNAc : N-acetyl-D-glucosamine

G(-) : Gram-negative bacteria

G(+) : Gram-positive bacteria

GNBP : Gram-negative bacteria-binding protein

HLS : hemocyte lysate supernatant

LGBP : lipopolysaccharide- and  $\beta$ -1,3-glucan binding protein

LPS : lipopolysaccharide

Mas : masquerade

MDM : molluscan defense molecule

PCR : polymerase chain reaction

PG : peptidoglycan

PGBP : peptidoglycan recognition protein

PO : phenoloxidase

ppA : . prophenoloxidase activating enzyme

proPO : prophenoloxidase

proPO system : prophenoloxidase activating system

PRP : pattern recognition protein

UTR : untranslated region

## **INTRODUCTION**

### **Evolution of immune system**

Immune systems have developed to protect multicellular organisms from foreign "non-self" substances. During evolution, two general immune systems have developed to detect foreign substances namely innate (natural) immunity and adaptive (acquired) immunity.

The innate immune system is phylogenetically a more ancient defense mechanism and can be found in all multicellular organisms. This system is the first-line of host defense that helps to limit infection in the early stage of infection and relies on germ line encoded receptors that recognize conserved molecular patterns found in microorganisms (Fearon and Locksley, 1996; Fearon, 1997; Medzhitov and Janeway, 1997).

The adaptive immune system evolved about 400 million years ago and is found only in vertebrates (Thompson, 1995). The immune response of vertebrates have developed more sophisticated and complicated mechanisms including an immunological memory with generation of a large repertoire of antigen-recognition receptors and innate immune systems such as phagocytosis, natural killer cells and complement system for both recognizing and eliminating foreign invaders (Hoffmann, 1995; Hoffmann *et al.*, 1996; Carroll, 1998). While adaptive immunity occurs only in vertebrates, invertebrates have adapted a rapid and efficient innate system to recognize and destroy non-self material, including pathogens. Although they cannot produce antibodies and hence have no immune memory, innate immune mechanisms are enough to protect and preserve themselves from intruding microorganisms. In addition to their rigid and wax-covered cuticle serving as a mechanical barrier they can also rapidly produce effective innate immune responses during infection. Since the discovery of inducible antimicrobial peptides from the moth *Hyalophora cecropia* by Boman and associates in 1980 (Hultmark *et al.*, 1980; Steiner *et al.*, 1981), great efforts have been made to investigate the function and mechanism of the innate immune system not only in invertebrates but also in all multicellular organisms ranging from humans to plants. It is

now clear that the innate immune system is very important for self or non-self recognition in vertebrates and plays an important role in adaptive immune systems (Medzhitov and Janeway, 1998a; b).

In this thesis, defense mechanisms of the freshwater crayfish *Pacifastacus leniusculus* have been studied with molecular and biochemical approaches to understand the innate immune system of invertebrates.

### **Immune responses in invertebrates**

Various cells in invertebrates respond to microorganisms by enclosing these infectious agents within aggregates and then destroying them. The innate immune system of invertebrates can respond to the presence of pathogens with cellular and humoral responses. The former includes phagocytosis, nodule formation, and encapsulation and these reactions are mediated by blood cells known as hemocytes.

In general, phagocytosis occurs against invading small microorganisms by a single hemocyte, whereas multicellular encapsulation or nodule formation are more complex processes that occur when invading pathogens or parasites are too large or too numerous to be ingested by a single phagocytic cell (Lanz-Mendoza *et al.*, 1996; Gillespie *et al.*, 1997; Johansson, 1999; Gillespie *et al.*, 2000).

Nodules represent aggregates of hemocytes entrapped in a sticky extracellular material and these aggregates are often melanized by the activity of the enzyme phenoloxidase. Encapsulation is similar to nodule formation, but occurs as a defense reaction against larger invaders such as fungi, nematodes, parasitoid eggs, or larvae (Gunnarsson and Lackie, 1985; Hoffmann *et al.*, 1996; Koizumi *et al.*, 1997; 1999; Gillespie *et al.*, 1997).

The cellular immune response is an important reaction for recognition of non-self material in an early stage of infection. Recently, several proteins involved in encapsulation and phagocytosis have been reported from a crayfish, *Pacifastacus leniusculus* (Kobayashi *et al.*, 1990; Thörnqvist *et al.*, 1994), a caterpillar, *Cotesia rubecula* (Asgari *et al.*, 1998), and a beetle, *Tenebrio molitor* (Cho *et al.*, 1999a; b).

The activation of the humoral immune system is contributed by several reactions such as proteolytic cascades leading to blood clotting, melanin formation, opsonization, and a transient synthesis of potent antibacterial peptides (Hoffmann *et al.*, 1999)

The clotting system is an important reaction in both vertebrates and in invertebrates to prevent blood loss through wounds. The clotting system of a chelicerate, the horseshoe crab *Tachypleus tridentatus*, has been characterized in great detail (Iwanaga *et al.*, 1998). The coagulation system of the horseshoe crab is composed of five protein components which make up a clotting cascade system (Iwanaga, 1993), the serine proteinase zymogen factors C, B, G, the proclotting enzyme and the clottable protein coagulogen. The factors C and G act as highly sensitive recognition proteins for LPS and  $\beta$ -1,3-glucan, respectively. Invading pathogens trigger these proteins, resulting in the sequential activation of clotting factors and subsequent clot formation. The extreme sensitivity of the clotting cascade for LPS is used in the so-called Limulus test, a widely employed assay method for the detection of bacterial endotoxins or LPS (Levin and Bang, 1964; Tanaka and Iwanaga, 1993). In crustaceans, the clotting protein was first cloned, isolated and characterized in crayfish (Hall *et al.*, 1999). Clotting in crayfish occurs through the polymerization of a clotting protein found in plasma and is catalysed by a calcium ion dependent transglutaminase released from hemocytes upon wounding (Hall *et al.*, 1999). Several clottable proteins have been partially characterized from penaid shrimps (Yeh *et al.*, 1998; 1999), lobster (Fuller *et al.*, 1971a; b), crayfish (Kopáček *et al.*, 1993), freshwater giant prawn (Yeh *et al.*, 1998), and sand crayfish (Komatsu and Ando, 1998). They have similar amino acid compositions and N-terminal sequences, but no homology to horseshoe crab coagulogens. The primary structure of crayfish clotting protein indicates that it belongs to the vitellogenin superfamily of protein (Hall *et al.*, 1999). This shows that two functionally similar processes in two related species of invertebrates are completely different and emphasize the large variation in related

processes in invertebrate animals. However, no insect clotting system has been characterized yet.

Antimicrobial peptides have become recognized as important components of the non-specific host defense or innate immune system in a variety of organisms ranging from vertebrates and invertebrates to plant species (Boman, 1995; Boman, 1998; Hetru et al., 1998; Hoffmann *et al.*, 1999). The primary structures of these positively charged antimicrobial peptides are highly diverse, yet their secondary structures share a common feature of amphipathicity (Ganz and Lehrer, 1994; Hancock and Lehrer, 1998). Although they exhibit great structural diversity, the peptides are often grouped into three families by certain common structural patterns: (i) linear peptides forming  $\alpha$ -helices and deprived of cysteine residues (cecropin and magainin family), (ii) cyclic peptides containing cysteine residues (defensin family), and (iii) peptides with an overrepresentation of proline and/or glycine residues.

Peptides in the  $\alpha$ -helix family generally have a random coil structure in aqueous solution and can penetrate bacterial membranes and disrupt the membrane structure by ion channel formation (Christensen *et al.*, 1988; Shai, 1998; Steiner *et al.*, 1988). This group of peptides has a broad spectrum of antibacterial activity ranging from gram-positive and gram-negative bacteria to fungi and protozoa. The defensin family peptides can be divided into two subgroups according to their structure, mammalian defensins having a triple-stranded  $\beta$ -sheet structure (Hill *et al.*, 1991) and insect defensins forming two stranded  $\beta$ -sheets with a flanking  $\alpha$ -helix (Cornet *et al.*, 1995). Although all defensins contain three disulfide bonds, the mammalian and insect defensins show different patterns. The antimicrobial activity spectrum of mammalian defensins includes gram-positive, gram-negative bacteria, mycobacteria, many fungi and some enveloped viruses, whereas insect defensins are active primarily against gram-positive bacteria and with little activity against gram-negative bacteria or eucaryotic cells. They affect the bacterial membrane structure by making pores (Hoffman and Hertu, 1992; White *et al.*, 1995). A third class of antimicrobial peptides have a high

percentage of proline or glycine residues. The proline-rich peptides are present in insects, crustaceans, and mammals and show activity against only a few species of gram-negative bacteria and are bacteriostatic rather than bacteriolytic. However, until now no glycine-rich molecules have been reported in mammals. Some of this group antibacterial peptides such as apidaecin, gloverins, and attacins have an immediate effect on protein synthesis, but little is known about the 3D structure of these peptides (Axén *et al.*, 1997; Shai, 1998; Bulet, 1999).

Several antimicrobial peptides have been characterized from insects and chelicerates, but a few peptides have been demonstrated in crustaceans from the shore crab *Carcinus maenas* (Relf *et al.*, 1999) and the shrimp *Penaeus vanamei* (Destoumieux *et al.*, 1997; 1999). Recently, two antibacterial peptides with low molecular masses named astacidin 1 and 2 were purified and characterized from crayfish *Pacifastacus leniusculus* hemolymph, (Lee and Söderhäll, unpublished). Astacidin 1 with 16 amino acid residues has no homology to other hitherto described antimicrobial peptides whereas, astacidin 2 with 14 amino acid residues has high similarity to the proline rich-peptide, metalnikowin 1 purified from the hemipteran insect, *Palomena prasina* (Chernysh *et al.*, 1996).

Many antimicrobial peptides show a broad activity against microorganisms and have a remarkable specific activity to prokaryotic cells and with low toxicity to eucaryotic cells. These phenomena and the mode of action demonstrate that antimicrobial peptides may be developed as therapeutic agents for use as pharmaceuticals or in agricultural applications (Zasloff, 1992).

### **Prophenoloxidase activating system (proPO system)**

The proPO system consists of several proteins involved in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Gillespie *et al.*, 1997; Söderhäll and Cerenius, 1998; Sritunyalucksana and Söderhäll, 2000). It is an efficient immune system for non-self recognition and is initiated by recognition of lipopolysaccharides or peptidoglycans from bacteria and  $\beta$ -1,3-glucans from fungi. This system contains a proteinase cascade

composed of pattern-recognition proteins (PRPs), several proteinases, their zymogens, and prophenoloxidase (proPO) (Figure 1) (Söderhäll and Cerenius, 1998).

Phylogenetically, proPO belongs to one of four well-supported subfamilies within: (i) the arylphorin subfamily (a storage protein in insects without copper binding function), (ii) the hemocyanins of branchiopod crustaceans (copper binding proteins involved in oxygen transport), (iii) the hemocyanins of chelicerates, (iv) and the prophenoloxidase of insects and crustaceans (copper binding proteins involved in immune responses). However, hemocyanins are found in chelicerates and crustaceans but not insects. In a recent study, chelicerates hemocyanins seem to be linked to proPO since upon a proteolytic cleavage in the spider, *Eurypelma californicum* (Decker and Rimke, 1998; Decker and Tuczec, 2000) and horseshoe crab, *Tachyplesus tridentatus* (Nagi and Kawabata, 2000) hemocyanins show PO activity. A phylogenetic analysis shows that the proPO subfamily has much higher evolutionary relationship with the chelicerate hemocyanins than the branchiopod hemocyanins (Hughes, 1999; Burmester, 2001).

The active form of proPO, phenoloxidase (PO; monophenol, dihydroxyphenylalanine: oxidoreductase; EC 1.14.18.1), also known as tyrosinase, catalyzes two successive reactions: hydroxylation of a monophenol to *o*-diphenol (monophenoloxidase activity) and the oxidation of the *o*-diphenol to *o*-quinone (diphenoloxidase activity) (Söderhäll and Cerenius, 1998; Decker and Tuczec, 2000). Production of *o*-quinones by PO is an initial step in the biochemical cascade of melanin biosynthesis. It is also important in cuticular sclerotization, wound healing, and encapsulation of foreign materials (Lai-Fook, 1966; Sugumaran, 1991; Söderhäll and Cerenius, 1998). Arthropod POs exist as an inactive zymogen under normal physiological conditions and then they can be activated by proteolytic cleavage except for a recently discovered proPO from the insect, wasp *Pimpla hypochondriaca*, which was active without any proteolytic cleavage (Parkison *et al.*, 2001).

Since the first primary structure of proPO was determined in crayfish (Aspán *et al.*, 1995), great numbers of proPO sequences were

reported from various invertebrates. In crayfish, proPO is synthesized and localized in granules of the blood cells, and released into plasma by an exocytosis triggered by the  $\beta$ -1,3-glucan binding protein. Crayfish hemocyanin is synthesized in the hepatopancreas (Aspán *et al.*, 1995; Söderhäll and Cerenius, 1998). In insects some proPOs exist in the plasma (Sugumaran and Kanost, 1993) and several isoforms encoded by different genes have been found (Ashida and Brey, 1997; Müller *et al.*, 1999), but the physiological significance of the presence of the different proPO polypeptides remains to be studied. Recently, two isoforms of cuticle proPOs of the silkworm, *Bombyx mori* have been characterized and one of them is shown to be transported from the hemolymph proPO to the cuticle. The transported cuticle proPO has different molecular mass from the hemolymph proPO because of the modification of one up to six methionine residue(s) (Asano and Ashida, 2000a; b).

PRPs are the triggering molecules of the proPO system, since they bind microbial components and then they induce activation of proteinases in the proPO system. Finally proPO is proteolytically converted to phenoloxidase by an endogenous trypsin-like serine proteinase, the so-called prophenoloxidase activating enzyme (ppA). So far, four ppAs and one cofactor have been characterized and cloned from four different animals; a beetle, *Holotrichia diomphalia* (Lee *et al.*, 1998a; b), a silkworm, *Bombyx mori* (Satoh *et al.*, 1999), a tobacco hornworm, *Manduca sexta* (Jiang *et al.*, 1998), and a crayfish, *Pacifastacus leniusculus* (Paper I). Their primary structure demonstrates that they all exist as zymogens of typical serine proteinases and are similar to *Drosophila* serine proteinases involved in the organization of the developing embryo (Chasan and Anderson, 1989; Jin and Anderson, 1990; Jiang and Kanost, 2000). They also contain one or two clip-domains, which have homologous amino acid sequences to the horseshoe crab big defensin (Saito *et al.*, 1995a) and mammalian  $\beta$ -defensin (Tang and Selsted, 1993). In crayfish the recombinant clip-domain of ppA has antibacterial activity *in vitro* against gram-positive bacteria suggesting a dual function of crayfish ppA and maybe also for other ppAs (paper I). Interestingly, a new type of prophenoloxidase activating factor has been

purified and cloned from the coleopteran, *Holotrichia diomphalia* (Kwon *et al.*, 2000). This protein has no proteinase activity but is an essential factor for the activation of the proPO system in this insect. The primary structure of this protein is similar to serine proteinase homologues lacking the complete catalytic triad necessary for serine proteinase activity, such as a crayfish (paper III and IV) and a *Drosophila* masquerade-like protein (Murugasu-Qei *et al.*, 1995), horseshoe crab factor D (Kawabata *et al.*, 1996), and mosquito infection-responsive serine protease-like protein (ispl5) (Dimopoulos *et al.*, 1997).

With the activation of the proPO system, other proteins will also gain their biological activity and can participate in cellular defense. One such molecule is a cell adhesion protein, peroxinectin, which has been purified, characterized and cloned from the crayfish, *Pacifastacus leniusculus* (Johansson and Söderhäll, 1988; 1989; 1993; 1995).

The proPO system has to be controlled and regulated to avoid the deleterious effects of active components of the system, and in particular PO, which can produce highly toxic intermediates. Several proteinase inhibitors for preventing over-activation of ppA (Hergenhahn *et al.*, 1987; Aspán *et al.*, 1990a) and a phenoloxidase inhibitor (POI), which can directly inhibit the activity of phenoloxidase (Daqing *et al.*, 1995; 1999; Sugumaran and Nellaiappan, 2000) have been reported from several arthropod species.

### **The non-self recognition system**

The innate immune system is activated by pathogens or environmental antigens and is mediated by interaction between receptors or pattern recognition molecules and pathogens. The recognition molecules for foreign material have been named as pattern-recognition proteins (PRPs) by Janeway (Medzhitov and Janeway, 1997; Carroll and Janeway, 1999) because the host primitive effector cells would recognize molecular patterns rather than particular structures of the invading microorganisms. Examples of pathogen-associated molecules, which are not found in other multicellular organisms, are LPS or peptidoglycans of bacterial cell walls,  $\beta$ -1,3-glucan of fungal cell walls, and double stranded

RNA of viruses. Most current research has emphasized the possible role of non-self recognition molecules in the vertebrate and the invertebrate immune system (Vasta *et al.*, 1994; Arason, 1996; Matsushita, 1996; Vasta *et al.*, 1999; Wilson *et al.*, 1999; Marques and Barracco, 2000).

Recognition of carbohydrates may have evolved because these common constituents of microbial cell walls have structures that are distinct from those of carbohydrates of eucaryotic cell surfaces. Therefore, LPS or/and  $\beta$ -1,3-glucan binding proteins (LBP,  $\beta$ GBP, or LGBP), peptidoglycan recognition protein (PGRP), lectins, and hemolin have been found in a variety of invertebrates and different biological functions have been proposed for these molecules after binding to their targets (Table 1).

Lectins/agglutinin are glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates and exist in almost all living organisms (Marques and Barracco, 2000). They can bind cells and an agglutination reaction occurs. Interaction between lectins and carbohydrates is involved in various biological activities, for instance the cellular and tissue transport of carbohydrates (Goldstein *et al.*, 1980; Ravindranath and Copper, 1984), glycoproteins (Vasta, 1992), cell adhesion (Kasai and Hirabayashi, 1996; Ni and Tizard, 1996; Vasta *et al.*, 1999), opsonization (Jomori and Natori, 1992; Cerenius *et al.*, 1994), and nodule formation (Koizumi *et al.*, 1999). Especially, C-type lectins, calcium dependent lectins are reported to be involved in immune recognition in invertebrates (Weis *et al.*, 1998; Vasta *et al.*, 1999). Constitutively expressed lectins that have an LPS-binding property have been characterized from the silkworm, *Bombyx mori* (Koizumi *et al.*, 1997; 1999) and the American cockroach, *Periplaneta americana* (Jomori and Natori, 1991; 1992; Kawasaki *et al.*, 1996; Natori *et al.*, 1999). The biological function of these LPS binding proteins was shown to be a bacterial clearance activity and an opsonic effect, respectively. Two kinds of inducible lectins (immulectins) were characterized and cloned in the tobacco hornworm, *Manduca sexta*, and were both found to stimulate the proPO system (Yu *et al.*, 1999; Yu and Kanost, 2000). A large number of carbohydrates binding proteins in invertebrates have

characters that make them lectins. Moreover, some LGBPs and  $\beta$ GBPs have a similar primary structure to bacterial glucanases, but they do not exhibit any glucanase activity and instead enhance or mediate activation of the proPO system (paper II; Cerenius *et al.*, 1994; Ochiai and Ashida, 2000; Ma and Kanost, 2000; Beschin *et al.*, 1998). Crayfish  $\beta$ GBP is present in plasma and interacts with  $\beta$ -1,3-glucan to enhance the activation of the proPO system and also to act as an opsonin to increase the rate of phagocytosis (Duvic and Söderhäll, 1990; 1992; 1993; Cerenius *et al.*, 1994; Thörnqvist *et al.*, 1994). In addition, a crayfish LBGP has been isolated, cloned, and characterized from hemocytes and it has binding activity to both LPS and  $\beta$ -1,3-glucans but not to peptidoglycans. The primary structure and cloning results showed that LBGP has significant homology with several gram-negative bacteria binding proteins and bacterial glucanases. The function of LBGP is to bind to LPS or  $\beta$ -1,3-glucans and then activate the proPO system in crayfish (paper II).

The crayfish masquerade-like protein (mas-like protein) is a pattern recognition protein from hemocytes, since it can bind to LPS,  $\beta$ -1,3-glucans, gram-negative bacteria, and yeast. The mas-like protein also has an opsonizing and cell adhesion activity. Its amino acid sequence showed homology to serine proteinases except for a substitution within the catalytic triad, which will render it without enzyme activity (paper III and IV).

Hemolin belongs to the immunoglobulin (Ig) superfamily containing Ig-like domains and binds to the lipid A part of LPS and gram-negative bacteria. It is the major inducible immune protein identified in the hemolymph, up-regulated about 18 fold upon immune challenge of *Hyalophora cecropia* pupae (Lanz-Mendoza *et al.*, 1996; Lanz-Mendoza and Faye, 1999) and 30-40 fold in *Manduca sexta* larvae (Zhoa and Kanost, 1996). The role of hemolin *in vivo* has not been fully clarified. However a number of data suggest a dual function in non-self recognition and signal transduction during the immune response and it also seems to have a role in neurogenesis (Lanz-Mendoza and Faye, 1999). Hemolin was shown to have similarity with transmembrane cell adhesion

molecules (Sun *et al.*, 1990; Bettencourt *et al.*, 1997). So far, hemolin has been reported from three lepidopteran species (Lanz-Mendoza *et al.*, 1996; Zhoa and Kanost, 1996) and a mollusc (Hoek *et al.*, 1996).

These data demonstrate that pattern recognition proteins are involved in various innate immune responses and this study will provide a background for understanding how invertebrates control their immune system against invading foreign pathogens.

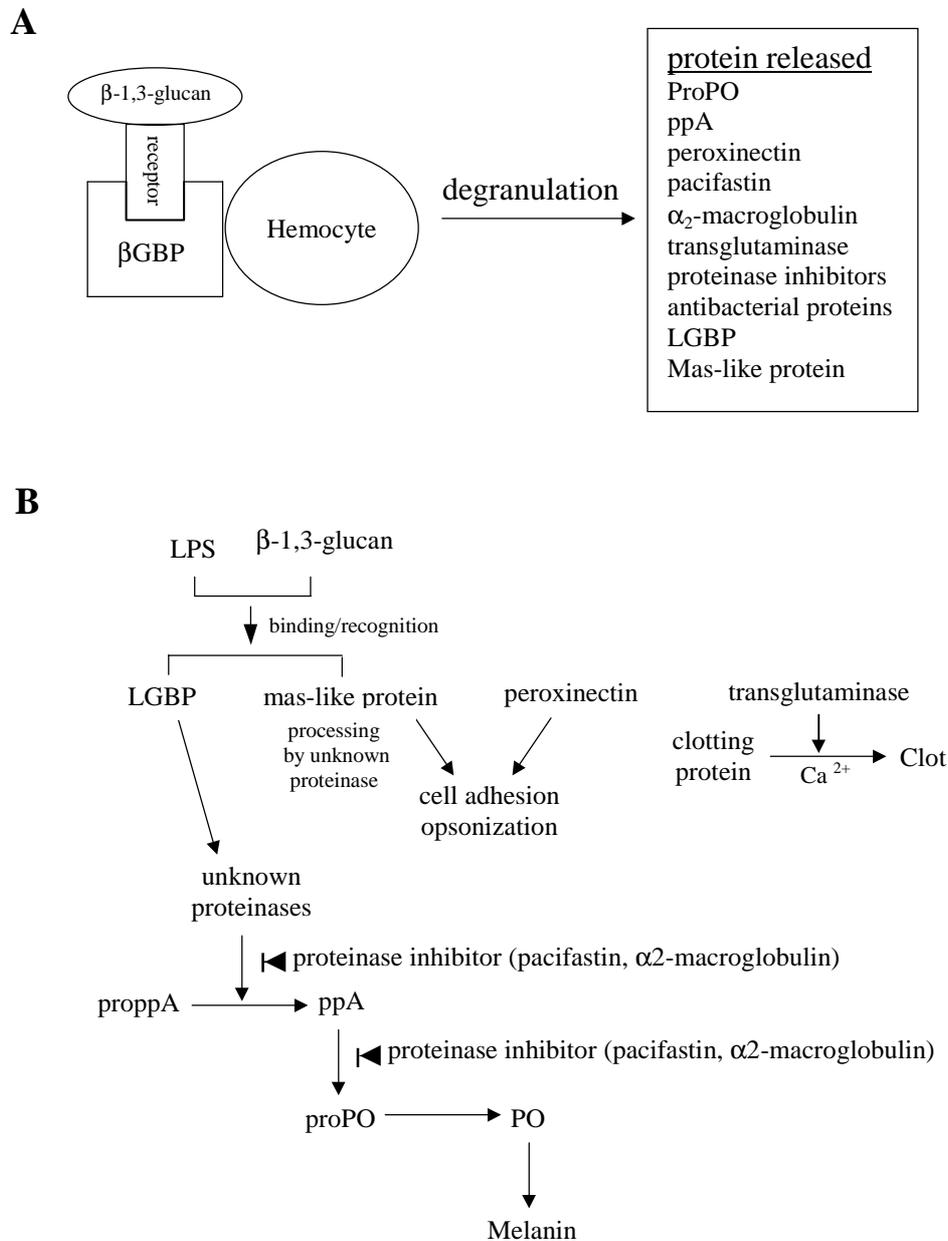


Figure 1. Schematic representation of innate immune systems in crayfish.  
 A:  $\beta$ GBP is activated by  $\beta$ -1,3-glucan and then induces the degranulation of hemocytes. Several proteins are released.  
 B: The proPO and clotting system in crayfish.

Proteins	Properties / References
<b>LPS-binding proteins</b>	
cockroach <i>Periplaneta americana</i> LBP	Opsonin (Jomori and Natori, 1991; 1992; Kawasaki <i>et al.</i> , 1996)
fall webworm <i>Hyphantria cunea</i> GNBP	Binding to LPS (Shin <i>et al.</i> , 1998; Shin <i>et al.</i> , 2000)
horseshoe crab <i>T. tridentatus</i> Factor C	Initiation of the coagulation system (Muta <i>et al.</i> , 1991; 1993a; Nakamura <i>et al.</i> , 1986; 1988)
horseshoe crab LPS-binding protein	Negative regulator of coagulation (Roth and Tobias, 1993)
mosquito <i>Anopheles gambiae</i> GNBP	LPS binding activity (Dimopoulos <i>et al.</i> , 1997)
silkworm <i>bombyx mori</i> BmLBP	Hemocyte nodule formation; bacterial clearance (Koizumi <i>et al.</i> , 1997; 1999)
silkworm <i>bombyx mori</i> GNBP	Binding to G(-) bacteria (Lee <i>et al.</i> , 1996)
<b>Ig-like proteins</b>	
moth <i>Hyalophora cecropia</i> Hemolin	IgG-like protein; Binding to LPD and G(-) bacteria; prevents hemocyte aggregation; enhances phagocytosis (Lanz-Mendoza <i>et al.</i> , 1996)
snail <i>Lymnaea stagnails</i> MDM	IgG-like protein (Hoek <i>et al.</i> , 1996)
tobacco hornworm <i>M. sexta</i> Hemolin	IgG-like protein; prevents hemocyte aggregation; enhances phagocytosis (Zhao and Kanost, 1996)
<b><math>\beta</math>-1,3-glucan binding proteins</b>	
black tiger shrimp <i>P. monodon</i> $\beta$ GBP	Binding to $\beta$ G (Sritunyalucksana <i>et al.</i> , unpublished)
brown shrimp <i>P. californiensis</i> $\beta$ GBP	Involvement in proPO system (Vargas-Albores <i>et al.</i> , 1996)
cockroach <i>Blaberus craniifer</i> $\beta$ GBP	Involvement in proPO system (Söderhäll <i>et al.</i> , 1988)
crayfish <i>P. leniusculus</i> $\beta$ GBP	Involvement in proPO system; plasma high-density lipoprotein; opsonin; degranulation factor (Duvic and Söderhäll, 1990; 1993; Barracco <i>et al.</i> , 1991; Cerenius <i>et al.</i> , 1994; Thörnqvist <i>et al.</i> , 1994; Hall <i>et al.</i> , 1995)
horseshoe crab <i>T. tridentatus</i> Factor G	Initiation of the coagulation system (Seki <i>et al.</i> , 1994; Muta <i>et al.</i> , 1995; 1996)
silkworm <i>Bombyx mori</i> $\beta$ GRP	Involvement in proPO system (Ochiai and Ashida, 2000)
tobacco hornworm <i>M. sexta</i> $\beta$ GBP	Involvement in proPO system, agglutinin (Ma and Kanost, 2000)
<b>LPS &amp; <math>\beta</math>-1,3-glucan binding proteins</b>	
crayfish <i>P. leniusculus</i> LGBP	Involvement in proPO system (paper II)
crayfish <i>P. leniusculus</i> mas-like protein	Opsonin; cell adhesion (paper III and IV)
earthworm <i>Eisenia foetida</i> CCF-1	Involvement in proPO system; cytolytic factor; opsonin; TNF- $\alpha$ function (Beschlin <i>et al.</i> , 1998; 1999)
fruit fly <i>Drosophila melanogaster</i> GNBP	Induction of antimicrobial peptide production (Kim <i>et al.</i> , 2000)
<b>Peptidoglycan binding proteins (T3-lysozyme-like protein)</b>	
fruit fly <i>D. melanogaste</i> PGRP-SA	Binding to PG
PGRP-SC1B	Binding to PG (Werner <i>et al.</i> , 2000)
moth <i>Trichoplusia ni</i> PGRP	Binding to PG and G(+) bacteria (Kang <i>et al.</i> , 1998)
silkworm <i>Bombyx mori</i> PGRP	Involvement in proPO system (Yoshida <i>et al.</i> , 1996; Ochiai and Ashida, 1999)
<b>Lectins</b>	
Cockroach <i>Blaberus craniifer</i> BDL 1	Binding to mannose
BDL 2	Opsonin; binding to D-GlcNAc/D-GalNAc
BDL 3	Opsonin; binding to D-GalNAc
GSL	Binding to $\beta$ -1,3-glucan (Wilson <i>et al.</i> , 1999)
fruit fly <i>Drosophila melanogaster</i> Lectin	Binding to galactose (Haq <i>et al.</i> , 1996)
flesh fly <i>S. peregrina</i> Granulocytin	Agglutinin; binding to mucin (Fujita <i>et al.</i> , 1998)
horseshoe crab <i>T. tridentatus</i> Lectin-L6	Agglutinin; antibacterial activity against G(-) (Saito <i>et al.</i> , 1995b)
Tachylectin-5	Enhancing the antibacterial activity of big defensin; binding to N-Acetyl group (Iwanaga <i>et al.</i> , 1998; Gokudan <i>et al.</i> , 1999)
tobacco hornworm <i>M. sexta</i> immulectin 1	Agglutinin; Involvement in proPO system (Yu <i>et al.</i> , 1999)
immulectin 2	Agglutinin; Involvement in proPO system (Yu <i>et al.</i> , 2000)

Table 1. Pattern recognition molecules of invertebrates.

## RESULTS AND DISCUSSION

### **The prophenoloxidase activating enzyme with a defensin-like domain has antibacterial activity *in vitro* (paper I)**

The major component of the proPO system is PO. *In vitro* studies have shown that PO exists as an inactive precursor, proPO, which is activated in a stepwise process involving serine proteinases triggered by microbial cell wall components, such as LPS,  $\beta$ -1,3-glucan or peptidoglycan.

The crayfish prophenoloxidase activating enzyme (ppA) was purified and partially characterized from hemocytes. The purified ppA has a molecular mass of about 36 kDa and is able to convert proPO to an active PO without any additional factors (Aspán *et al.*, 1990b). This protein was cloned from a blood cell cDNA library and its corresponding cDNA of 1,736 base pairs encodes a zymogenic protein (proppA) of 468 amino acids. A transcript of 1.8 kilobase was detected in mRNA preparations from crayfish hemocytes by Northern blot analysis.

The proppA has a putative signal sequence of 23 amino acids and the mature protein consists of 445 amino acids with a calculated molecular mass of 48,107 Da and an estimated pI of 9.04. The cleavage site for the activation of proppA was identified by amino acid sequencing as an internal peptide bond between Arg236 and Ile237, which separates a 23 kDa cationic N-terminal half (pI=11.9) and a 25 kDa anionic C-terminal half, serine proteinase domain (pI=4.6). The C-terminal half of the proppA is composed of a typical serine proteinase domain, with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half contains a basic glycine-rich domain, a basic proline-rich domain and a clip-domain, in which the disulfide-bonding pattern is likely to be identical to those of the horseshoe crab big defensin and mammalian  $\beta$ -defensins.

Two kinds of antibodies were raised against synthetic peptides derived from a region of C- (anti-C antibody) and the N-terminal halves (anti-N antibody). Antibodies made against both the C- and the N-

terminal halves recognize two proppAs with molecular masses of 58 kDa and 56 kDa under reducing conditions, 46 kDa and 44 kDa under non-reducing conditions, which are probably isoforms of proppA. However, under non-reducing conditions only the anti-C antibody recognized the two proppAs and it could efficiently inhibit the laminarin ( $\beta$ -1,3-glucan)-triggered activation of prophenoloxidase *in vitro*, which suggests that a conformational change take place upon reduction that allows the anti-N to react with the N-terminal half of proppA. The purified ppA has a molecular mass of 36 kDa, which is probably a result of that the C- and N-terminal halves are still linked by a disulfide bridge after proteolytic cleavage.

The clip domain structure of the crayfish proppA is similar to the corresponding domain in three insect proppAs (paper I; Jiang *et al.*, 1998; Satoh *et al.*, 1999), as well as in the horseshoe crab proclotting enzyme (Muta *et al.*, 1993b). Moreover, the disulfide bonding pattern in the clip-domain shows similarity to those of horseshoe crab big defensin (Saito *et al.*, 1995a) and the  $\beta$ -defensin-12 of the bovine neutrophils (Tang and Selsted, 1993). To ascertain the role of this domain, the recombinant clip-domain in crayfish proppA was overexpressed in *E. coli* and the resulting peptide exhibited anti-bacterial activity against gram-positive bacterial strains such as *Micrococcus luteus* M11 and *Bacillus megaterium* Bm11 with 50 % growth inhibitory concentrations of 1.43  $\mu$ M and 17.9  $\mu$ M, respectively. These results suggest that the proppAs may have a dual function; one is to activate proPO and another one to generate antibacterial peptides.

### **A lipopolysaccharide- and $\beta$ -1,3-glucan binding protein involved in the proPO system (paper II)**

A lipopolysaccharide- and  $\beta$ -1,3-glucan-binding protein (LGBP) was isolated and characterized from blood cells (hemocytes) of the freshwater crayfish *Pacifastacus leniusculus*. The LGBP was purified by chromatography on Blue-Sepharose and Phenyl-Sepharose, followed by Sephacryl S-200. The homogeneous LGBP has a molecular mass of 36

kDa and 40 kDa on 10 % SDS-PAGE under reducing and non-reducing conditions, respectively. The calculated mass of LGBP is 39,492 Da, which corresponds to the native size of LGBP and the estimated pI of the mature LGBP is 5.80.

The LGBP was cloned from a crayfish hemocyte cDNA library. The result shows that LGBP has a signal peptide of 15 amino acid residues and the mature LGBP has 346 amino acid. The 3'-UTR contains more than 17 tandem repeats of 32 nucleotides. Northern blot analysis shows that mRNA of LGBP is constitutively expressed as a single band of 5.3 kilobases in hemocytes. Two probes, one spanning the coding region and another the 3'-untranslated region with 17 repeated sequences, gave the same band, which indicates that the repeated sequences are not an artifact.

By using fluorescein isothiocyanate-labeled LPS, we could detect that the LPS binding activity of immobilized LGBP increased gradually in a dose-dependent manner. In solution, LGBP was also shown to bind to LPS as well as to  $\beta$ -1,3-glucans such as laminarin and curdlan, but not to peptidoglycan.

To evaluate the involvement of LGBP in the proPO system, a polyclonal antibody against LGBP was made and used for the inhibition of phenoloxidase (PO) activity triggered by the  $\beta$ -1,3-glucan, laminarin. The PO activity was completely blocked by the anti-LGBP antibody. The inhibited PO activity could be recovered by the addition of laminarin-preincubated LGBP, but not by laminarin itself or laminarin-preincubated BSA. These results suggest that the 36kDa LGBP plays a role in the activation of the proPO system in crayfish as a PRP.

The primary structure of LGBP shows significant homology with several putative gram-negative bacteria binding proteins and  $\beta$ -1,3-glucanases (see Table 1).

### **Properties of the mas-like protein (paper III and IV)**

Previous studies have suggested that serine proteinases are involved in invertebrate innate immune response. Several clones

encoding putative serine proteinases were isolated from a crayfish hemocyte cDNA library. One of these clones is a crayfish masquerade-like protein. This protein has a similar primary structure to *Drosophila melanogaster* masquerade (mas) (Murugasu-Qui *et al.*, 1995), a serine proteinase homologue expressed during embryogenesis and larval and pupal development.

Typical structure of serine proteinases, such as the catalytic triad, a substrate binding pocket, the position of six conserved cysteine residues and putative activation site of the proenzyme, are present in the mas-like protein except that a serine residue is replaced by glycine in the catalytic triad resulting in a protein without proteinase activity.

The crayfish mas-like protein consists of a modified serine proteinase domain, seven disulfide-knotted motifs, and a repeated glycine-rich region present in the N-terminal half. Several serine proteinase homologues have already been identified in vertebrates and invertebrates. Although the proteins have no enzyme activity, they have been suggested to have different biological functions such as; a cell adhesion activity, antimicrobial activity, LPS binding activity, to function as a growth factor, and a component of proPO system (paper III; IV; Murugasu-Qui *et al.*, 1995; Olson *et al.*, 1990; Barthalay *et al.*, 1990; Almeida *et al.*, 1996; Kawabata *et al.*, 1996; Nakamura *et al.*, 1989; Kwon *et al.*, 2000; Dimopoulos *et al.*, 1997).

The mas-like protein could also be purified by its *E.coli* binding property. The mas-like protein recognized formaldehyde treated gram-negative bacteria, *i.e.* *E. coli*, *P. vulgaris*, and *S. flexneri* as well as yeast, *Saccharomyces cerevisiae*, whereas it does not bind to formaldehyde fixed gram-positive bacteria.

The intact mas-like protein is present in crayfish hemocytes as a heterodimer composed of two subunits with molecular masses of 134 kDa and 129 kDa. Under reducing conditions the molecular masses of the intact proteins are not changed. The 134 kDa and 129 kDa proteins are isomers encoded by one cDNA, and these masses are higher than predicted from the open reading frame, *i.e.* 98.8 kDa, and it is therefore possible that the difference in molecular masses is a result of

glycosylation. After binding to bacteria or yeast cell walls, the mas-like protein is processed by a proteolytic enzyme, which cleaves at a specific site, LDL/YR.

The 134 kDa of the processed protein yields four subunits of 65 kDa, 47 kDa, 33 kDa, and 29 kDa and the 129 kDa protein results in four subunits of 63 kDa, 47 kDa, 33 kDa, and 29 kDa in 10 % SDS-PAGE under reducing conditions. Obviously, the 65 kDa and 63 kDa subunits are not completely processed into the 47 kDa and 29 kDa subunits, since these two molecules always exist in a fairly high concentration. Moreover, the 65 kDa and 63 kDa subunits showed identical N-terminal amino acid sequences, but have slightly different molecular masses on SDS-PAGE. We propose that glycosylation or cleavage of the C-terminal part might give rise to this difference in masses. The 47 kDa and 33 kDa subunits also have different masses from the calculated masses 31.6 kDa and 29.8 kDa, respectively, since they have glycosylation sites and high amount of cysteines. The intact form of the mas-like protein contains seven putative disulfide-knotted motifs and two of these disulfide motifs are involved in inter-disulfide bonding.

A 27 kDa subunit of the mas-like protein can be purified using a high concentration of calcium ion buffer and an affinity column with the anti-mas-like protein antibody. It could be detected by immunoblot analysis using an affinity-purified antibody against a synthetic peptide in the C-terminal domain of 27 kDa (paper III). The 33 kDa subunit was also detected using the same antibody when this protein was purified by its *E. coli* binding property using crayfish phosphate-buffered saline without calcium ions. The 29 kDa subunit (a part of 65 kDa and 63 kDa subunit) as well as other subunits were not detected by this anti-mas-like protein antibody, since the antibody was made to a synthetic peptide, corresponding to a portion of the 27 kDa subunit (paper III and IV). Interestingly, the N-terminal amino acid sequences of 27 kDa and 33 kDa subunit exhibit identical sequences showing that the 27 kD and 33 kDa subunits are the same protein. Therefore, high concentration of calcium ions seems to cause a conformational change of the protein or alternatively the protein may be cleaved differently at the C-terminus.

The 27 kDa mas-like protein exhibited cell adhesion activity *in vitro*, indicating that the C-terminal domain of the crayfish mas-like protein mediates cell adhesion, whereas the two intact proteins, 134 kDa and 129 kDa, have binding activity to LPS, glucans, gram-negative bacteria, and yeast. The processed form of the mas-like protein was unable to bind any microorganism. The processing of the mas-like protein is similar to that of human hepatocyte growth factor (Nakamura *et al.*, 1989) (HGF), which also has a serine proteinase-like domain. This protein is processed to a mature form by cleavage with an unknown trypsin-like enzyme (Nakamura *et al.*, 1989).

*E. coli* coated with the mas-like protein were more rapidly cleared in crayfish than only *E. coli* suggesting this protein is an opsonin. Therefore, the cell adhesion and opsonic activities of the mas-like protein, which will greatly accelerate the rate of phagocytosis suggest that it plays a role as an innate immune protein.

## CONCLUSIONS

The aim of this work is to understand innate immune responses in crayfish. Therefore, several related proteins were purified and characterized both using molecular and biological methods and their functions investigated.

Invertebrates do not contain antigen-specific lymphocytes and do not produce immunoglobulin molecules. However, they contain a number of soluble molecules that bind to and lyse microorganisms. Typical such molecules are lectin-like proteins, which bind to carbohydrates present on microbial cell walls and hence initiate several immune responses as well as agglutinate the invading microorganisms. Invertebrates also produce numerous antimicrobial peptides.

Prophenoloxidase activating system (proPO system) is an innate immune system of invertebrates and is activated by microbial cell wall components such as LPS, peptidoglycan, or  $\beta$ -1,3-glucan recognized PRP. Prophenoloxidase (proPO) is an essential enzyme for producing melanin and is activated by the prophenoloxidase activating enzyme (ppA).

The ppA was purified and cloned from crayfish hemocyte and has been shown to have serine proteinase activity. Arthropod ppAs have a clip-like domain as a common structure and this clip-domain might have antimicrobial activity, because their structure shows high homology to horseshoe crab big defensin and human  $\beta$ -defensin. The clip-like domain within crayfish proppA is the first, which has been expressed and shown to exhibit antibacterial activity *in vitro* against gram-negative bacteria. Although low concentration of antibacterial fragments is generated from a proppA *in vivo*, it may be of importance at localized places where the proPO system is activated by invading microorganisms, for example in melanized nodules. This suggests that arthropod proppAs have a dual function, that is, the activation of proPO and antibacterial activity. However, further investigations need to be done to understand the dual function of proppA *in vivo* and the interaction between the other domains in the N-terminal half, the proline- and glycine-rich domain.

For activation of the proPO system, LPS,  $\beta$ -1,3-glucan, and peptidoglycan binding/recognition proteins recognize common structures of carbohydrates in microorganisms. Several carbohydrate recognition proteins were reported to be involved in the activation of proPO system. Crayfish LGBP was also demonstrated to have a binding activity to LPS and  $\beta$ -1,3-glucan, and hence to play a role as initiator of proPO system. However, the relationship between LGBP and proppA is yet unknown in arthropods. It has to be investigated in more detail whether LGBP can directly activate proppA or if LGBP interacts with another proteinase to activate proppA in the proPO system. Thus an important future study would be to isolate and characterize the remaining proteinases involved in the proPO system.

The crayfish mas-like protein is also characterized as a PRP. The processing of crayfish mas-like protein is interesting and the processed form of mas-like protein was purified by its *E. coli*-binding activity in crayfish hemocyte. The mas-like protein exists in an intact form, but while the mas-like protein binds to microorganisms, it is processed by an unknown proteolytic enzyme. It would be interesting to see which proteinase is involved in the processing of the mas-like protein and the role of its other subunits after cleavage.

After processing, the mas-like protein was separated into 5 subunits (65 kDa, 63 kDa, 47 kDa, 33 kDa, and 29 kDa) on SDS-PAGE. Interestingly, 33 kDa subunit changed its molecular mass to 27 kDa under a high calcium concentration. This suggests that a calcium ion might effect protein conformational change and the processing of the mas-like protein. It would be interesting to investigate the effect of calcium concentration on the mas-like protein processing. The 27 kDa protein has cell adhesion activity, whereas the intact mas-like protein has binding activity to various gram-negative bacteria, LPS, yeast, and  $\beta$ -1,3-glucan.

An interesting future study would also be to reveal whether any proteinase of the proPO system is involved in the cleavage of Spätzle resulting in induction of the antifungal peptide, drosomycin in *Drosophila*. It seems logic that there is some connection between the

proPO system and induction of antibacterial synthesis, since the proPO system is so sensitive to activation by microbial polysaccharide. In crayfish for example this proPO system can be activated by picogram of LPS or  $\beta$ -1,3-glucans making this system extremely sensitive to microbial polysaccharides.

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