THESIS

CELLULAR IMMUNE REACTION IN RESPONSE TO WHITE SPOT SYNDROME VIRUS (WSSV) INFECTION IN FRESHWATER CRAYFISH, *Pacifastacus leniusculus*

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GRADUATE SCHOOL, KASETSART UNIVERSITY

2005

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Agricultural Biotechnology)
DEGREE

Agricultural Biotechnology
FIELD

Interdisciplinary Graduate Program
PROGRAM

TITLE: Cellular Immune Reaction in Response to White Spot Syndrome Virus (WSSV) Infection in Freshwater Crayfish, *Pacifastacus leniusculus*

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THESIS

CELLULAR IMMUNE REACTION IN RESPONSE TO WHITE SPOT SYNDROME VIRUS (WSSV) INFECTION IN FRESHWATER CRAYFISH,
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A Thesis Submitted in Partial Fulfilment of
the Requirements for the Degree of
Doctor of Philosophy (Agricultural Biotechnology)
Graduate School, Kasetsart University
2005

ISBN 974-16-1037-8

Siripavee Sricharoen 2005: Cellular Immune Reaction in Response to White Spot Syndrome Virus (WSSV) Infection in Freshwater Crayfish, *Pacifastacus leniusculus*. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Mr. Suriyan Tunkijjanukij, Dr. Scient. 87 pages.

ISBN 974-16-1037-8

Degranulation of crayfish granular cells was enhanced by the Ca^{2+} ionophore A23187, lipopolysaccharide-peptidoglycan (LPS-PGN), and peroxinectin. Nine proteins were released upon Ca^{2+} ionophore A23187 stimulation. Six of them were characterized and found to be a masquerade-like protein (cMas I), a masquerade-like serine proteinase (cMas II), a mannose receptor protein (MRP), a vitelline membrane outer layer protein I (VMO-I), and two antimicrobial peptides (AMPs). Seven and six proteins were found when using peroxinectin and LPS-PGN as triggers, respectively, and all of them were also released by the Ca^{2+} ionophore A23187 treatment.

White spot syndrome virus (WSSV) has caused mortality in a variety of crustaceans, including shrimp, crabs and crayfish. WSSV-injected crayfish showed similar symptoms as infected shrimp, but no appearance of white spots on the cuticle or reddish body color were observed. WSSV interaction with crayfish hemocytes, the semigranular cells (SGCs) were found to be more susceptible to WSSV than granular cells (GCs). Infected GCs were resistant to degranulation induced by phorbol 12-myristate 13-acetate (PMA) or hemocyte lysate supernatant (HLS), but not the Ca^{2+} ionophore A23187. Therefore, the protein kinase C (PKC) pathway might be affected by WSSV during its replication inside the cells. It was also found that WSSV inhibited the proPO system and apoptosis in hemocytes. WSSV might produce some anti-apoptotic factors during its infection cells. However, the mechanism of this phenomenon has still to be elucidated.

Siripavee Sricharoen 27 / Dec / 2005

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I wish to express my gratitude to all of my supervisors, Assist. Prof. Dr. Suriyan Tunkijjanukij, Assist. Prof. Dr. Supawadee Poompuang, and Assist. Prof. Dr. Julapark Chunwongse of Kasetasart University (KU) for theirs encouragements, comments and guidance for my study. Sincerely thanks to Prof. Kenneth Söderhäll, who have provided the stimulation and invaluable help to perform my research work throughout one and half year at Comparative Physiology Department (CPD), Evolutionary Biology Center (EBC), Uppsala University, Sweden. Particularly thanks for his spending time on correcting my papers that published in international journals, accordingly.

I also wish to thank Dr. Irene Söderhäll, Comparative Physiology Department, for her advice to start with crayfish innate immunity, cloning, sequencing, especially for her kindly discussions, suggestions and critical reading of the manuscripts.

Thanks go to my Thai friends in Uppsala, Dr. Paiboon Nuannin who always makes me enjoy my life with delicious traditional Thai food, unforgettable trips as well as valuable advisory, Dr. Pikul Jiravanichpaisal for her care and help since I arrived Uppsala until returned home, my lovely friends (Nuch, Kaew, Pee Addy, Pee Rat and Pee Ruk), and all unnamed friends in Thailand for their cooperation and friendship.

I also would like to thank to Ragnar Ajaxon, Anbar Khodabandeh, and Maria Lind Karlberg who taught me about laboratory techniques and all unnamed kindly friends at people at CPD for theirs support and warm hospitality during my stay in Uppsala.

Special thanks to Khun Kowit Charoenwattanasak, who always being with, taking care of my life, giving me a wonderful time, and never say no whatever I need to be.

Finally, I wish to express my deepest appreciation to my mother and my lovely family for theirs inspiration, thoughtfulness and patience whilst I have worked on my research. And the most important person, Mr. Promma Sricharoen, who never dies from my heart, is my best father.

The scholarship for me was funded by Faculty of Agriculture, KKU and Center for Agricultural Biotechnology (CAB), KU, Thailand. The work was financially supported by The Swedish Science Research Council and Formas through Prof. Kenneth Söderhäll.

Siripavee Sricharoen
December 2005

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Cont'd)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP	=	Antimicrobial peptide
βG	=	*β*-1,3-glucan
βGBP	=	*β*-1,3-glucan binding protein
cMas I	=	Crayfish Masquerade-like protein I
cMas II	=	Crayfish Masquerade-like protein II
CFS	=	Crayfish saline
GCs	=	Granular cells
GNBP	=	Gram-negative bacteria binding protein
HC	=	Hyaline cells
HLS	=	Hemocyte lysate supernatant
LBP	=	Lipopolysaccharide binding protein
LPS	=	Lipopolysaccharide
LPS-PGN	=	Lipopolysaccharide-peptidoglycan
MRP	=	Mannose receptor protein
PAMP	=	Pathogen associated molecular pattern
PGN	=	Peptidoglycan
PGBP	=	Peptidoglycan binding protein
PKC	=	Protein kinase C
PMA	=	Phorbol 12-myristate 13-acetate
Prnx	=	Peroxinectin
PO	=	Phenoloxidase
ppA	=	Prophenoloxidase activating enzyme
PPAF	=	Prophenoloxidase activation factor
proPO	=	Prophenoloxidase
PRR	=	Pattern recognition receptor
SGCs	=	Semigranular cells
TEM	=	Transmission electron microscopy
VMO-I	=	Vitelline membrane outer layer protein I
WSSV	=	White spot syndrome virus

CELLULAR IMMUNE REACTION IN RESPONSE TO WHITE SPOT SYNDROME VIRUS (WSSV) INFECTION IN FRESHWATER CRAYFISH, *Pacifastacus leniusculus*

INTRODUCTION

Farming of freshwater crayfish is growing in popularity around the world. Currently, the major producers are China and USA. The main species is the redswamp crayfish, *Procambarus clarkii*, which is native to USA and used in crayfish culture throughout the world. In Europe, native freshwater crayfish such as the noble crayfish (*Astacus astacus*) and the introduced signal crayfish (*P. leniusculus*) are produced in semi-intensive aquaculture systems, natural lakes and waterways. Marron (*Cherax tenuimanus*), yabby (*C. destructor*) and the red claw (*C. quadricarinatus*) are Australian freshwater crayfish species, which have shown high potential for semi-intensive aquaculture in many countries. For example, in China current annual production in 2001 was up to 1000 tones (Edgerton, 2004).

For crustacean aquaculture, disease is the most serious problem and this affects the population by killing animals or reducing their ability to breed. Although little is known about viral infections in crustaceans, white spot syndrome virus (WSSV) is known to be highly pathogenic to freshwater crayfish. The virus has caused massive losses in shrimp aquaculture around the world (Lightner *et al*., 1997). Several prevention methods including screening for the presence of virus in hatchery stocks, eliminating potential vectors, and reducing environmental stresses, have been employed to control the spread of the diseases. In the long run, however, the most effective way to ensure sustainability and development of crustacean aquaculture will depend on the selection of disease resistant animals. Molecular techniques provide powerful tools to study genetic control of defense and resistant mechanisms at the DNA level. Within this field, crustacean immunology and biotechnology applications would be key elements in developing new approaches for control of disease in aquatic farming industry.

Freshwater crayfish, *P. leniusculus*, as in invertebrates, lack adaptive immune response, therefore they have to rely solely on innate immune mechanisms. The prophenoloxidase (proPO) system is a very efficient non-self recognition system in invertebrates which can recognize and respond to picograms per liter of lipopolysaccharides (LPS) from bacteria or β-1, 3-glucans from fungi showing that the recognition of foreign matter in invertebrates is much more efficient than in vertebrates. If a parasitic organism succeeds in gaining entry into the body cavity, specific pattern recognition proteins in the hemolymph will bind to polysaccharides from the invading microbe. These complexes will bind to specific membrane receptors on the hemocytes and cause an exocytotic release of the proPO system. Outside the cell, the proPO system will be activated and as a result several biological factors will be produced which will participate in the immune defence. The structure and function of the components of the proPO system have been studied in greatest detail in crayfish. The proPO system is contained within secretory granules in semigranular and granular hemocytes. These granules also loaded with various antimicrobial peptides and proteinase inhibitors, the secretion of which can be induced by treatment with LPS or the endogenous "degranulation" cell adhesion protein peroxinectin.

The purpose of this work will focus on characterization of different proteins released from granular hemocytes upon immune challenge by various activators and by WSSV infection in freshwater crayfish.

OBJECTIVES

1. To determine the secretion pattern of proteins released from crayfish granular hemocytes upon immune challenge by Ca^{2+} ionophore A23187, bacterial lipopolysaccharides and peroxinectin.

2. To characterize proteins and to clone genes related to the released proteins from granular hemocytes of crayfish after treatment with Ca^{2+} ionophore A23187, peroxinectin and lipopolysaccharides.

3. To investigate the effects of WSSV on circulating hemocytes of freshwater crayfish.

LITERATURE REVIEW

Crayfish Immunity

Invertebrates, including arthropods, lack adaptive immunity, instead, they have a very sensitive innate immune system. The innate immune system includes both cellular and humoral mechanisms. Crayfish have a hard, wax-covered cuticulum serving as a first mechanical barrier to intruders, in addition to their rapidly responding and sensitive innate immune system. Cellular immune responses are mediated by the hemocytes in the hemolymph, and are important in early non-self recognition. The cellular immune responses consist primarily of phagocytosis of small microbes, encapsulation of larger parasites and nodule formation (Ratcliff *et al*., 1985; Johansson and Söderhäll, 1989). The humoral response involves coagulation and exocytosis of different proteins to the plasma, for example, components of the proPO activating cascade, leading to opsonization and melanization, (Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004), and synthesis of antimicrobial peptides (Dimarq *et al*., 1997).

Recognition of the microbe occurs through pattern recognition molecules in the hemolymph of invertebrates (Janeway, 1989; Söderhäll and Cerenius, 1998). Microbial molecules, such as β-1, 3-glucans from fungi (Ochiai and Ashida, 1988; Söderhäll *et al*., 1988), lipopolysaccharides (Sun *et al*., 1990), peptidoglycans and lipoteichoic acids from bacteria (Yosida *et al*., 1996; Ochiai and Ashida, 1999) and double-stranded RNA from virus, are targets for the pattern recognition proteins. Presence and recognition of these microbial molecules allow the host to choose a defence mechanism optimised against a certain class of pathogens (Medzhitov and Janeway, 2000). The biological roles of pattern recognition proteins in crayfish are to trigger the proteinase cascade, the pro-PO system and subsequent clearance of microbial invaders from the hemolymph.

The Prophenoloxidase Activating System (proPO System)

The proPO activating system localized inside the circulating hemocytes is a recognition system that can be activated by microbial cell wall components, such as β-1, 3-glucans (βG), lipopolysaccharides (LPS) and peptidoglycans (PGN). These structures are recognized by a group of germ-line encoded receptors, usually termed pattern recognition receptors (PRR). When βG binding protein binds to a βG, it becomes activated and can bind to a cell-surface associated protein, a superoxide dismutase (SOD) on the semigranular cells (SGCs) and granular cells (GCs) (Johansson *et al*., 1999). The recognition of nonself leads to degranulation of SGCs and GCs. Phenoloxidase activating enzyme (proppA) is released which becomes activated by the presence of pathogen-associated molecular patterns (PAMPs). The proPO is converted to phenoloxidase (PO) by the active phenoloxidase activating enzyme (ppA) and leading to the synthesis of melanin, which can be seen as dark spots in the cuticle of crustaceans (Figure 1) (Cerenius and Söderhäll, 2004). Since the first cloning of an invertebrate proPO from the freshwater crayfish *P. leniusculus* in 1995 (Aspán *et al*., 1995), proPO has been cloned from about 20 different arthropod species, such as for example, *Sarcophaga bullata* (Chase *et al*., 2000), black tiger shrimp *Penaeus monodon* (Sritunyalucksana *et al*., 1999), malaria mosquito *Anopheles strephensis* (Cui *et al*., 2000), parasitoid wasp *Pimpla hypochondriaca* (Parkinson *et al*., 2001), and *Drosophila melanogaster* (Asada *et al*., 2003).

In freshwater crayfish, the proPO system is stored in the granules of the hemocytes and released into the plasma by a regulated exocytosis (Johansson and Söderhäll, 1985). Corresponding to this study, induced exocytosis of crayfish granular cells by LPS-PGN, Ca^{2+} ionophore A23187, and peroxinectin and nine proteins were released in large amount. Six of the nine proteins were characterized and one of them was found to be a masquerade-like serine proteinase (cMas II) (Sricharoen *et al*., 2005). This protein, cMas II, is similar to several masquerade-like serine proteinases in insects such as that of *Tenebrio* prophenoloxidase activating factor (Lee *et al*., 2002), *Bombyx* masquerade-like serine proteinase homologue, *Holotrichia* prophenoloxidase activating factor-2 (PPAF-2) (Lee *et al*., 1998), *Manduca* serine proteinase-like protein-1(Yu *et al*., 2003) and *Anopheles* serine protease like protein Agmas1 (Cui *et al*., 2000). The masquerade-like protein is a pattern recognition molecule, which has a primary structure similar to serine proteinases. Serine proteinases belong to a diverse multigene family that shares a common catalytic mechanism and structural characteristics such as the presence of three conserved amino acid residues, His, Asp, and Ser, within the active site. In animals, these enzymes are involved in several biological processes, including digestion, proenzyme, prohormone, and complement activation, as well as participate in defense mechanisms (Neurath, 1986). Serine proteinases are typically synthesized as zymogens or inactive proenzymes, which then will induce activation by a specific and limited proteolytic cleavage at a specific peptide bond (Furie and Furie, 1988). The N-terminal domain of the protein has been shown to be important in the activation and also may play a key role for the normal regulation of enzymatic activity (Smith, *et al*., 1994). Several serine proteinase-inactive homologues have been identified in animals and have been shown to have different biological functions, for example, a cell adhesion activity, *e.g*. *Pacifastacus* masquerade (mas)-like protein (Huang *et al*., 2000) and *Drosophila* mas (Murugasu *et al.*, 1995), antimicrobial and LPS binding activities, *e.g*., human azurocidin (Almeida *et al*., 1991) and horseshoe crab factor D (Kawabata *et al*., 1996), a component of proPO system, *e.g*., 45-kDa serine proteinase homologue protein of coleopteran insect *H. diomphalia* (Kwon *et al*., 2000). These molecules show homology to serine proteinases except for a substitution of usually one of the catalytic residues. The proPO system, an enzyme cascade, which carries out recognition and defense responses in invertebrates, is composed of several serine proteinases and their inhibitors and terminates with the zymogen, prophenoloxidase (Söderhäll and Cerenius, 1998). The active form phenoloxidase is produced by serine proteinase such as the proPO activating enzyme. Several insect serine proteinases have been found to be involved in the activation of the proPO system (Söderhäll and Cerenius, 1998), such as the masquerade-like serine proteinase homologues from *Tenebrio* (Lee *et al*., 2002) and *Holotrichia* (Kwon *et al*., 2000), named prophenoloxidase activating factors (PPAFs).

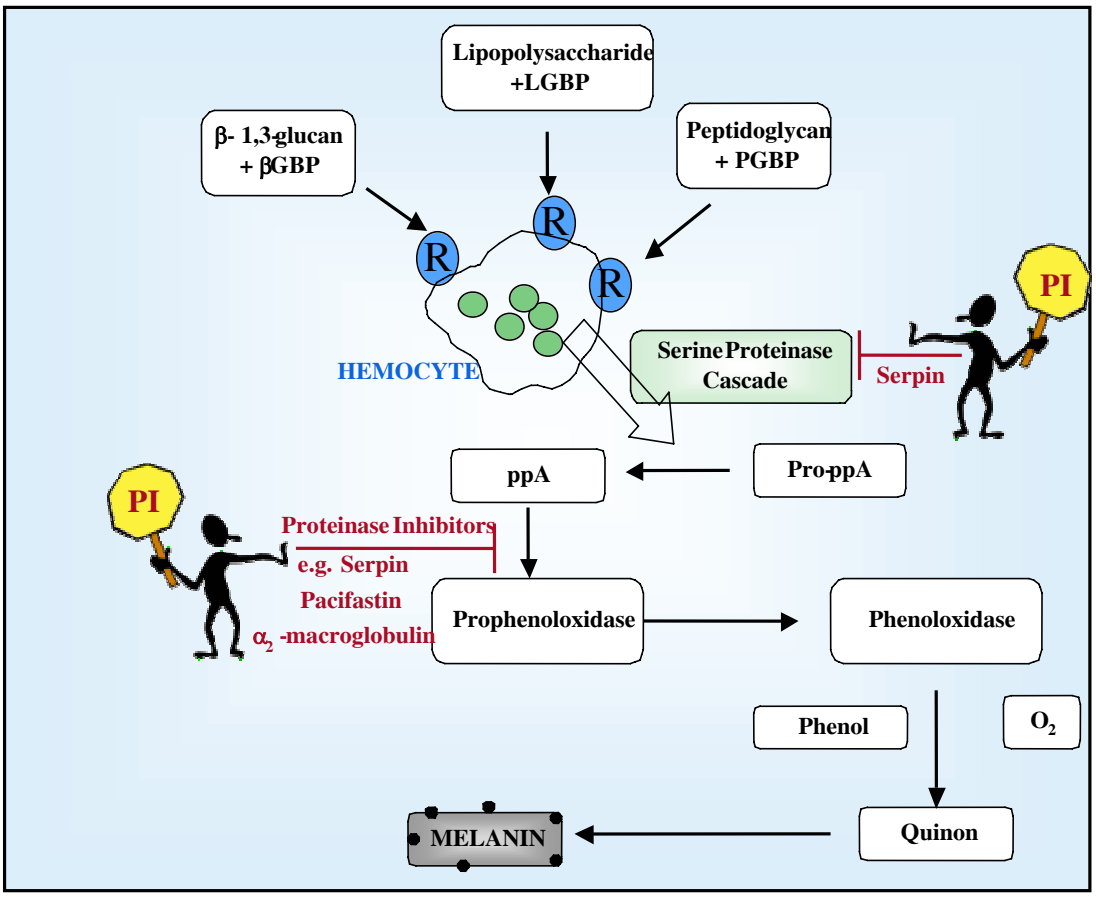


Figure 1 Overview of the arthropod prophenoloxidase (proPO) activating system. The system is activated by pattern-recognition proteins bound to β-1, 3-glucans, lipopolysaccharides, peptidoglycans (i.e. βGBP, LGBP, and PGBP) or by other compounds such as endogenous factors produced upon tissue damage. A cascade of serine proteinases, which are not yet fully characterized, will result in the cleavage of the pro-form of the prophenoloxidase-activating enzyme (pro-ppA) into active ppA. The box containing serine proteinase homologs is dotted, as some ppAs require them and some ppAs are capable of cleaving proPO directly into active PO.

Peroxinectin

Peroxinectin, a 76-kDa cell adhesion factor, was the first cell adhesion protein isolated from invertebrate blood (Johansson and Söderhäll, 1988). Besides having cell adhesion and peroxidase activity, crayfish peroxinectin also acts as a degranulation factor, an encapsulation-promoting factor and an opsonin (Johansson and Söderhäll, 1988; Thörnqvist *et al.*, 1994; Johansson *et al.*, 1995). Crayfish peroxinectin is synthesized and stored, in an inactive form, within granular and semi-granular haemocytes. It is released and activated concomitant with activation of the proPO activating system (Johansson and Söderhäll, 1988).

Peroxinectin structurally belongs to a family of heme-containing animal peroxidases, including human myeloperoxidase, which is present in high concentration in granules of neutrophiles (Everse *et al.*, 1991). Myeloperoxidase was found to in addition to produce microbicidal agents (Klebanoff, 1988) also to support cell adhesion of leukocytes (Johansson *et al*., 1997). Myeloperoxidase can also trigger attachment, spreading and degranulation of crayfish blood in the same manner as crayfish peroxinectin (Johansson *et al*., 1997). The sequence of human myeloperoxidase (Morishita *et al*., 1987) and peroxinectin are similar, and 32 % of the residues are identical (Johansson *et al*., 1997).

Peroxinectin-like proteins have been found in other arthropods than crayfish. From blood cells of black tiger shrimp (*P. monodon*), a cDNA clone with 69 % amino acid identity to crayfish peroxinectin has been found (Sritunyalucksana *et al*., 2001). This peroxinectin is also shown to have cell adhesion properties to shrimp hemocytes. The cell adhesion activity of shrimp peroxinectin was generated upon activation of the proPO system in shrimp, suggesting that the protein is a proPO system activated protein. Several peroxidase isozymes have been described in *Drosophila melanogaster,* one of these, peroxidasin, is shown to be present as an extracellular matrix protein in the larval hematopoietic lymph gland (Nelson *et al.*, 1994) and was proposed to participate in extracellular matrix consolidation and in defence mechanisms (Nelson *et al*., 1994; Lanot *et al.*, 2001).

Peroxinectin was first characterized and cloned from the hemocytes of crayfish *P. leniusculus* (Johansson *et al*., 1995) and recently from black tiger prawn *P. monodon* (Sritunyalucksana *et al*., 2001) and white shrimp *L. vannamei* (Liu *et al*., 2004). A peroxinectin-like gene (*Dpxt*) has been sequenced in *Drosophila* and it has a putative RGD-integrin binding tripeptides. Like peroxinectin, the *Dpxt* predicted protein has two different domains: one is 17% identical to the crayfish peroxinectin residues 1-230 and the other is a hemoperoxidase-like domain, which has peroxidase activity and 31.9 % identity to crayfish peroxinectin and it may be involved in cell adhesion processes (Vázquez *et al*., 2002).

Table 1 Arthropod blood cell adhesion molecules

Species (protein)	Cloned	Cell adhesion activity	Reference
Carcinus maenus	No	Yes[a]	Thomqvist *et al*. (1994)
***Bombyx mori* (Hemocytin)**	Yes	Yes[c]	Kotani *et al*. (1995)
Penaeus paulensis	No	Yes[b]	Perazzolo and Baracco (1997)
***Psudomonas includens* (Plasmatacyte spreading peptide)**	Yes	Yes[a]	Clark *et al*. (1997)
***P. leniusculus* (Peroxinectin)**	Yes	Yes[a]	Johansson and Söderhäll (1988)
***P. monodon* (Peroxinectin)**	Yes	Yes[b]	Sritunyalucksana *et al*. (2001)
***Drosophila melanogaster* (Peroxinectin –related precusor)**	Yes	Yes[a]	Vázquez *et al*. (2002)
***L. vannamei* (Peroxinectin)**	Yes	Yes[a]	Liu *et al*. (2004)

[a] Cell adhesion activity detected from purified protein.
[b] Cell adhesion activity detected in hemolymph
[c] Cell adhesion activity detected in recombinant protein expressed in baculovirus vector.

Antimicrobial Peptides

The production of antimicrobial peptides is an important and widespread mechanism of host defence throughout the living kingdom, present in bacteria, plants, and invertebrates to vertebrates (Boman, 1995; Hoffmann *et al*., 1999). These peptides were initially characterized in insects as small, cationic molecules involved in the innate immune system. The cationic molecules are still the largest group of antimicrobial peptides and so far, over 400 peptides from this group have been characterized, primarily from insects. The cationic antimicrobial peptides have been grouped into four distinct classes according to their primary and secondary structures (Hétru *et al*., 1998):

1. Linear peptides forming amphipatic α-helices and deprived cystein residues, for example cecropin and magainin. The cytotoxic effect of cecropins has been attributed to their ability to interact with lipid membranes to form channels of different sizes. They have also been shown to inhibit protein import and respiration (Bartlett *et al*., 2002).

2. Cystein-rich peptides with intramolecular disulfide bridges, for example drosomycin and defensin. Drocomycin is only found in *Drosophila* but defensin is widely distributed throughout divergent taxa. The defensins display a rapid bactericidal effect involving pore formation.

3. Proline-rich peptides, for example drosocin and apidaecin. Most of the proline-rich peptides are active against gram-negative bacteria and appears to involve stereospecific recognition of cellular targets.

4. Glycine-rich peptides or polypeptides, such as diptericin and attacin. These peptides are mainly active against Gram-negative bacteria by inhibiting the synthesis of outer membrane proteins, thus increasing the permeability of the outer membrane.

In addition to the cationic peptides these groups of non-cationic antimicrobial peptides have been identified (Lee *et al*., 2003):

1. The anionic peptides, for example peptide B and dermcidin.
2. The aromatic dipeptides, for example *p*-Hydroxycinnamaldehyde.
3. Peptides derived from oxygen-binding proteins, represented by lactoferrin and Astacidin 1.

The anionic antimicrobial peptides were generally isolated from mammalian epithelia. Hemocyanin derivatives, the first representatives of the peptides from oxygen-binding proteins have been isolated from hemolymph of shrimp and crayfish (Destoumieux-Garzon *et al*., 2001; Lee *et al*., 2004).

Inaddition, a new family of antimicrobial peptides named penaeidins were characterized and cloned from the hemocytes of shrimp *L. vannamei* (Destomieux *et al*., 1997). These peptides are ubiquitous in crustaceans. Originally, the penaeidin family of antimicrobial peptides consist of three distinct classes (1-3) with multiple isoforms within class 3 (Destomieux *et al*., 1997), but now a novel class, designated as penaeidin 4 have been described in two shrimp species, *L. vannamei* and *L. setiferus* (Cuthbertson *et al*., 2002). The penaeidins display both antifungal and anti-Gram positive bacterial activities (Destoumieux *et al*., 2000) and chitin binding properties (Bachère *et al*., 2000). The penaeidins are synthesized and stored within granules of the circulating haemocytes of shrimp. The response to microbial challenge results in an increase of the plasmatic penaeidin concentration with a subsequent binding of the peptides to cuticular epithelia (Destomieux *et al*., 2000). It is assumed that the dual functions of penaeidin, chitin-binding property and antimicrobial activity, may be important in chitin assembly and wound healing, and in shrimp protection in developmental and molting stages.

The production of antimicrobial substances has been intensively studied, mostly in *Drosophila*. The *Drosophila* antimicrobial peptides are active against both Gram-positive bacteria (drosomycin, defensin and metchnikowin) and Gram-negative bacteria (diptericin, drococin, attacin and cecropin) and against fungi (drosomycin and metchnikowin). These peptides are synthesized by the fat body (equivalent to the vertebrate liver) and secreted to the hemolymph. The genes coding for these antimicrobial peptides are regulated by at least two distinct pathways, the Toll and Imd (immune deficiency) pathways, reviewed in Imler and Hoffmann (2000) and Hoffmann and Reichart (2002).

In crustaceans, hemolymph antimicrobial activities have been demonstrated in different species. Antibacterial peptides and polypeptides have been isolated from the hemocytes of the green crabs, *Carcinus maenas* and blue crabs, *Callinectes sapidus* (Schnapp *et al*., 1996; Relf *et al*., 1999) and freshwater crayfish, *P. leniusculus* (Lee *et al*., 2003, 2004). In *C. maenas*, two constitutive hemocytic molecules with antimicrobial activities have been characterized, one a 6.5-kDa peptide rich in proline residues (Schnapp *et al*., 1996), and the other a cysteine-rich 11.5-kDa polypeptide, carcinin or crustin Cm1 (Relf *et al*., 1999). This peptide exhibits specific activity for only gram-positive and salt-tolerant bacteria (Relf *et al*., 1999). A putative homologue of the 11.5-kDa polypeptide has been characterized from the hemocytes of the Pacific white shrimp, *L. vannamei,* the Atlantic white shrimp, *L. setiferus* (Gross *et al*., 2001) and the Black tiger shrimp, *P. monodon* (Chen *et al*., 2004). The full nucleotide sequences have been determined for several isoforms that appear in both shrimp species and these new families are called the *crustins* (Bartlett *et al*., 2002). Like the 11.5-kDa from *C. maenas,* crustins from shrimp show no homology with other known antibacterial peptides, but do have sequence identity with members of the Whey Acidic Protein (WAP) family of proteinase inhibitory proteins.

Proteinase inhibitors in the hemolymph may defend the host against microbial proteinases and are necessary in the regulation of many immune-related cascades, such as coagulation and the proPO cascade (Bartlett *et al*., 2002). Previously, it was reported that crayfish hemocyanin could be processed to produce an antibacterial peptide, astacidin 1 (Lee *et al*., 2003). Astacidin 1 was expressed in the crayfish hepatopancreas and its expression was induced during bacterial infection. Hemocyanin is a blue copper-containing oxygen-transporting molecule and is the predominant protein in the plasma of many crustaceans. A structurally similar protein, the molting protein cryptocyanin, has been identified in crab (*Cancer magister*). During the molt cycle it is shown that both the level of cryptocyanin and hemocyanin fluctuate, with a peak during the late premolt (Terwilliger *et al*., 1999).

Hemocytes

The hemocytes are very important in the immune system, since they participate in recognition, phagocytosis, melanization, and cytotoxicity. In the freshwater crayfish, *P. leniusculus*, like in other crustaceans, three different circulating hemocytes can be identified and separated: hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs) (Söderhäll and Smith, 1983). The hemocytes carry out different functions in immunity. The hyaline cells are the main phagocytic cells. The semi-granular cells are involved in early non-self recognition, phagocytose, encapsulation and nodule formation. Semi-granular and granular cells contain the prophenoloxidase activating system including the cell adhesive and opsonic protein, peroxinectin (Söderhäll and Häll, 1984; Johansson and Söderhäll, 1988; Johansson *et al.*, 1995). Phagocytosis, encapsulation and nodule formation are the most common mechanisms of cell-mediated defense reactions. Phagocytosis involves the attachment of hemocytes to a parasite or an intruder followed by ingestion and destruction. Encapsulation occurs when a foreign particle is too large to be consumed by phagocytosis and then the hemocytes will collaborate by sealing off the foreign body. Nodule formation is a process similar to encapsulation, in which tissues become melanized due to the host's phenoloxidase activity (Söderhäll and Cerenius, 1992, 1998).

Degranulation or Exocytosis of Crayfish Blood Cells

The release of proteins from cellular vesicles to the external milieu by exocytosis in response to various stimuli is a basic process, found through out the animal kingdom (Rasmussen, 1970; Palade, 1975). It has been proposed that degranulation and the release of previously cell-bound factors are essential elements of a cellular reaction to foreignness in arthropods (Smith and Söderhäll, 1983a). Exocytosis from the blood cells would be a suitable way to deliver to the plasma the components of the prophenoloxidase (proPO) activating system.

The role of exocytosis in the cellular defence reactions of arthropods was investigated using *in vitro* cultures of isolated haemocytes (blood cells) from the freshwater crayfish and the shore crab *C. maenas* (Söderhäll *et al*., 1986). In the both species, activated lysates of those cell types that contained the prophenoloxidase activating system (granular cells of crab and crayfish and semigranular cells of crayfish) were found to induce degranulation of semigranular and granular cells. It is well established that, in arthropods, the defence of the host against invasive or opportunistic micro-organisms is affected principally through the phagocytic, encapsulating and agglutinating activities of the circulating haemocytes as well as by antimicrobial factors in the plasma (Gotz and Boman, 1985).

Recently, evidence has been presented that recognition of non-self and cellular activation in these animals may be mediated through the prophenoloxidase activating system of the blood. Degranulation has been observed in crayfish as a result of exposure of the haemocytes to either β -1,3-glucans (Smith and Söderhäll, 1983b) and or Lipopolysaccharides (Johansson and Söderhäll, l985) and it has recently been shown that the degranulation of crayfish haemocytes involves the secretion of the proenzymes proPO and protease (Johansson and Söderhäll, l985).

White Spot Syndrome Virus (WSSV)

WSSV is a rod-shaped, enveloped, non-occluded baculovirus (Wang *et al*., 1995) which contains a 305,107 bp double stranded circular DNA (Zhang *et al*., 2001). Although viruses have caused major losses in the farming of other aquatic animals, including shrimp, little is known about viral infections in crustaceans. To date, no virus has been associated with widespread, serious mortality in either wild or farmed freshwater crayfish populations. However, white spot syndrome virus (WSSV) is known to be highly pathogenic to freshwater crayfish (Richman, *et al*., 1997) and extremely pathogenic to many species of penaeid shrimp (*P. japonicus*, *P. monodon*, *P. semisulcatus* and *P. penicillatus*), and other crustaceans (Wang *et al*., 1998; Sahul Hameed *et al*., 1998, 2001). WSSV can cause up to 100% mortality in *P. monodon* ponds 2-7 days after infection (Chou *et al*., 1995). A clinical sign of WSSV infection is discoloration and spot marking of the exoskeleton, primarily on the carapace and occasionally on the chelipeds of *Orconectes punctimanus* and *Procambarus sp*. (Richman *et al.*, 1997). Characteristic small white spots develop on the inside surface of the cuticle of the carapace and appendages of infected prawns and the bodies often turn red in color (Chou *et al*., 1995). WSSV-infected freshwater crayfish *P. leniusculus* showed similar symptoms as infected prawn, but without the appearance of white spots on the cuticle or reddish body color

(Jiravanichpaisal *et al*., 2001). WSSV infects hemocytes and other tissues of mesodermal and ectodermal origin.

In situ hybridization techniques and PCR methods have been developed for WSSV diagnostic test (Wongteerasupaya *et al*., 1996). Shi *et al*. (2005) demonstrated that WSSV replicated only in the connective tissue cells that surround the lymphoid organ tubules, whereas the hemocytes within the lymphoid organ tubules did not support WSSV replication in the crayfish *P. clarkii*. WSSV particles were phagocytosed by the hemocytes of *P. clarkii* but were not destroyed by the cell defence systems even when the cells were finally autolysed. Jiravanichpaisal *et al*. (2001) has also reported that the epithelial cells of tubules in the hepatopancreas were not affected in WSSV infected crayfish *P. leniusculus* even in the last stage of infection. WSSV-infected crayfish showed a high number of granular cells until the last period of infection. There are several possible explanations for this event. Firstly, the granular cells are more resistant to viral infection than semi-granular cells. Secondly, WSSV may affect hematopoietic tissue development and lead to changes in the proportions of different circulating blood cell types.

Two species of freshwater crayfish *P. leniusculus* and *A. astacus* were found to be susceptible to WSSV in which high mortality was observed at high temperature. The proliferation of the hematopoietic tissue at high temperatures may support replication of WSSV and cause mortality in WSSV infection at high temperature (Jiravanichpaisal *et al*., 2004).

WSSV infection can trigger the host cell to activate its genetically programmed cell death pathway, leading to the morphological features of apoptosis. It has been suggested that apoptosis may be the cause of death in shrimp with lethal viral infections and that it may be an integral part of a process for adaptive tolerance to viruses in crustaceans. The idea of tolerance subsequently led to the proposal that crustaceans respond to viral pathogens by using a mechanism of adaptive accommodation (Flegel and Pasharawipas, 1998).

MATERIALS AND METHODS

Ca^{2+} Ionophore A23187, LPS-PGN, and Peroxinectin Induce Exocytosis

Animals

Freshwater crayfish, *P. leniusculus,* were collected from Berga kräftodling Södermanland, Sweden, and kept in aquaria in aerated tap water at 10°C. Only intermolt animals in average size 2.5-7.5 centimeter-long were used in the experiments.

Separation of Hemocytes

The different hemocyte populations of *P. leniusculus* were separated and harvested by a density gradient centrifugation method described by Söderhäll and Smith (1983). Continuous density gradients of 70 % Percoll in 0.15 M NaCl were centrifuged at 25,000 g for 30 min at 4°C. About 0.5 ml of crayfish hemolymph was bled from the abdominal haemocoel through a needle (0.8 mm) into 1 ml of anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 4.6) and then immediately put on top of a preformed Percoll gradient. After centrifugation at 2,900 g for 18 min at 4°C, granular cells (G-cells) were harvested with a Pasteur pipette and diluted with 0.15 M NaCl to 1: 1.

Purification and Immunoblotting of Peroxinectin

Hemocyte lysate supernatant (HLS) was prepared by collecting hemolymph from 150 crayfish with 0.8 x 50 mm needles into 10 ml ice-cold buffer of 10 mM Na cacodylate, 0.25 M sucrose and 100 mM $CaCl_2$, pH 7.0. The hemocytes were spun down at 800 g for 10 min at 4°C and the supernatant was removed. The resulting cell pellet was washed with the same buffer and homogenized with a glass homogenizer in 12.5 ml cacodylate buffer containing 5 mM $CaCl_2$. The cell lysate was precipitated with ammonium sulfate (50% saturation) at 0°C for 2 h and was collected by centrifugation at 10,000 g for 15 min. The pellet was resuspended in 1.5 ml cacodylate buffer (10 mM Na cacodylate, pH 7.0) and then dialyzed against of the same buffer overnight. The dialyzed fraction was spun down at 5,000 g for 15 min. The supernatant was applied to a carboxymethyl-cellulose column (0.8x 3.0 cm) (Sigma) previously equilibrated with cacodylate buffer and then eluted with 0.25 M NaCl in cacodylate buffer. The fractions containing peroxinectin were pooled and 1mM $CaCl_2$, 1mM $MnCl_2$ and 0.5 M NaCl were added. A Con A-Sepharose 4B (Pharmacia) was equilibrated with 10 mM Na cacodylate, 0.5 NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0, and the peroxinectin fractions were applied to this column, and then the column was washed with the same buffer until $A_{280} = 0$ is achieved. The column was equilibrated for 2 h with 0.25 M α-methylamannoside in 10 mM Na cacodylate, 0.5 M NaCl pH 7.0, and then washed with the same buffer until $A_{280} = 0$. The peroxinectin was eluted by incubation the column overnight in 0.75 α - methylamannoside in 10 mM Na cacodylate, 0.5 M NaCl, pH 7.0. The fractions containing peroxinectin were pooled and stored at -20°C.

For immunoblotting, the purified peroxinectin was subjected to 15 % SDS-PAGE under reducing conditions and then electrotransferred to nitrocellulose membranes in transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol) for 2 h at 280 mA on ice. The membrane was subsequently blocked in TTBS (0.1% Tween 20 in mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 3 % BSA for 1 hr and incubated with Ab TTBS containing 0.1 % BSA overnight. An affinity-purified Ab (10μg/ml) to peroxinectin was used for immunoblotting. The membrane was washed with TTBS once for 15 min and three times for 5 min each time. The anti-rabbit IgG peroxidase-conjugated IgG diluted 1/1,000 with TTBS containing 0.1% BSA was incubated for 1 h and washed with TTBS for 15 min and three times for 5 min each time. The ECL Western blotting reagent kit (Amersham Pharmacia Biotech) was used for the detection and the resulting peroxinectin was used in the experiments.

Degranulation Assay

Isolated suspended granular cells were treated with $CaCl_2$ 20 mM final concentration to allow the cells to attach to sterile well plates for 25 min at room temperature (20^{o}C). The resulting cell monolayers were washed with crayfish saline (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) and then treated with triggers of degranulation : Ca^{2+} ionophore A23187 (Sigma) (10 μM, diluted from a stock solution of 5 mM in DMSO containing 10 mM $CaCl_2$), lipopolysaccharides peptidoglycan (LPS-PGN) (10 μg/ml) and peroxinectin (20 μg/ml) for 30 min to 1 h at 20^{o}C. Control monolayers were incubated with only CFS. For inhibition studies, monolayers of attached cells were pretreated with SITS (4-acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid disodium salt) at 20^{o}C for 30 min. The cells were then stimulated with LPS-PGN at a final concentration of 10 μg/ml in CFS at room temperature for 45 min. The exocytosed fluid was collected and precipitated with trichloroacetic acid (TCA) precipitation to recover proteins. The precipitated proteins were subjected to SDS-PAGE and stained with 0.2 % Coomassie blue in 50 % methanol.

Electrophoresis

SDS-PAGE was conducted by the method of Laemmli (1970). Samples were denatured by heating on a hot plate at 95^{o}C for 4 min in SDS-PAGE sample loading buffer with 0.1% DTT. The gels were stained with coomassie blue and destained with destaining solution. A low molecular mass calibration kit for electrophoresis (Amersham Pharmacia Biotech) was used: rabbit muscel phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa).

Proteomic Analysis of the Vesicle Content of Granular Hemocytes

Determination of the Amino Acid Sequence and Mass Spectrometry Analysis

Nine protein bands were identified using an Applied Biosystem 476A automated amino acid sequencer. To confirm amino acid sequences mass spectrometry analysis, MALDI-TOF-MS (Matrix- Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) was performed in a Q-tof tandem mass spectrometer (Micromass, Manchester UK) equipped with nanospray interphase and using MassLynx program (Micromass, Manchester UK) for interpretion of mass spectra. (Uppsala Genome Center Sequencing Service, Uppsala University, Sweden)

CDNA Cloning and Sequencing

Partial cDNA sequences corresponding to the protein bands were obtained by random sequencing of a ZAP Express cDNA library constructed from mRNA of crayfish hemocytes. Degenerated primers were synthesized according to amino acid sequences of proteins and polymerase chain reaction (PCR) were followed by 94^{o}C for 2 min, and 35 cycles of 94^{o}C for 20 sec, 45^{o}C for 20 sec, and 70^{o}C for 1 min, followed by one 7 min extension period at 70^{o}C. The PCR products were purified by QIA quick-spin PCR Purification Kit (Qiagen) and subcloned into TOPO cloning vector (Invitrogen). The plasmids were released according to the manufacturer's instructions (Sigma).The insert was digested out by the restriction enzyme *Eco*RI (Amersham Pharmacia Biotech) for 1 h at 37^{o}C and run on 1 % agarose gel to confirm its size and finally sequenced with an Applied Biosystems PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Life Sciences ABI 310). If the clones lacked the 5' and 3' region of the cDNA, this remaining region was amplified by 5' RACE and 3' RACE with a specific primer which designed from the consensus of sequences, using hemocyte cDNA as template.

Total RNA was isolated from hemocytes by a total RNA isolation kit (Sigma) as described by the manufacturer followed by treatment with DNase I. One microgram RNA was used to synthesize the first strand cDNA (Thermo script RT) (Invitrogen) and oligo dT primers according to the manufacturer's instructions. The cDNA was amplified in a 50 μl PCR reaction containing 2 μl cDNA, 1 μl of company supplied PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 μM of each primer and 2.5 unit of *taq* DNA polymerase (Invitrogen). The fragment was generated by PCR using a specific primer and an anchor primer (SMART IIA oligo). The PCR conditions were 94^{o}C for 2 min followed by 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 1 min, 72^{o}C for 1 min and one cycle of final extension at 72^{o}C for 7 min. The amplified products were separated on 1 % agarose gel and visualized under UV illumination.

The PCR fragments were purified using the same protocol as above using QIA quick-spin PCR Purification Kit (Qiagen) and subcloned into TOPO cloning vector (Invitrogen).

Sequence Analysis

The cDNA sequence was analyzed with the Mac Vector 4.1.4 software (Kodak Scientific Imaging Systems, New Haven, CT, USA). The nucleotide sequence and the deduced amino acid sequence were compared with the BLAST program (National Center Biotechnology International, Bethesda, MD, USA).

White Spot Syndrome Virus (WSSV) Interaction with Crayfish Hemocytes

Experimental Animals

Freshwater crayfish *P. leniusculus* were purchased from Berga kräftodling, Södermanland, Sweden. The crayfish were held in tanks with running aerated water at 16ºC. Only adult intermolt crayfish were used in the experiments.

Preparation of Hemocytes Lysate Supernatant (HLS)

Hemolymph from crayfish was collected with 1.2 mm needles in ice-cold buffer of 10 mM Na cacodylate, 0.25 M sucrose and 100 mM $CaCl_2$, pH 7.0. Hemocytes were spun down at 800 g for 10 min (4ºC). The cell pellet was washed with the same buffer and homogenized with a glass piston homogenizer in 1-3 ml of 10 mM Na cacodylate, pH 7.0, containing either 5mM $CaCl_2$, a Ca^{2+} concentration that yields spontaneously activated serine protease and phenoloxidase in a haemocyte lysate or containing 100 mM $CaCl_2$, to keep the enzymes of the proPO system in their inactivate form. These homogenates were then centrifuged at 70,000 g for 20 min at 4ºC and the resulting HLS was used in the experiments.

Preparation of Monolayers of Hemocytes

Granular cells (GCs) from WSSV infected (moribund stage) and normal crayfish were separated by a density gradient centrifugation method described by Söderhäll and Smith (1983). Monolayers of pure granular cells were prepared on clean, pyrogen-free glass coverslips. Briefly, isolated GCs were diluted 1: 2 with 0.15 M NaCl and then treated with $CaCl_2$ (20 mM final concentration) to allow the cells to attach to sterile coverslips for 30 min at room temperature.

Experiments with Cell Monolayers

Effect of Dilution of HLS on Degranulation of Granular Cells *in Vitro*

The effect of HLS on crayfish hemocytes *in vitro* was examined by overlaying freshly prepared crayfish GCs monolayers with 100 µl of crayfish HLS containing the degranulation factor, peroxinectin, at different dilutions (Johansson *et al*., 1995). The cell monolayers were then incubated for 40 min at 20 ºC , washed gently with crayfish saline (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2mM $NaHCO_3$, pH 6.8) (Smith and Söderhäll, 1983a), and then fixed in 10 % formalin in CFS. Control monolayers were incubated with CFS. After fixation, the cells were observed by use of phase contrast microscope and the percentage of degranulated, spread or non-spread (unaffected) cells were determined for at least 200 cells on each coverslip.

Effect of HLS on SGCs and GCs

The SGCs and GCs were separated and treated as described above. After fixation, the cells were observed under the microscope, and the presence of WSSV was detected by *in situ* hybridization using a digoxigenin labelled WSSV specific probe as earlier described (Jiravanichpaisal *et al*., 2001).

Degranulation of Granular Cells

The GCs monolayers were washed three times with CFS and then treated with the Ca^{2+} ionophore A23187 (Sigma) at a final concentration of 10 μM in CFS and 10 mM $CaCl_2$ (final concentration) for 30 min or phorbol 12-myristate 13-acetate (PMA) at a final concentration of 4 μM in CFS for 30 min. The Ca^{2+} ionophore A23187 was diluted from a stock solution of 5 mM in dimethyl sulfoxide (DMSO) as described in Johansson and Söderhäll (1985) and the same amount of DMSO was always present in the controls. The cells were fixed and counted as above.

Melanization of Granular Cells

Granular cells monolayers were incubated with modified Leibovitz's L-15 medium (L-15 M81) at room temperature for at least 16 h, then observed and the percentages of melanized cells were determined under the microscope.

RNA Isolation and RT-PCR

After isolated GCs from WSSV infected and normal crayfish were allowed to attach to the well at room temperature for 30 min, the cells were washed with CFS three times. Total RNA was isolated by using total RNA isolation kit (Sigma) according to manufacture's instructions. 100 ng of RNA from each treatment was analyzed by RT-PCR using Thermoscript (Invitrogen) and Oligo (dT) primer according to the manufacturer's instructions. The following primers were used for the final PCR step: proPO 5'-AGT GAA CAG GAC TCC ACC TAC TGC-3' and 5'-ACT GAT GTC TAT GAA ATC CAG CCC-3' corresponding to residues 622+/1045- (Accession number CAA58471), and a control RT-PCR analysis of expression of the housekeeping gene small ribosomal protein was also undertaken 40S forward and reverse, 5'-CCA GGA CCC CCA AAC TTC TTA G-3' and 5'-GAA AAC TGC CAC AGC CGT TG-3' respectively. The products were then analyzed on an agarose gel, stained with ethidium bromide, and visualized by ultraviolet light. The expected size of proPO and 40S were 423 and 359 nucleotides, respectively.

Cell Death Assay

Hemolymph from WSSV infected crayfish 3 and 5 days post infection was collected with 1.2 mm needle in ice-cold anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 4.6). The cells were spun down and resuspended in CFS. The cell suspension was smeared on the glass slides and allowed to dry at room temperature. Air dried cell samples were fixed with freshly prepared paraformaldehyde (4% in CPBS, pH 7.4) for 1 h at room temperature.

For detection of apoptotic cells a direct TUNEL assay was performed using the *in situ* cell death detection Kit, Florescein (Boeringer Mannheim) according to the manufacturer's instructions.

In Situ Hybridization to Monitor WSSV Infected Hemocytes

Some samples of fixed hemocytes on coverslip as prepared above were carried out using *in situ* hybridization to determine percentage of WSSV infection. A digoxigenin (DIG)-labelled WSSV-specific probe was prepared from a WSSV genomic squence (Genbank accession number AF178573), Hybridization and staining procedure were as previously described by Jiravanichpaisal *et al*. (2001) except that the concentration of Proteinase K (Roche) was decreased to 10 μg per ml.

Statistical Analysis

The mortality data were compared using Student's *t*-test; meanwhile the comparison of multiple groups in the haemocyte counts experiments were performed by one-way analysis of variance. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Ca^{2+} Ionophore A23187, LPS-PGN, and Peroxinectin Induce Exocytosis

Degranulation occurred on the *in vitro* monolayers of separated granular cells of *P. leniusculus* when the cells were incubated with activators; the Ca^{2+} ionophore A23187 (10 μM), LPS-PGN (10 μg/ml) and peroxinectin (20 μg/ml) (Table 2). All triggers caused degranulation except LPS-PGN caused the granular cells to round up and degranulate but not undergo lysis (Figure 2). The control cells in CFS appeared to spread on the glass without degranulation (Figure 3). These activators of degranulation induced the hemocytes to secrete their granular contents in a concentration-dependent manner. The secreted proteins had molecular masses of 134, 42, 38, 32, 28, 20, 16, 14 and 12 kDa in SDS-PAGE (Figure 4). Six strong bands, L_1-L_6, were observed using LPS-PGN as a trigger with molecular masses of 134, 32, 28, 20, 16 and 14 kDa as shown in SDS-PAGE (Figure 5). Seven strong bands, P_1-P_7, were obtained when peroxinectin was used as a trigger of degranulation with masses of 134, 42, 32, 28, 20, 16, and 14 kDa, as shown in the SDS-PAGE (Figure 6). The first band with a mass of 134 kDa was found to be the masquerade-like protein (Lee and Söderhäll, 2001). The second band, I_2, with a mass of 42 kDa is a masquerade-like serine protease. The third and fourth band with masses of 38 and 32 kDa (Figure 4, I_3 and I_4) were found to contain glycine-rich sequences (GGGGFGGGGFGGG or LGGGGGGLGGGGFGGG). These glycine rich sequences were also found in the released band with a mass of 32 kDa ($I_4 = L_2 = P_3$) after LPS-PGN or peroxinectin treated cells. The fifth band, I_5, with a mass of 28 kDa was strong following Ca^{2+} ionophore A23187 treatment and weaker when peroxinectin (P_4) was used. It is identical to the third band when LPS-PGN was used ($I_5 = P_4 = L_3$). The sixth band (I_6) with a mass of 20 kDa is the same as the fourth LPS-PGN induced band (L_4) and is identical as the fifth band when peroxinectin was used ($I_6 = L_4 = P_5$). The seventh band (I_7) with a mass of 16 kDa is the same as the fifth band (L_5) of LPS-PGN and the sixth band (P_6) of peroxinectin treatment ($I_7 = L_5 = P_6$), while the eighth band (I_8) with a mass of 14 kDa is the same as the seventh band (P_7) of peroxinectin treatment and the sixth band (L_6) of LPS-PGN ($I_8 = P_7 = L_6$). The last band, I_9, with a mass of 12 kDa was only detected after the Ca^{2+} ionophore A23187 treatment. In summary, all proteins secreted as a result of peroxinectin and LPS-PGN treatment of the cells were detected after degranulation induced by the Ca^{2+} ionophore A23187.

Table 2 Exocytosis of granular cells induced by activators: Ca^{2+} ionophore A23187, LPS-PGN and peroxinectin

Treatment[a]	% degranulated cells (±SD)[b]	Number of experiments
Ca^{2+} ionophore A23187 10 μM + $CaCl_2$ 10 mM	90 (±12)	5
LPS-PGN (10 μg/ml)	23 (± 8)	5
Peroxinectin (20 μg/ml)	10 (± 4)	6
Control (CFS)	0 (± 0)	4
Ca^{2+} ionophore A23187 10 μM + 10 mM $CaCl_2$ + SITS 100 μM	4 (±1)	3
LPS (10 μg/ml) + SITS 100 μM	2 (± 1)	3

[a] Bands of separated granular cells from Percoll gradients were pooled and collected by centrifugation. The cell pellets was resuspended in 0.15 M NaCl. The cell suspension was incubated with Ca^{2+} ionophore A23187 10 μM and 10 mM $CaCl_2$ (final concentration) for 30 min at room temperature, LPS-PGN 10 μg/ml for 40 min at room temperature, peroxinectin 20 μg/ml for 1 h at room temperature. Control cell suspension was kept in crayfish saline (CFS). The cell suspension was preincubated with 100 μM SITS for 30 min at 20°C before addition of Ca^{2+} ionophore A23187 10 μM+10 mM $CaCl_2$ and LPS (20 μg/ml).

[b] At least 200 cells on each coverslip were counted under the phase contrast microscope; the percentage of lysed cells in these experiments was always less than 5%

SITS: 4-acetamino-4'-isothiocyanatostilbene-2, 2'-disulfonic acid disodium salt

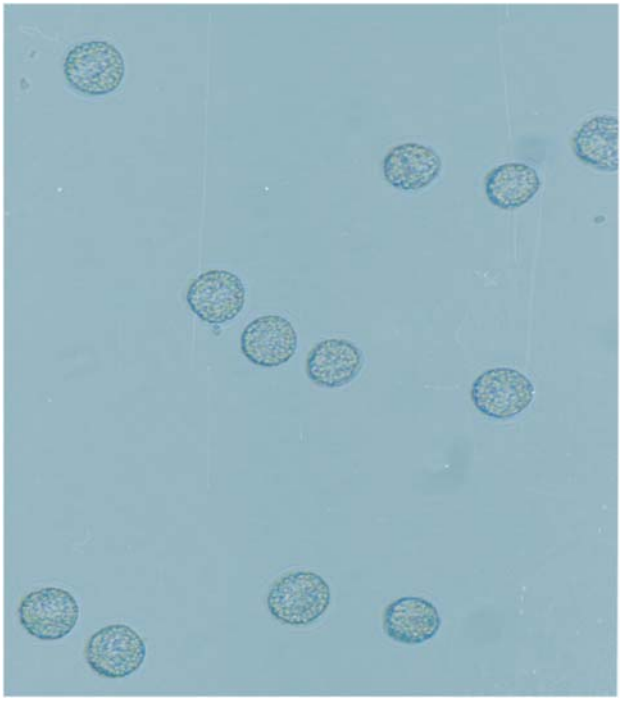


Figure 2 Degranulated granular cells, incubated with LPS-PGN (10 μg/ml)

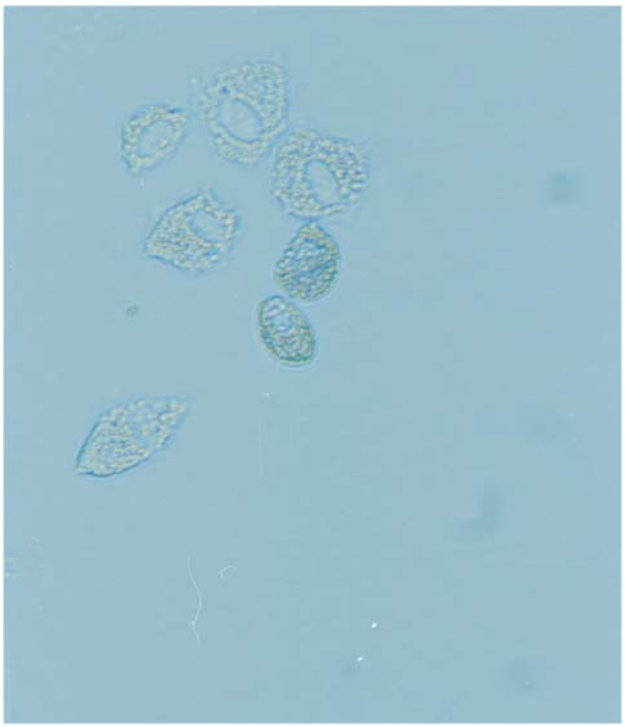


Figure 3 Unaffected granular cells, control; incubated with CFS

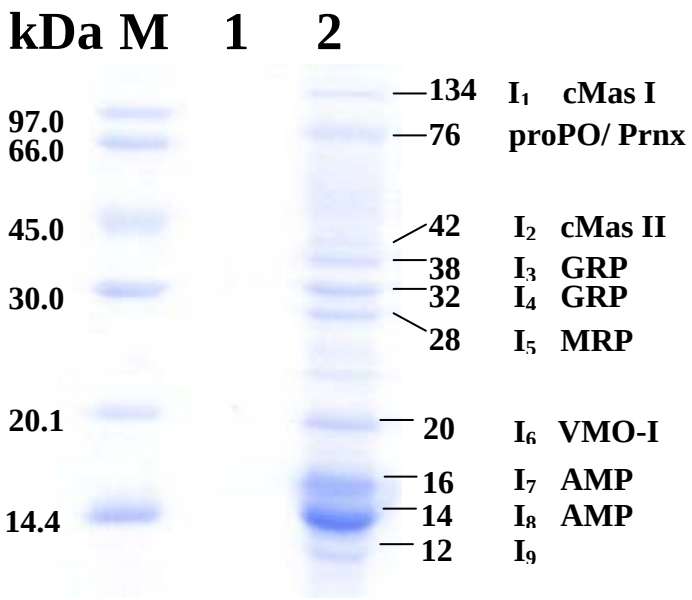


Figure 4 Analysis of the exocytosed fluid from crayfish granular cells resulting after treatment with Ca^{2+} ionophore A23187 10 μM and 10 mM $CaCl_2$ (final concentration) for 30 min at room temperature). Proteins were precipitated with trichloroacetic acid (TCA) of the exocytosed fluid. The precipitated proteins were subjected to SDS-PAGE under reducing conditions and then stained with 0.2 % Coomassie blue in 50 % methanol. Lane M, Size marker; lane 1, The precipitated protein of the control cells after incubated with CFS; lane 2, 12 μg precipitated protein after treated with Ca^{2+} ionophore A23187 for 30 min at room temperature. The first band of molecular masses 134 kDa was named band I_1 and 42, 38, 32, 28, 20, 16, 14 and 12 kDa were named I_2, I_3, I_4, I_5, I_6, I_7, I_8, and I_9, respectively.

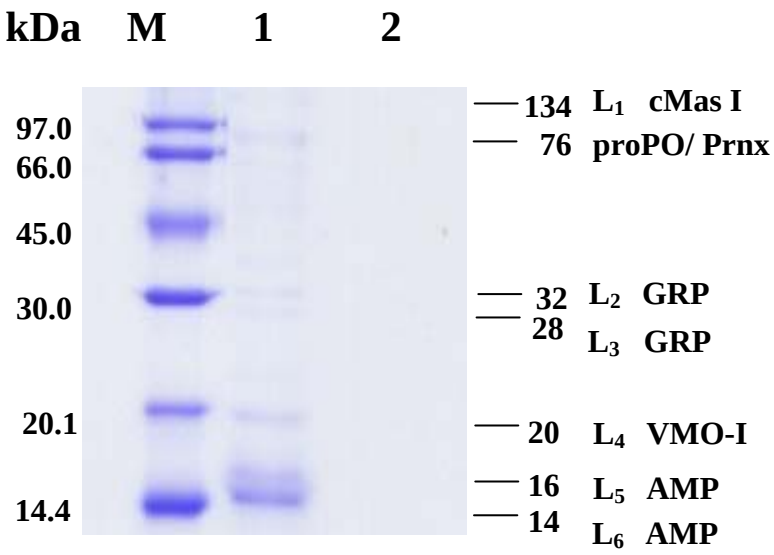


Figure 5 Analysis of the exocytosed fluid from crayfish granular cells after lipopolysaccharide treatment. Proteins were recovered by trichloroacetic acid (TCA) precipitation of the exocytosed fluid. The proteins precipitated were subjected to SDS-PAGE under reducing conditions stained with 0.2 % Coomassie blue in 50 % methanol. Lane M, Size marker; lane 1, 12 μg precipitated protein after treated LPS-PGN for 40 min at room temperature; lane 2, The precipitated protein from the control cells after incubated with CFS. The first band of molecular masses 134 kDa was named L_1 and 32, 28, 20, 16 and 14 kDa were named L_2, L_3, L_4, L_5, and L_6, respectively.

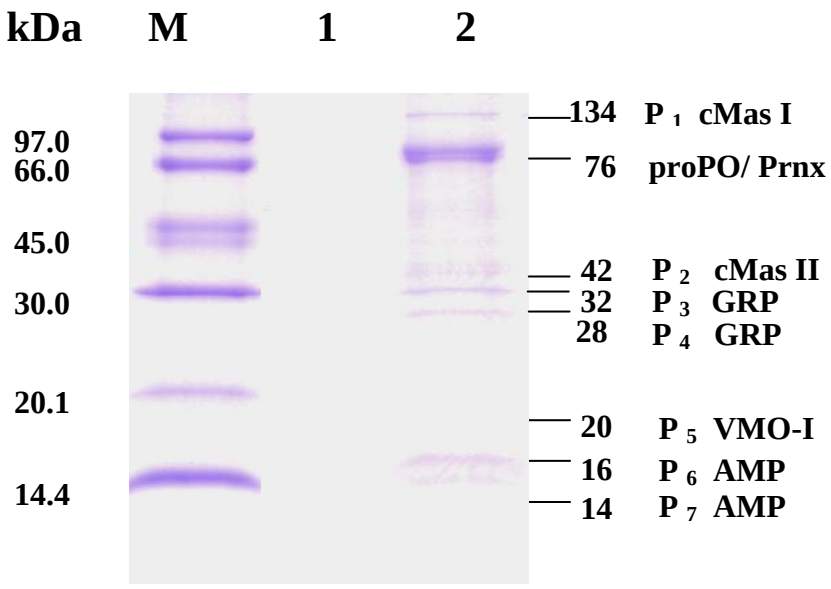


Figure 6 Analysis of the exocytosed fluid from crayfish granular cells resulting after peroxinectin (20 μg/ml) treatment. Proteins were recovered by trichloroacetic acid (TCA) precipitation of the exocytosed fluid. The proteins precipitated were subjected to SDS-PAGE under reducing condition stained with 0.2 % Coomassie blue in 50 % methanol. Lane M, Size marker; lane 1, Control monolayers were incubated with only CFS; lane 2, 12 μg precipitated protein after treated with peroxinectin for 1 h at room temperature. The first band of molecular masses 134 kDa was named P1 and 42, 32, 28, 20, 16 and 14 kDa were named P_2, P_3, P_4, P_5, P_6, and P_7, respectively.

Proteomic Analysis of the Vesicle Content of Granular Hemocytes

The partial amino acid sequences of the band with a molecular mass of 134 kDa showed that it was the masquerade-like protein (cMas I) (Lee and Söderhäll, 2001), whereas the partial amino acid sequences of the protein with a mass of 42 kDa protein were determined as K/RFPVEDPYAGQLK, K/RDAFEGVGEFQR, K/RLVNL LTGGQCAONK, K/RLGQTFTLDR, K/RDVTLQNDVGLLNLQR and PVQDPFV. Degenerate primers were designed based on those partial amino acid sequences and they were used for screening a hemocyte first-strand cDNA library. One of the clones derived from this hemocyte cDNA was identified as a masquerade-like serine proteinase (cMas II). To obtain a full sequence, gene specific primers were designed and were then used to screen a hemocyte cDNA by 5' and 3' RACE (Rapid Amplification of cDNA Ends) (Invitrogen). The obtained full-length cDNA sequence had an open reading frame of 1466 nucleotides corresponding to 535 amino acid residues with a predicted mass of 42 kDa and an isoelectric point of 6.4 (Figure 7a). Figure 7b shows a putative catalytic domain, E_{150} to I_{385} (a trypsin-like serine proteinase) of cMas II. However, a serine residue in the catalytic site is replaced by a glycine residue and it belongs to the family of masquerade-like proteins presumably without any proteinase activity.

```
1    -     CAGACTGCTACACCTTCCAGTTACTGTGTTTAGTGCTCTCCTAACATTACCATCTACACACTACGATGAGAGTAT -  75
                                                                          M  R  V

76   -     GGGCGAGTGTATGCTTGGTGCTGGCGGTGACAGTGGAGAGTCAGAGGCTAGGAGTCACCACAAGGCTGGGTCTGT - 150
           W  A  S  V  C  L  V  L  A  V  T  V  E  S  Q  R  L  G  V  T  T  R  L  G  L

151  -     TGGGCCCAGAGATCGGCTTGGATCCCGTTCCCGGCAGCAACTTCAACCCCCCACCCAGGGACGCTGGGATCACCA - 225
           L  G  P  E  I  G  L  D  P  V  P  G  S  N  F  N  P  P  P  R  D  A  G  I  T

226  -     GGTGTGTCTGCCTCCCCGTTAACCAGGTGTGTCCCGAAGGCCAAGCTACTCCTCCACAGAGGCCTGAAGGGGTAG - 300
           R  C  V  C  L  P  V  N  Q  V  C  P  E  G  Q  A  T  P  P  Q  R  P  E  G  V

301  -     CAATCAACCATGGAGCTGGTCAGATCGACGTCCGCATAGTTAACTTGCTTACAGGAGGGCAGTGTCCGGGTCAGA - 375
           A  I  N  H  G  A  G  Q  I  D  V  R  I  V  N  L  L  T  G  G  Q  C  P  G  Q

376  -     AGATGTGCTGCCCTGGAGGTGAACTCTCCACAGGACAAGGGACGAACCCTGTACTTCCCAACAAATTGCCGATCA - 450
           K  M  C  C  P  G  G  E  L  S  T  G  Q  G  T  N  P  V  L  P  N  K  L  P  I

451  -     ATACTGGTGGCTGCGGCTTCCAGAATCCTTTACCTGTACCCAACCAACCAGCCAAGTTTGCCGAGGCAGAGTTCG - 525
           N  T  G  G  C  G  F  Q  N  P  L  P  V  P  N  Q  P  A  K  F  A  E  A  E  F

526  -     GAGAGTATCCATGGATGGCGGTGGTGCTGGACAATGGTAACAACTACAAGGGTGGTGGAGTCCTCATCAGCGAGA - 600
           G  E  Y  P  W  M  A  V  V  L  D  N  G  N  N  Y  K  G  G  G  V  L  I  S  E

601  -     ACTGGGTGCTCACCGCCGCGCACAAGGTCAACAATGAGAGGAACCTGAAAGTGCGGTTGGGCGAGCACGATGTCA - 675
           N  W  V  L  T  A  A  H  K  V  N  N  E  R  N  L  K  V  R  L  G  E  H  D  V

676  -     CTAAGCCAAAGGACCACCCAAATTTTGATCACATCGAGATACCAGTCGGAAGAATCATCATTCACCCAGAGCTCA - 750
           T  K  P  K  D  H  P  N  F  D  H  I  E  I  P  V  G  R  I  I  I  H  P  E  L

751  -     AAGTTGACACCTTACAGAACGACGTGGGCCTCCTGAACCTCCAGAGACCGGTCAACACAAACAGGTTCCCACACA - 825
           K  V  D  T  L  Q  N  D  V  G  L  L  N  L  Q  R  P  V  N  T  N  R  F  P  H

826  -     TCGGGACAGCCTGTCTCCCTCGCCAGGGCCAGATCTTCGCTGGCGAAAACCAGTGCTGGGTGACCGGGTTCGGGA - 900
           I  G  T  A  C  L  P  R  Q  G  Q  I  F  A  G  E  N  Q  C  W  V  T  G  F  G

901  -     AGGACGCCTTCGAAGGTGTGGGAGAGTTCCAGCGCATCCTGAAGGAGGTGGACGTGCCCGTACAGGACCCCTTCG - 975
           K  D  A  F  E  G  V  G  E  F  Q  R  I  L  K  E  V  D  V  P  V  Q  D  P  F

976  -     TNTGCCAGGAAAGGCTCAGGAGCACTCGCCTCGGGCAGACATTCACTCTAGATAGAAATTCATTCTTGTGCGCTG - 1050
           V  C  Q  E  R  L  R  S  T  R  L  G  Q  T  F  T  L  D  R  N  S  F  L  C  A

1051-     GAGGTATCGAAGGGAAGGACGCCTGCACGGGTGACGGAGGGGCCCCCTTGGTGTGTAGGCCGGAGAGAGGGCAGT - 1125
           G  G  I  E  G  K  D  A  C  T  G  D  G  G  A  P  L  V  C  R  P  E  R  G  Q

1126-     GGACAGTAGCTGGTCTGGTCGCATGGGGTATTGGCTGCGCCACCAGCGAAGTCCCAGGCGTCTACGTCAACATCG - 1200
           W  T  V  A  G  L  V  A  W  G  I  G  C  A  T  S  E  V  P  G  V  Y  V  N  I

1201-     CCTCCTACGCTGACTTCATCCGTCGGTACGTCAGGTAAGACATACTCGCCTCTCCACCAGCTCAGAGTTAAGACA - 1275
           A  S  Y  A  D  F  I  R  R  Y  V  R  *

1276-     CTGCTAACATGTGGTATTCGTTATAAATTTTACAGTAAATCCTGGCAATTCTTGTGTATAGTAATAAGACGAACT - 1350

1351-     TAATTTGTTCAACAGATTTTATTGATTTGTATTACTTTTCATAACTCTGACCCCCTGTTAAAAAGTGGGAAACAG - 1425

1426-     TCGCCAGCAAAGCGGGCAGTAAATAAAAAAAAAAAAAAAAA                                  - 1466
```

Figure 7a Nucleotide sequence of masquerade-like serine proteinase (cMas II) and its deduced amino acid sequence. Bold amino acids are start codon ATG and stop codon TAA. Circles indicate the residues corresponding to the catalytic triad. Underlined are the deduced amino acid sequences which match the MALDI-TOF-MS sequences.

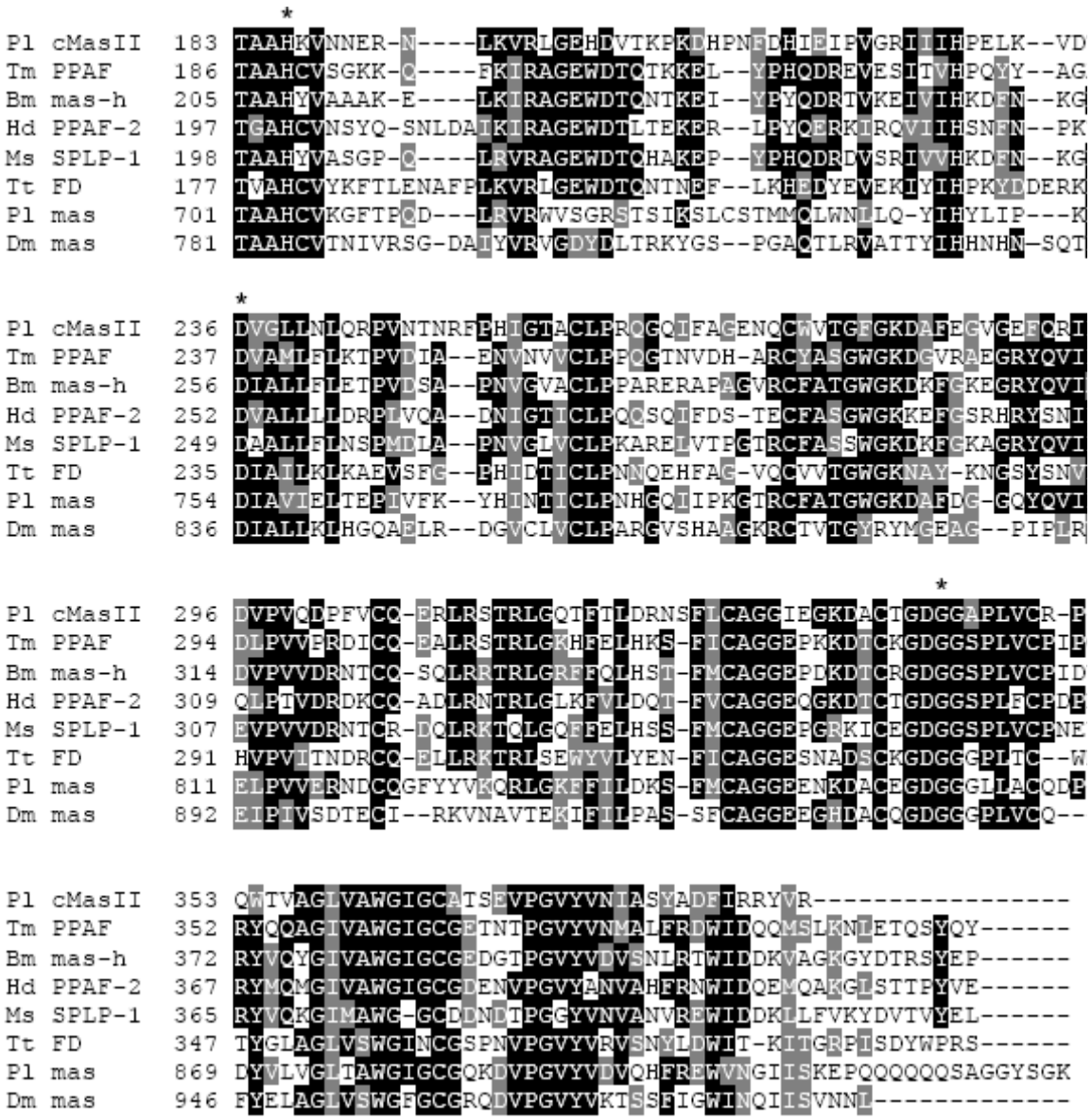


Figure 7b The partial amino acid sequence alignment of the masquerade-like serine proteinase from the crayfish, *P. leniusculus* (cMas II) with other serine proteinases: *Tenebrio* PPAF(*Tm PPAF*), *Bombyx* masquerade-like serine proteinase homolog (*Bm mas-h*), *Holotrichia* PPAF-2(*Hd PPAF-2*), *Manduca* serine proteinase-like protein-1(*Ms SPLP-1*), *T. tridentatus* big defensin (*Tt FD*), *P. leniusculus* mas-like protein (*Pl mas*) and *Drosophila melanogaster* mas (*Dm mas*). The numbers show the order of amino acid sequence residues from each protein. The conserved residues of catalytic triad are indicated by asterisks. The alignment was performed using ClustalW.

Partial internal amino acid sequences of the protein with a mass of 38 kDa were LGGGGGGGLGGGGLGGGFGGGK, RDYSNPCFLALAACR, RDLGFTEQQFLGK and K/RQFWTLQLN. The band with a mass of 32 kDa had partial amino acid sequences as SGGGFGGGGGGFGGSSGDK, LGGGGGGLGGGGAVGGGYGGGK and GGGGGGLGGGGLGGGYGGGK and show some similarities with glycine-rich antimicrobial peptides from the spider *A. gomesiana* (Lorenzini *et al*., 2003). No full cDNA-clones were obtained for these proteins.

The internal amino acid residues with the sequences K/RYVWQQANSYCK, K/RHSLPYLWLMDR and ESPTENQFLSTLLDK of the 28 kDa band were used to design and synthesize degenerate primers. A corresponding cDNA was found to code for the complete amino acid sequence of a mannose receptor protein. It includes 1398 nucleotides that encode an open reading frame of 273 amino acids. The amino acid sequence from Q_{142} to K_{267} corresponds exactly to the deduced amino acid sequence of the mannose receptor from house mouse (*Mus musculus*) (Accession number NP 032651) (Harris *et al*., 1992). A calculated molecular mass of the deduced sequence of the mannose receptor protein is 28.3 kDa with an isoelectric point of 8.74. The nucleotide sequence and deduced amino acid sequence are shown in Figure 8.

For the protein with a mass of 20 kDa, three partial amino acid residues sequences SDDTSLDGLK, DVLFLCCR and FLVDNLA was observed. These sequences were used to design and synthesize degenerate primers. Several positive clones were sequenced and were found to encode a protein similar to a vitelline membrane outer layer protein I (VMO-I). The amino acid residues from S_{20} to R_{184} correspond to the deduced amino acid of a VMO-I homologue from *Gallus gallus* (chicken)(Accession number BAA05086)(Uyeda *et al*., 1994)*, Cyprinus carpio* (common carp) (Accession number AAD23572) (Chang *et al*., 1999), and also from the crayfish hemoctye EST-library (Accession number CF542389) (Irene Söderhäll, unpublished). The mature protein consists of 1368 nucleotides that encode an open reading frame of 185 amino acids with a calculated molecular mass of 20 kDa and an isoelectric point of 4.44. The nucleotide sequence and deduced amino acid sequence are shown in Figure 9.

The 16 kDa protein (I_8=L_6=P_7) had amino acid sequences; YLPDVHQLLCR, LPGCVNTCQK, NYPNHLNCQDDED and K/RGSNYCCGPEYLPLKR that perfectly match a putative antimicrobial peptide found in the crayfish hemocyte EST-library (Accession number CF542515, Figure 10). This peptide shows some similarities with a putative carcinin-like antibacterial protein from *H. gammarus* (Accession number CAH 10349). Similarly the 14 kDa (I_9=L_7=P_8) band turned out to match another putative antimicrobial peptide from the crayfish hemocyte EST-library (Accession number CF542483, Figure 11), that shows high similarity (45% identity and 53% similarity) with another carcinin-like protein from *C. maenas* (Accession number CAH25401).

```
1     -   ATCACAACACCGCATTTCAAGATGAAGTGTGTGTTGCTGGTCCTGAGCGTGGTAGTGGCG  -   60
                            M  K  C  V  L  L  V  L  S  V  V  V  A

61    -   GTGGCCCAGAGTCAGGTCCCCTACGGTAGCTTCGGGGGGAGGCGGCGGCTTCCCTGGGAGA  -  120
           V  A  Q  S  Q  V  P  Y  G  S  F  G  G  G  G  G  F  P  G  R

121   -   CCCGGTATTCACCCCGGGAGACATGGCGGCGGGGGACAGATTCAGTTCCCCGGTTCAAGA  -  180
           P  G  I  H  P  G  R  H  G  G  G  G  Q  I  Q  F  P  G  S  R

181   -   CCCGGGCTCCATGGGAAGCCGGGAGGGATTAGTGGATTTGGTGGTGCAGGGGGAGTCGGT  -  240
           P  G  L  H  G  K  P  G  G  I  S  G  F  G  G  A  G  G  V  G

241   -   GGATTTGGCGGGGCAGGAGGAGTTGGTGGTGCAGGAGGATTTGGTGGTGCGGGAGGAGTC  -  300
           G  F  G  G  A  G  G  V  G  G  A  G  G  F  G  G  A  G  G  V

301   -   GGTGGATTGGTTGGTACTGGAGGCGGTGGACAGGCACAGGAGCAGCCTCTCTCAACCAAG  -  360
           G  G  L  V  G  T  G  G  G  G  Q  A  Q  E  Q  P  L  S  T  K

361   -   TTCTATCCGGCGTACATCAGTCCCCAGGTTCACGTATCTCTGGGTGGAAGCAACTACCAC  -  420
           F  Y  P  A  Y  I  S  P  Q  V  H  V  S  L  G  G  S  N  Y  H

421   -   TTCTCCTGGTGCGTTGACGGTGGGCAGAAGTACGTGTGGCAGCAAGCCAACAGTTACTGC  -  480
           F  S  W  C  V  D  G  G  Q  K  Y  V  W  Q  Q  A  N  S  Y  C

481   -   AAGCAGCTGGGACCTGGGTGGGGCGCTGTGAGCATAGAGTCCCCGACTGAGAACCAGTTC  -  540
           K  Q  L  G  P  G  W  G  A  V  S  I  E  S  P  T  E  N  Q  F

541   -   ATCTCCACCATCATCGACAAGCACAGCCTACCATACATCTGGACCAGCGGGAACCGTTTG  -  600
           I  S  T  I  I  D  K  H  S  L  P  Y  I  W  T  S  G  N  R  L

601   -   AGTGGTGGTCTCAACGACTGGAGGTGGGGCACAGGCCAGCCTCTCAAATATGAAAACTGG  -  660
           S  G  G  L  N  D  W  R  W  G  T  G  Q  P  L  K  Y  E  N  W

661   -   GCTCGCACTGGATTCATTCCCGGCAATCCTCAGCCCGACAACCAGGAGGACAACAATGAG  -  720
           A  R  T  G  F  I  P  G  N  P  Q  P  D  N  Q  E  D  N  N  E

721   -   CAATGCCTCTCAGTGCTCAACCGGTTCTACCCCAACGATGGCATTACCTGGCATGACGTC  -  780
           Q  C  L  S  V  L  N  R  F  Y  P  N  D  G  I  T  W  H  D  V

781   -   GGTTGCCACCACGTCAAGCCTACCATCTGCGAGTATAGCAAAGTCCAGAGCTACGCTGGA  -  840
           G  C  H  H  V  K  P  T  I  C  E  Y  S  K  V  Q  S  Y  A  G

841   -   TAAACCTGCCGAGTGGTATAACTTATTTGAATGACAAATCTTTATTCCTATTTTTTAGCA  -  900
           *

901   -   GTCTGTGCAGTCTTACAGAGAACATTAAAGTTCGAGTTTGCTTAAAATCACAAGTTACTC  -  960
961   -   CTTATAACAGAAAACGGATATTAAATTCTTTCACATGCCGTTCTCTCTCATTGGTGCTGT  - 1020
1021  -   AAATCTGTAGAAGACGATATGAAAAAATAATCAGGGATATATATATATATACACCGAGAG  - 1080
1081  -   GTGTGCCCCGTCTCTCGTTGTATATATACAGAGTTTACTTTGGTCCTGGACTATGATCAG  - 1140
1141  -   AACTAAAGTGTACTTGTTGGTTGGTCAGTAAGCTCCTGGGGGTGGCCATAATAACGCTCCT  - 1200
1201  -   GCGGTGGCCATAATAACGCTCCTGGGGTTAGAAAGCCTTTGGACATTCGAAAAGCTTCCA  - 1260
1261  -   GGATTGAATTATGTGTTTCGTGATAGAAAAATGGCATTATCTTTTTCTGAATTTCTCTTA  - 1320
1321  -   ATTTACCCATTAATTAATTTAAGTCCTTCCTGTTATCTTGAATTGTAATAAAAAAAATAT  - 1380
1381  -   TCTTCAAAAAAAAAAAAA                                            - 1398
```

Figure 8 Nucleotide sequence of mannose receptor protein and its deduced amino acid sequences. Bold-italics are the deduced amino acid sequences which match the MALDI-TOF-MS sequences. The bold-underline are corresponds to the sequence used for the design of the primer for 5' and 3'- RACE-mannose receptor protein. The polyadenylation signal is underlined (AATAAA).

```
1    -     ACAGATTTGTCTTCGCGGTGGTCCAGACTAAGTCTTCAGACGAGGTTCTAGCGAGGATGA  -   60
                                                                   M

61   -     CGAACACTTTCATAATCCTTCTCTGCGTCGCAGGTGCTGTGCTGGGGAAGGACTCCACCA  -  120
           T  N  T  F  I  I  L  L  C  V  A  G  A  V  L  G  K  D  S  T

121  -     TCCGGTCTAACAATGGAGGGCCTTGGGGTGACTGGGGGTCAGAATCTCACTGTCCCCCTA  -  180
           I  R  S  N  N  G  G  P  W  G  D  W  G  S  E  S  H  C  P  P

181  -     ACAGCTTTGCCACCGGCTTTGCTATTAAGGTGGAGCGACCAGTTGGCGATTCGGACGACA  -  240
           N  S  F  A  T  G  F  A  I  K  V  E  R  P  V  G  D  S  D  D

241  -     CGTCCATAAACGGGATCAAGTTGTTCTGCACGTCTGGCAACGACGGCTCCGAGACGGAAG  -  300
           T  S  I  N  G  I  K  L  F  C  T  S  G  N  D  G  S  E  T  E

301  -     TCACCTCCAACCAGCAGCAGTGGGGCAGCTGGACCGATAGGCGTCAATGCCCCCACGGTC  -  360
           V  T  S  N  Q  Q  Q  W  G  S  W  T  D  R  R  Q  C  P  H  G

361  -     GCTTGACTAGCATGAGGCTGCGAGTTGAGGGGAGGCAAGGATCCGGTGATGACACGGCCG  -  420
           R  L  T  S  M  R  L  R  V  E  G  R  Q  G  S  G  D  D  T  A

421  -     CCAACAACCTGGACATGCGCTGCCAGAACGGTCAAGAACTTGGCGGAGGAGGCAACAACT  -  480
           A  N  N  L  D  M  R  C  Q  N  G  Q  E  L  G  G  G  G  N  N

481  -     GGGGCGACTGGAGCCCGTGGGAGACCTGCCAACTTGGCCAAGCGATCTGTGGGCTGCAGA  -  540
           W  G  D  W  S  P  W  E  T  C  Q  L  G  Q  A  I  C  G  L  Q

541  -     CCCGGGTTGAAGGCACTCAAGCAGGTGACGACACAGCTCTCAACGATGTCATATTCTTAT  -  600
           T  R  V  E  G  T  Q  A  G  D  D  T  A  L  N  D  V  I  F  L

601  -     GCTGCAGGCAGTAGGCCAGAGATAAAACCAAACATCCTGGTCATAACTTCCAAAAGGGCT  -  660
           C  C  R  Q  *

661  -     TCAAGAAGTATATTGATATATGACATTACGAGGTTCCCTATTGTATGATTTAGTTCTTGT  -  720
721  -     TCATTCGATATTTTTACAGTTTTTAGGCTTCCAAGAATGATTACCATGGCTTCATTTTGT  -  780
781  -     ATGCTTTATTCATGTTGATTATACAGCATTTTCCCAATGTACACATCAATAAAGACGGCA  -  840
841  -     CCCACATACAATGACTTAGTGTCCACTATCAAGAGATGCAATTATATGATCATTTCCGGA  -  900
901  -     ATCTTTTGTTGTAGGAAGATACGGCAGAATTTTGAGCTTTGAATGTTTTGTTTATTGTGG  -  960
961  -     TTTACTTATGGAGAACATTGCATACGCAGCTGAATTGTTGTGTAGGGATCTCCAAAAATG  -1020
1021-     CGAATAAAGACATCTGCAACCATTATAGGGAAAGTAATTTTAGATGAATTGGTAAATGTT  -1080
1081-     TCAGAGGCACAAGTGTAACATATCAAATACATCTTGTACTAGAAAGAAACCCTTGCAATC  -1140
1141-     CCTTTTGAATGATATATAGCACTGGATTTTATCATTATCAAATAAAAGTACACAAAATGT  -1200
1201-     AAAGCAGAAATGTGGAATATAAGGTTTTAGAAAGACACCTTCAAACTTTATTATTAAAGA  -1260
1261-     GAGCCTCATGCTAGGCTCTGAATAAACTTATCCAGATAAAATGTTTTTTGTGCCAAATCT  -1320
1321-     TGACATGAAGCATCAACTGACACGAACTTCTGTTGTTGACAAAATAAATATCCTGCTTTA  -1380
1381-     AAAAAAAAAAAAAAAAAAAA                                          -1400
```

Figure 9 Nucleotide and deduced amino acid sequences of VMO-I protein. The number of nucleotides is shown in both of right and left in the column. The polyadenylation signals (AATAAA) are underlined. Bold–italics are the deduced amino acid sequences which match the MALDI-TOF-MS analyzed sequences.

```
1       ATGCTGCGTGTACTGATGATGTCGCTGCTGGTGGTGGCGGCGCTCGGCCACATCTCCCCG  60
1        M  L  R  V  L  M  M  S  L  L  V  V  A  A  L  G  H  I  S  P   20

61      CCCCGGCCGGAGGGCTGCAACTACTACTGCAAGAAGCCTGAAGGTCCTAACAAGGGCTCT  120
21       P  R  P  E  G  C  N  Y  Y  C  K  K  P  E  G  P  N  K  G  S   40

121     AACTACTGCTGCGGCCCGGAGTACATCCCGCTGAAGCGGGAAGAGAAGCACGCTGGTAAT  180
41       N  Y  C  C  G  P  E  Y  I  P  L  K  R  E  E  K  H  A  G  N   60

181     TGCCCGCCTCCTCTCAAGGAATGCACAAGGTTCCCAAGACCACCTCAGGTGTGCCCCCAT  240
61       C  P  P  P  L  K  E  C  T  R  F  P  R  P  P  Q  V  C  P  H   80

241     GATGGACATTGTCCCTACAACCAGAAGTGTTGCTTCGACACCTGTCTCGACATCCACACC  300
81       D  G  H  C  P  Y  N  Q  K  C  C  F  D  T  C  L  D  I  H  T   100

301     TGCAAGCCAGCTCACTTCTATATTAACTAG                                 330
101      C  K  P  A  H  F  Y  I  N                                     109
```

Figure 10 The deduced amino acid sequences of the 16 kDa protein shown perfect nucleotide match with a putative antimicrobial peptide found in a crayfish hemocyte EST-library (Accession number CF542515). The umbers of nucleotides are shown in both of right and left in the column.

```
1    -    GGCACGAGGCATCAGCTCACCTGCGCCTCCAGCACTTCCTCTCACTAAACATGCTGCGTG -60
                                                              M  L  R

61   -    TACTGATGGTGTCGCTGCTGGTGGTGGCGGCGCTCGGCCACATCTCCCCGCCCCGGCCGG -120
          V  L  M  V  S  L  L  V  V  A  A  L  G  H  I  S  P  P  R  P

121  -    AGGGCTGCAACTACTACTGCAAGAAGCCTGAAGGTCCTAACAAGGGCTCTAACTACTGCT -180
          E  G  C  N  Y  Y  C  K  K  P  E  G  P  N  K  G  S  N  Y  C

181  -    GCGGCCCGGAGTACATCCCGCTGAAGAGGGAAGAGAAGCACGCTGGTAATTGCCCGCCTC -240
          C  G  P  E  Y  I  P  L  K  R  E  E  K  H  A  G  N  C  P  P

241  -    CTCTCAAGGAATGCACAAGGTTCCCAAGACCACCTCAGGTGTGCCCCCATGATGGACATT -300
          P  L  K  E  C  T  R  F  P  R  P  P  Q  V  C  P  H  D  G  H

301  -    GCCCCTACAACCAGAAGTGTTGCTTCGACACCTGTCTCGACATCCACACCTGCAAGCCAG -360
          C  P  Y  N  Q  K  C  C  F  D  T  C  L  D  I  H  T  C  K  P

361  -    CTCACTTCTATATTAACTAGCTTGAGCGGGCGAGAGCGTACCCGCAGCTCGACTGTGTCC -420
          A  H  F  Y  I  N  *  L  E  R  A  R  A  Y  P  Q  L  D  C  V

421  -    CTTCAAGACGGAGCCACACTTCGCTGAACTGAACTACCGTGCTATGCTTGTTTAGTGGCT -480
          P  S  R  R  S  H  T  S  L  N  *  T  T  V  L  C  L  F  S  G

481  -    GACTTGCTTGTTATGCTCACTTGTCTTTCTTATTGTTTATTTAAAAGGAACATCGGTTTC -540
          *  L  A  C  Y  A  H  L  S  F  L  L  F  I  *  K  E  H  R  F

541  -    ATGTATAAATGAGAGGAGAATTGTTTATTCAATAAAAAAATACTTTGTTAAAAAAAAAAA -600
          H  V  *  M  R  G  E  L  F  I  Q  *  K  N  T  L  L  K  K  K

601  -    AAAAAA                                                    -606
```

Figure 11 The deduced amino acid sequences of the 14 kDa protein. The numbers of nucleotides are shown in both of right and left in the column. These nucleotides match with the MALDI-TOF-MS.

Invertebrate animals, such as the crayfish, *P. leniusculus*, mount extremely efficient cellular and humoral innate immune response against invading pathogens and the circulating hemocytes are important in this defence (Söderhäll and Cerenius, 1992). The semigranular hemocytes display limited phagocytotic ability and are involved in encapsulation reactions (Smith and Söderhäll, 1983a; Söderhäll and Cerenius, 1992). The release of proteins from cellular vesicles in response to various stimuli is a fundamental process (Palade, 1975). The exocytosis of granular contents from hemocytes into the external milieu in response to endotoxin, peptidoglycans, and β -1,3-glucans confers humoral immunity to invertebrates (Söderhäll and Cerenius, 1992). For example, the proPO-activating system and antibacterial proteins and peptides are released into the plasma (Smith and Söderhäll, 1983a; Söderhäll and Cerenius, 1992). In this study, the Ca^{2+} ionophore A23187, LPS-PGN, and peroxinectin were able to induce degranulation *in vitro* of isolated granular cells from crayfish, resulting in nine, six and seven exocytosed proteins respectively. Six identical proteins, $I_{1,4,5,6,7,8}$ and $L_{1,2,3,4,5,6,}$ and $P_{1,3,4,5,6,7}$ were found to be released by the three exocytosis triggers. One protein was released upon Ca^{2+} ionophore A23187 and peroxinectin (I_3 and P_2) treatment whereas bands I_4 and I_9 were strongly released only from Ca^{2+} ionophore A23187.

The cMas I has been isolated, purified, and characterized from hemocytes of the crayfish by Lee and Söderhäll (2001). This protein is present in crayfish hemocytes as a heterodimer composed of two subunits with molecular masses of 134 and 129 kDa. The cMas I is functionally similar to human azurocidin, which is a multifunctional protein with antimicrobial activity against both Gram-negative, Gram-positive bacteria and *Candida albicans*. It also exhibits both LPS and heparin binding activity (Lee and Söderhäll, 2001). Several serine proteinase homologue proteins have been characterized from different animals and the inactive serine proteinase-like domains show significant functions, for example cell adhesion activity (Huang *et al*., 2000), LPS binding activity (Gabay *et al*., 1990), and antimicrobial activity (Almeida *et al*., 1996). The cMas I protein plays a key role in innate immunity as a pattern recognition protein that recognizes Gram-negative bacteria and yeast and is involved in the clearance of microorganisms from the hemolymph (Lee and Söderhäll, 2001).

Also, in this study, a significant band released with a mass of about 76 kDa was observed. It is supposed to be a peroxinectin (Johansson *et al*.,1995) and/or prophenoloxidase (Söderhäll *et al*., 1994; Söderhäll and Cerenius, 1998) and those bands were not analyzed. Previously the results have shown that prophenoloxidase activating system is released from hemocytes by the Ca^{2+} ionophore A23187 (Johansson and Söderhäll, 1985) and that components of the system are present in their vesicles (Liang *et al*., 1992).

The protein with a mass of 42 kDa is a masquerade-like serine proteinase which contains a non-catalytic serine proteinase domain from E_{150} to I_{385}. This protein, cMas II, is similar to several masquerade-like serine proteinases in insects such as that of *Tenebrio* prophenoloxidase activating factor (Lee *et al*., 2002), *Bombyx* masquerade-like serine proteinase homologue (Accession number AAN77090), *Holotrichia* prophenoloxidase activating factor-2 (PPAF-2) (Liang *et al*., 2002), *Manduca* serine proteinase- like protein-1(Yu *et al*., 2003) and *Anopheles* serine protease like protein Agmas1(Accession number CAB93496). It is also similar to *Tachypleus* factor-D involved in the clotting system in horseshoe crab.

The masquerade-like serine proteinase homologues from *Tenebrio* and *Holotrichia* are involved in the proPO activating cascade and are therefore named prophenoloxidase activating factors (PPAFs). These PPAFs have some common characteristics in structure and function. They possess a carboxy-terminal serine proteinase domain and a clip domain in the amino terminus and they are involved in cleaving proPO (Lee *et al*., 1998, 2002). In *Holotrichia*, the serine proteinase PPAF-I in the absence of a protein cofactor PPAF-II, converts the 79 kDa proPO to an intermediate form with a mass of 76 kDa, without exhibiting PO activity. However, in the presence of PPAF-II, and PPAF-I the 79 kDa proPO is converted to active PO with a mass of 60 kDa (Lee *et al*., 1998). In crayfish, a prophenoloxidase activating enzyme (Wang *et al*., 2001) can cleave proPO by itself without interaction of a masquerade-like protein. The new masquerade-like protein in this study is similar to that of insect PPAFs and may be required in the upper steps of the proPO-cascade.

The two proteins with masses of 38 and 32 kDa contain high glycine-rich sequences similar to a novel glycine-rich antimicrobial peptide constitutively expressed in the hemocytes of the spider, *A. gomesiana* (Lorenzini *et al*., 2003). Acanthoscurrins are linear cationic peptides with a high glycine (72-73%) amino acid composition and presence of the three-fold repeats of 26 amino acids (GGGLGGGGLGGGGLGGGK GLGGGGLG) (Lorenzini *et al*., 2003).

The protein with a mass of 28 kDa show similarities with a mannose receptor protein from *M. musculus* (house mouse) (Harris *et al*., 1992) which mediates macrophage phagocytosis of bacteria, fungi and some enveloped viruses in the vertebrate innate immune response (Neth *et al*., 2000). Mannose binding proteins (MBP), or MBL (mannose binding lectin) are C-type lectins, which bind to mannose or N-acetylglucosamine (GlcNAc) on the surfaces of pathogens in a calcium- dependent manner (Weis *et al*., 1998). MBP contains collagen-like regions and the Ca^{2+}-dependent carbohydrate recognition domain (CRD), and acts as an opsonin and also activates the complement system by forming a complex with a novel serine protease termed MBL-associated serine protease (MASP) in serum (Matsushita *et al*., 1992).

The ~20 kDa protein, VMO-I, was found to be similar to a vitelline membrane outer layer protein I (VMO-I) from common carp (*C. carpio*) (Chang *et al*., 1999) and chicken (*G. gallus*) (Uyeda *et al*., 1994) . VMO-I is a basic protein composed of 163 amino acids, with a molecular mass of ~ 18 kDa in quail and other poultry eggs (Burley *et al*., 1987; Kido *et al*., 1995), whereas the mature protein of an outer layer protein of carp fertilization envelope is 21 kDa and has 58 % identity to the outer layer protein of chick vitelline membrane (Chang *et al*., 1999). In crustaceans, VMO-I proteins are known to be synthesized in females by extraovarian tissue and then transported via the hemolymph to the developing oocytes (Byrne *et al*., 1989). The major role of the vitelline membrane is to prevent the mixing of the yolk and albumen and also act as an important antimicrobial barrier, as indicated by the high content of lysozyme in the outer layer (Back *et al*., 1982). Sugihara *et al.* (1995) demonstrated the presence of a substance with an ovomucin-like property, viral antihaemagglutinin activity, in the vitelline membrane. In crayfish, this protein is stored in granules of the granular hemocytes and is released by exocytosis. However, the specific role of this protein in crayfish immune defense still remains to be elucidated.

Proteins with masses of 16 and 14 kDa are anti-microbial peptides. Antimicrobial peptides are known to be involved in the innate immune response of vertebrates and invertebrates as well as plants (Cammue *et al*., 1994; Lehrer and Ganz, 1999). In horseshoe crab, antimicrobial peptides are mainly synthesized in hemocytes, stored in the granules and released into the hemolymph upon microbial stimulation (Iwanaga, 2002). Antimicrobial peptides from crustaceans have previously been isolated from the hemocytes of the green crab *C. maenas* (Schnapp *et al*., 1996), blue crab *Callinectes sapidus* (Khoo *et al*., 1999), freshwater crayfish, *P. leniusculus* (Lee *et al*., 2003, 2004), and the shrimp *P. vannamei* (Destoumieux *et al*., 1997).

Exocytosis of granular cells *in vitro* could be evoked by the Ca^{2+} ionophore A23187, LPS-PGN, and also peroxinectin. LPS-PGN is a component of bacterial cell walls which was found to induce exocytosis *in vitro* of granular hemocytes from horseshoe crab. It has been reported to occur through a G protein-mediating pathway that activates a phospholipase C on hemocytes surface, leading to the LPS-induced exocytosis (Ariki *et al*., 2004). Peroxinectin is a cell adhesive factor and also acts as a degranulation factor (Johansson and Söderhäll, 1988, 1989) and this is brought about by triggering an intracellular signaling pathway involving protein kinase C (Johansson and Söderhäll, 1985). An interesting finding was that a majority of the proteins in the vesicles is antimicrobial peptides and this is logic, since microorganisms release LPS-PGN or β -1, 3-glucans which can cause exocytosis of arthropod hemocytes and hence these microorganisms eliciting this release will be killed in proximity to the hemocytes. Further two masquerade-like proteins, one of which functioning as an opsonin, and the other may be involved in the proPO system are also abundant and released from the hemocytes upon microbial challenge.

White Spot Syndrome Virus (WSSV) Interaction with Crayfish Hemocytes

Jiravanichpaisal *et al*. (2001) have demonstrated that WSSV had a significant effect on the proportion of different hemocyte types in crayfish, *P. leniusculus*. The number of GCs was significantly higher in WSSV infected crayfish compared to sham-injected and non-injected animals. In the current study, WSSV particles were detected by *in situ* hybridization in separated GCs and SGCs. The prevalence of WSSV-infected cells from moribund crayfish was for SGCs 22 % while only 5 % of the GCs contained the virus (Figure12). This suggests that GCs are more resistant to WSSV than SGCs and the SGCs are more susceptible and gradually disappeared from the blood circulation. The susceptibility of SGCs to WSSV was also observed in the shrimp, *P. merguiensis*, during WSSV infection. The results showed that the SGCs were highly susceptible to WSSV infection, had higher virus load and infection rate compared to GCs (Van de Braak *et al*., 2002). In contrast to crayfish, the number of GCs and SGCs continuously increased during the infection particularly, the percentage of infected SGCs increased rapidly from 21 % on day 2 to 65 % on day 3, while the increase in percentage of the infected GCs was 7 % and 23 % respectively. In other studies, the total number of circulating hemocytes in shrimp were dramatically decreased after being infected with WSSV (Van de Braak *et al*., 2002; Guan *et al*., 2003; Wongprasert *et al*., 2003). Söderhäll *et al*. (1986) proposed that SGCs are the first hemocyte type to react to foreign particles *in vivo* and that they respond by degranulation and thus release of the proPO system into the plasma. The system will be activated and triggered more degranulation by peroxinectin, which then causes an amplified secretion of the proPO system from both SGCs and GCs.

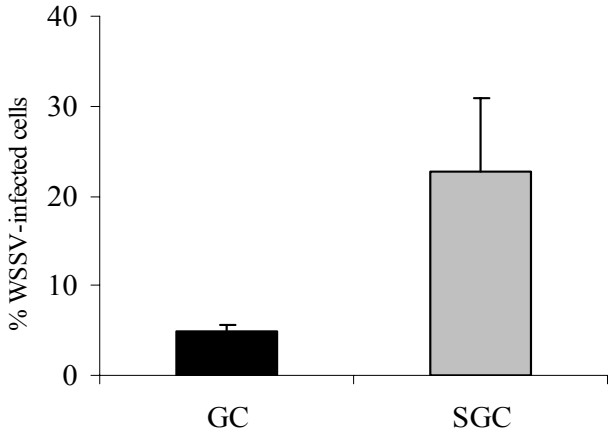


Figure 12 The percentage of WSSV-infected cells from moribund crayfish detected by *in situ* hybridization of SGCs and GCs.

In order to understand more of the virus hemocyte interaction, the effect of hemocyte lysate supernatant (HLS), containing the degranulation factor peroxinectin, on isolated granular cells was performed. As shown in Figure 13, the percentage of degranulated cells of WSSV-infected crayfish was significantly lower ($p < 0.05$) than those of sham-injected crayfish. In addition, the Ca^{2+} ionophore A23187 and the PKC-activating PMA was also used as secretagogues (Johansson and Söderhäll, 1985). As a result, PMA in a dose-dependent manner affected the degranulation of GCs from WSSV infected crayfish as shown in Figure 14. The percentage of degranulated cells from WSSV-infected crayfish was significantly lower than those from the sham-injected crayfish, whereas the non-spread cells were significantly higher. There was no significant difference in the percentage of cell degranulation when treated with both Ca^{2+} ionophore A23187 and CFS in both sham and WSSV-infected crayfish. The active 76-kDa protein, peroxinectin in HLS, which mediates attachment and spreading of the hemocytes, also triggers degranulation (regulated exocytosis) of granular cells (Johansson *et al*., 2002). Peroxinectin can bind to its receptor superoxide dismutase (Holmblad and Söderhäll, 1999) on the hemocyte surface, but little is known how binding of the peroxinectin ligands leads to the biological effects and cellular activities of degranulation, cell spreading, and phagocytosis in arthropod blood cells. However, it is shown that binding of peroxinectin triggers cellular responses via a pathway that includes protein kinase C (PKC) activation and elevated protein tyrosine phosphorylation of a cellular protein of ~80 kDa (Johansson and Söderhäll, 1993). PKC is a family of enzymes that are physiologically activated by 1, 2-diacylglycerol (DAG) or its surrogate, phorbol 12-myristate 13-acetate (PMA) and other lipids. It was shown that PMA alone caused a dose-dependent degranulation (Johansson and Söderhäll, 1993).

The Ca^{2+} ionophore A23187 has been widely used to induce degranulation in amebocytes of *Limulus polyphemus* and crayfish hemocytes and proteins released are for example protease inhibitors (Armstrong, 1985), the proPO system (Johansson and Söderhäll, 1985) and other proteins (Sricharoen *et al*., 2005). HLS-treatment of GCs and SGCs from WSSV-infected crayfish resulted in three different cell-reactions; non-spread, spread and degranulated cells and these different ‘‘cell-reactions’’ were investigated for presence of virus infection by *in situ* hybridization. The non-spread cell group from both GCs and SGCs had more WSSV positive cells than the degranulated cells (Figure 15 and Figure 16). Based on these results, it was reasonable to assume that the PKC pathway might be somehow affected by WSSV during its replication inside the cells.

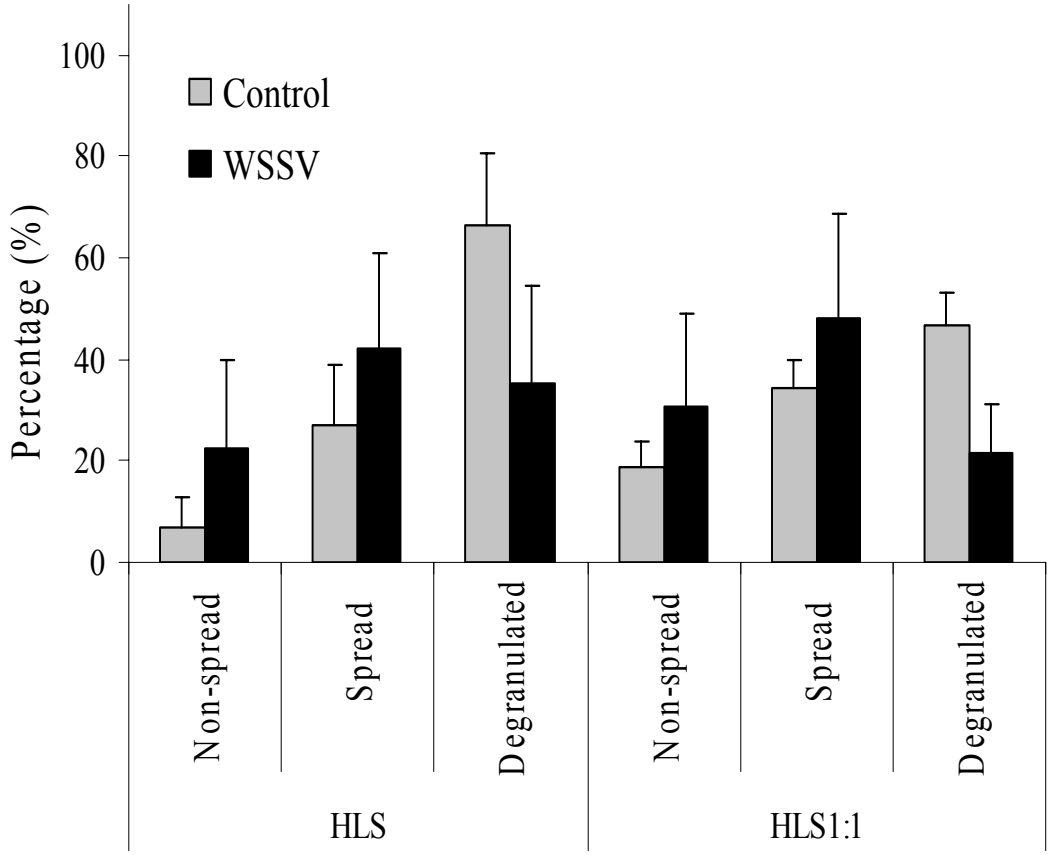


Figure 13 Percentage of degranulated, spread and non-spread GCs from WSSV-infected and sham-infected crayfish after treatment with HLS. (WSSV= Black columns; Control= grey columns)

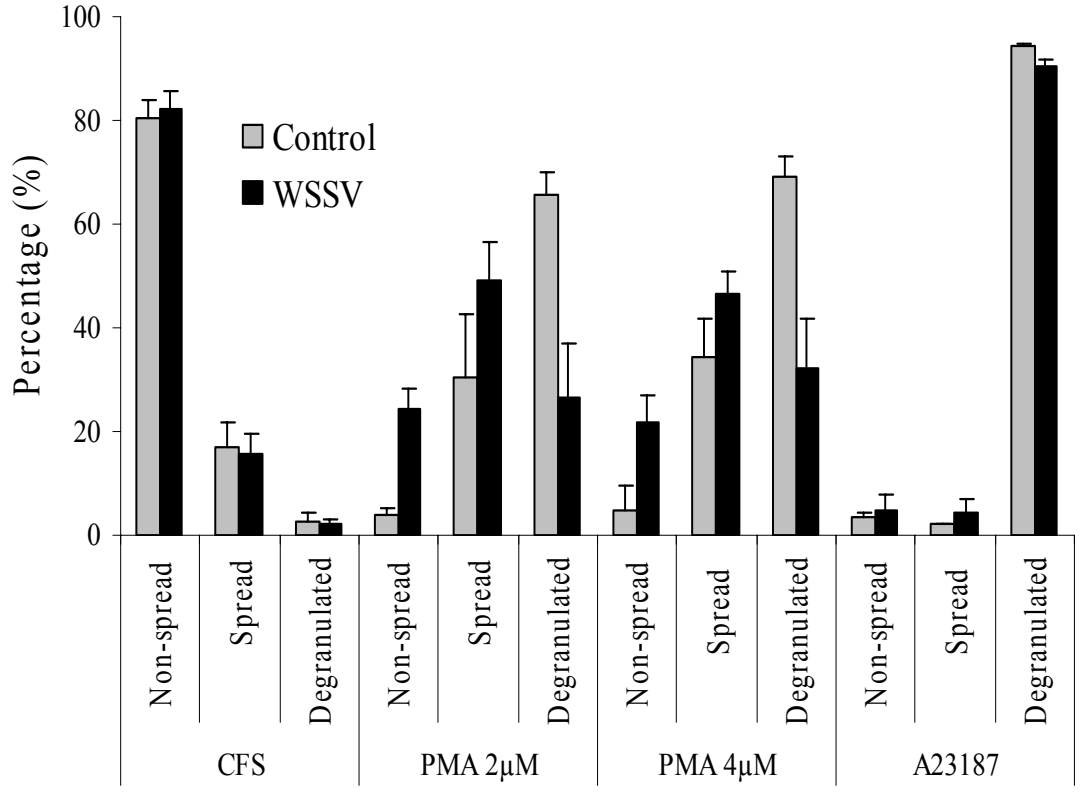


Figure 14 Percentage of degranulated, spread and non-spread GCs of WSSV infected and sham-infected crayfish after treatment with PMA (2µM and 4µM), the Ca^{2+} ionophore A23187 and CFS as control.

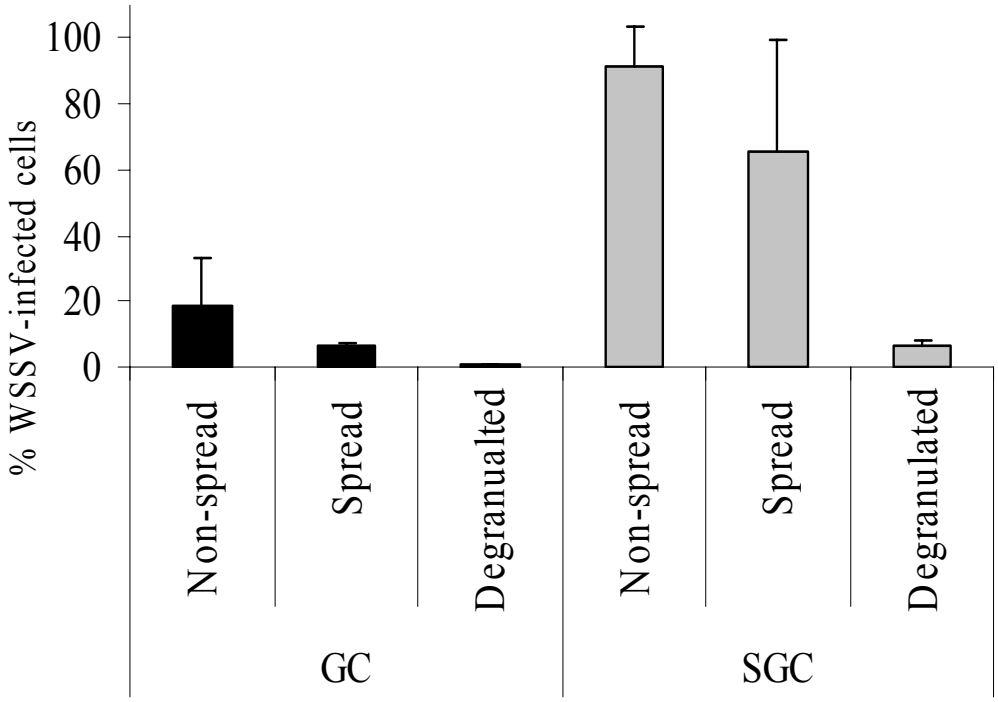
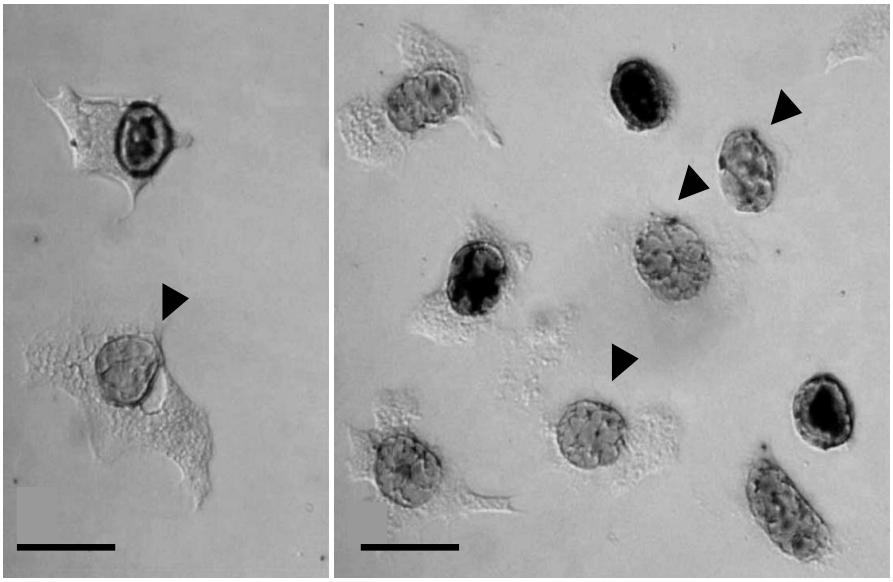


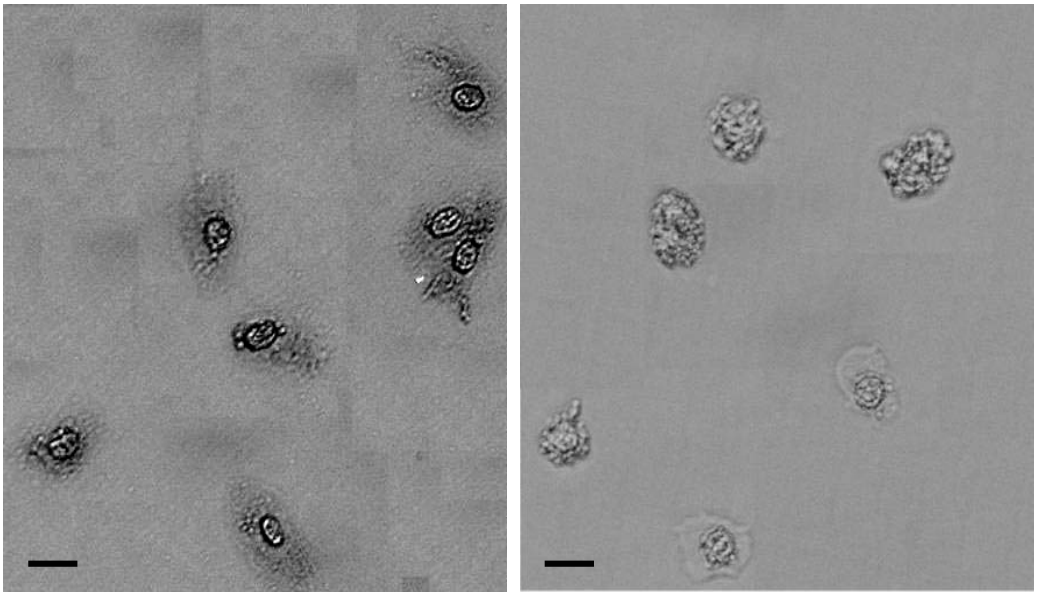
Figure 15 Percentage of WSSV-infected cells in non-spread, spread and degranulated cells after treatment with HLS (GCs = Black columns; SGCs = grey columns).



(a) (b)

Figure 16 The presence of WSSV in HLS-treated hemocytes as detected by *In situ* hybridisation (a) GCs, WSSV is present in non-spread cells (dark nuclei) but absent in degranulated cells (arrows). (b) SGCs, WSSV is present in non-spread cells (dark nuclei) but absent in completely degranulated cells (arrows). Scale bars = 10 μM

Isolated GCs from sham-injected and WSSV-injected crayfish were incubated in modified L15 medium at room temperature for at least 16 h and the GCs from sham-injected crayfish melanized after degranulation, while no melanization was found in cells from WSSV infected crayfish regardless of whether the cells were or were not degranulated as shown in Figure 17. So far, little is known about the interactions of pathogenic viruses with the immune system of other invertebrates except for one study of polydnaviruses (PDVs) in insect. In many species, parasitized by braconid and ichneumonid wasps, host immunosuppression appears to be mediated by PDVs injected by the female parasitoid into the host hemocoel. The parasitoids likely exploit other mechanisms of immunoevasion via antigen masking, antigen mimicry, or production of active inhibitors of the hemocyte-mediated encapsulation response as well as to inhibit the melanization reaction directly (Lavine and Beckage, 1995). However, the phenoloxidase activities in hemocyte lysate supernatant of both sham-injected and WSSV-injected crayfish remained similar as well as proPO expression as detected by RT-PCR. This suggests that the observed inhibition of melanization in cells is affected upstream of phenoloxidase or alternatively by depletion of the native substrate for PO.



(a) (b)

Figure 17 Melanization in isolated GCs after incubation in L-15 medium at room temperature for at least 16 h; (a) from sham-injected crayfish and (b) from WSSV-injected crayfish. Scale bars = 10 μM

Successful viral replication requires not only the efficient production and spread of progeny, but also evasion of host defence mechanisms that could limit replication by killing infected cells. In addition to inducing immune and inflammatory responses, infection by most viruses triggers apoptosis or programmed cell death of the infected cell. Some viruses seem to use apoptosis as a mechanism of cell killing and enhanced virus spread. In both cases, successful replication relies on the ability of certain viral products to block or delay apoptosis until sufficient progeny have been produced.

The hall mark characteristic of apoptotic cells was described by Roulston *et al*. (1999) that the membrane of an apoptotic cell actively blebs, but remains intact, ultimately blebbing the cell apart into membrane-bound apoptotic bodies that contain cytoplasmic and/or nuclear material. According to this description, many small cells were found scattering among the hemocytes from WSSV-infected crayfish. These small apoptotic bodies varied in size and contained nuclear material confirmed by propidium iodide staining. Moreover, WSSV-DNA was detected in these bodies by *in situ* hybridization as shown in Figure 18. This study also detected the presence of apoptotic cells in circulating hemocytes by TdT-mediated X-dUTP nick end labeling (TUNEL) assay in sham-injected and WSSV-injected crayfish. Surprisingly, the percentage of apoptotic cells was low in both groups (Table 3). In infected crayfish, however, about 1.5 % and 1 % of the hemocytes were apoptotic at day 3 and day 5 post injections, respectively and significantly higher than in the control crayfish, which were about 0.15 % and 0.1 %, respectively at day 3 and day 5.

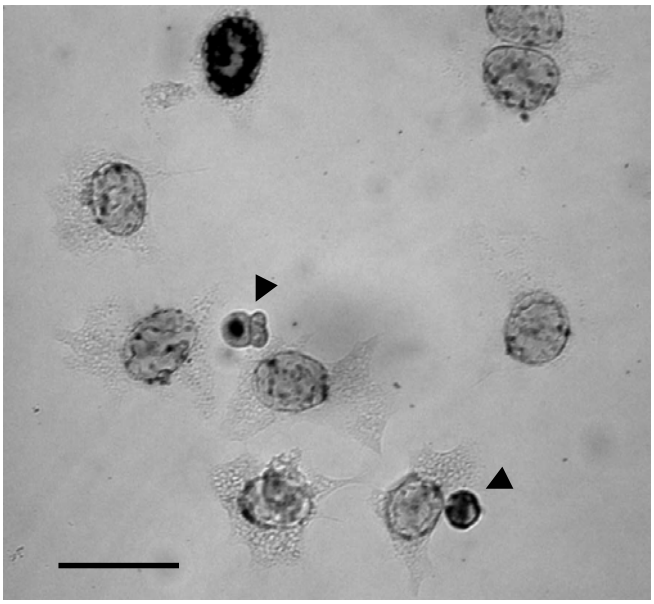


Figure 18 Percentage of apoptotic cells among circulating hemocytes analyzed by TUNEL assay at 3 and 5 days post-injection of WSSV. Scale bars = 10 μM

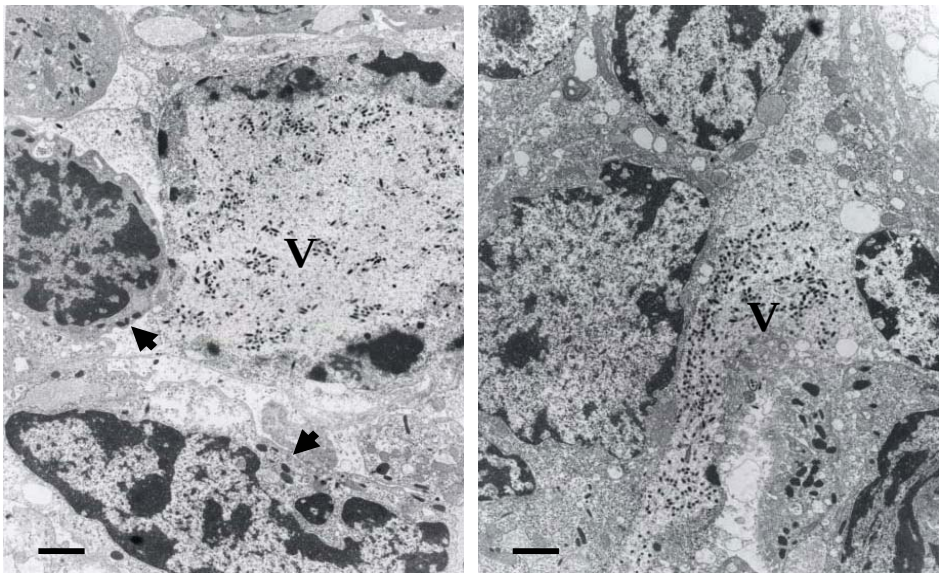
Table 3 The percentage of apoptotic cells in both control and WSSV-infected crayfish

	% Apoptotic Cells	
	3 days post injection	5 days post injection
Control crayfish	0.15	0.1
WSSV-infected	1.50	1.0

A low percentage of apoptosis was also found in the lymphoid organ of *P. japonicus* following WSSV-infection, and the percentage of apoptosis from infected-shrimp with high dose of WSSV were 2.9 % and 1.6 % on day 3 and 5, respectively, whereas with a low dose were 1.7 % and 0.4% on day 3 and day 5, respectively (Wu and Muroga, 2004) and around 3 % in the hematopoietic tissue of *P. monodon* at 36 h post infection (Wongprasert *et al*., 2003). However, as detected by DAPI-staining, the percent of apoptotic hemocytes was low at 24 h post virus infection but reached about 20 % at 60 h (Wongprasert *et al*., 2003). Anggraeni and Owens (2000) considered apoptosis as the defence responsible for eliminating the virus, whereas Khanobdee *et al*. (2002) suggested that progressive occurrence of apoptosis may be the cause of death in *P. monodon* infected with yellow head virus (YHV). Wongprasert *et al*. (2003) clearly demonstrated that apoptosis occurs following WSSV infection in *P. monodon*, but the importance of this for the mortality needs further investigation.

Since the prevalence of apoptotic cells was very low, it may indicate that WSSV may induce anti-apoptotic mechanisms. In baculovirus, P35 and IAPs (inhibitors of apoptosis proteins) proteins were found to be involved in inhibition of host cell apoptosis. The function of IAPs in insect and vertebrates is not only to inhibit apoptosis during development but also to prevent a defensive apoptotic response of the host cells. IAPs have been cloned and characterized in silkworm, *Bombyx mori* (Kim *et al*., 2001), and very recently, the ORF 390 of WSSV genome was identified as a novel anti-apoptotic gene (Wang *et al*., 2004). They also confirmed that the WSSV could infect shrimp primary cells derived from lymphoid tissue and block the cell apoptosis induced by the actinomycin D, in turn; the cells infected with virus prior to actinomycin D treatment did not show characteristics of apoptosis.

Finally, WSSV-infected cells in hematopoietic tissues from moribund WSSV-infected crayfish were observed by transmission electron microscope (TEM). As shown in Figure 19 (a) an infected cell with enlarge nuclei containing massive WSSV particles had no fine granules, whereas the cells with presence of fine granules adjacent to the infected cells did not contain WSSV. This suggests that WSSV infects specific cell types in the hematopoietic tissue. A cluster of intact WSSV virions was found outside the cells as shown in Figure 19 (b). In shrimp, intact virus particles were found in the cytoplasmic vacuoles of GCs but not in nuclei (Wang *et al*., 2002) possibly due to virus infection/replication, phagocytosis or endocytosis. Intact WSSV in vacuoles of hematopoietic cells with heavy infection were also found in this study.



(a) (b)

Figure 19 Electron micrographs of WSSV-infected hematopoietic tissue (a) mature WSSV virions in the enlarged nucleus of a heavily infected hematopoietic cell. No fine granules were found in the cytoplasm, whereas adjacent cells with define granules (arrows) were not infected. (b) Massive mature WSSV virions were found outside the nucleus. Scale bars = 500 nM

In conclusion, these studies suggested that the SGCs were more susceptible to WSSV infection. WSSV infected GCs were resistant to degranulation induced by PMA or HLS, but not by the Ca^{2+} ionophore A23187. Therefore, the PKC pathway might affected by WSSV during its replication inside the cells. It was also found that WSSV inhibited the proPO system in hemocytes. However, the mechanism of this phenomenon has still to be elucidate

CONCLUSION

Different activators: lipopolysaccharide-peptidoglycan (LPS-PGN), the Ca^{2+} ionophore A23187, and peroxinectin (a cell adhesion and degranulation factor from the hemocytes) were used to induce degranulation *in vitro* of isolated granular cells from the freshwater crayfish *P. leniusculus*. Nine major protein bands which released from the isolated granular cells after challenge with the Ca^{2+} ionophore A23187 were observed. Six of them were characterized as a masquerade-like protein (cMas I), a masquerade-like protein (cMas II), a mannose receptor protein (MRP), a vitelline membrane outer layer protein I (VMO-I), and two antimicrobial peptides (AMPs). The released protein band with a mass of 76 kDa is more likely prophenoloxidase and/or peroxinectin and was not analyzed (Johansson and Söderhäll, 1988). When peroxinectin was used as a trigger of exocytosis seven strong protein bands could be identified and for the lipopolysaccharide-peptidoglycan six proteins could be identified and all of them were also released by the Ca^{2+} ionophore A23187 treatment.

The interactions between WSSV and crayfish hemocytes have been studied. The hemocyte lysate supernatant (HLS) containing the degranulation factor, peroxinectin, phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore A23187 were used as a triggers for inducing degranulation in both GCs of non-injected and WSSV-infected crayfish. The results showed that the percentage of degranulated cells from WSSV-infected crayfish was significantly lower than those from non-injected crayfish when HLS or PMA was used, whereas no significant difference was found using the Ca^{2+} ionophore A23187. Peroxinectin and PMA have a degranulation effect via an intracellular signaling involving protein kinase C (PKC) (Johansson and Söderhäll, 1993), which might be affected by WSSV during infection, whereas the Ca^{2+} ionophore A23187 uses an alternative pathway.

When GCs are incubated for longer times in a culture medium used for crayfish hematopoietic cells, melanization can be observed around the cells. However when GCs from WSSV infected cells were used melanization did not occur indicating that the virus in some way interfere with the melanization process. In this study, experiment was designed to elucidate whether this phenomenon was due to lack of active PO or lower proPO expression in virus infected animals. However no difference in PO activity could be detected in HLS from WSSV-infected and sham-injected crayfish, and as shown by RT-PCR no difference in proPO expression that could explain lack of melanization in WSSV infected animals was found. This suggests that the observed inhibition of melanization in cells is affected upstream of phenoloxidase, or that the endogenous substrate is depleted by some unknown mechanism in virus infected crayfish.

The presence of apoptotic cells in circulating hemocytes was detected by TUNEL assay in sham-injected and WSSV-injected crayfish. The results show that the percentage of apoptotic cells was low in both groups, but significantly higher in WSSV-infected animal. It has been found that the mechanisms of apoptosis are conserved across animal species. For example, the black tiger shrimp *P. monodon*, apoptosis occurred in the lymphoid organ cells and apoptosis acts as the mechanism responsible for eliminating the virus-infected cells (Anggraeni and Owens, 2000).

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APPENDIX

Appendix A

Exocytosis and Proteomic Analysis of the Vesicle Content of Granular Hemocytes from a Crayfish

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Developmental & Comparative Immunology
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Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish

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Received 16 February 2005; revised 14 March 2005; accepted 31 March 2005
Available online 17 May 2005

Abstract

The circulating blood cells (hemocytes) of invertebrates are important in cellular immune reactions and to deliver immune factors synthesized in these cells to the external milieu. Previously, we have shown that release of vesicle contents is involved in a regulated exocytosis and here we show which proteins in the vesicles are most abundant and which are released by triggering of exocytosis using a calcium ionophore, lipopolysaccharides–peptidoglycan and peroxinectin, a cell adhesion and degranulation factor from the hemocytes. The ionophore caused release of nine proteins and six of them were characterized and found to be a masquerade-like protein, a masquerade-like serine proteinase, a mannose receptor protein, a vitelline membrane outer layer protein I, and two anti-microbial peptides. The released protein band with a mass of 76 kDa is more likely pro-phenoloxidase and/or peroxinectin. When peroxinectin was used as a trigger of exocytosis, seven proteins could be identified and for the lipopolysaccharides–peptidoglycan six proteins could be identified and all of them were also released by the ionophore treatment. Interestingly, several anti-microbial peptides were the most abundant proteins and were efficiently released by all treatments as were two masquerade-like proteins one of which is functioning as an opsonic protein.

Keywords: *Pacifastacus leniusculus*; Crayfish; Exocytosis; Calcium (Ca^{2+}) ionophore A23187; Lipopolysaccharide–peptidoglycan; Peroxinectin; Granular hemocyte; Electrophoresis

1. Introduction

Crayfish have an innate immune system, which consists of cellular and humoral responses. Cellular immune responses are mediated by the hemocytes (blood cells) in the hemolymph (blood) and consist of phagocytosis of small microbes, encapsulation of larger parasites and nodule formation. Humoral immune responses include hemolymph coagulation, exocytosis from the hemocytes of different proteins to

Abbreviations proPO, pro-phenoloxidase; PO, phenoloxidase; LPS–PGN, lipopolysaccharide–peptidoglycan; Prnx, peroxinectin; cMas I, crayfish masquerade-like protein; AMP, anti-microbial peptide; VMO-I, vitelline membrane outer layer protein I; cMas II, crayfish masquerade-like serine proteinase; MRP, mannose receptor protein.

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doi:10.1016/j.dci.2005.03.010

the plasma, proteolytic cascades leading to opsonization and melanization [1], and anti-microbial peptides [2]. Within the granules of the hemocytes several immune factors, such as the pro-phenoloxidase activating system, are present and release of these contents occurs by exocytosis (degranulation) [3]. The calcium ionophore A23187 was also found to induce degranulation of granular hemocytes from the crayfish, *Pacifastacus leniusculus* [4]. The secreted components released from the hemocytes to the plasma by lipopolysaccharide and β-1, 3-glucan are anti-microbial substances [5] and components of the pro-phenoloxidase (proPO), activating system [4]. Crayfish peroxinectin, a 76-kDa cell adhesion factor, was the first cell adhesion protein isolated from invertebrate blood [6] and it has both peroxidase and cell adhesion activity, and also acts as a degranulation factor, an encapsulation-promoting factor and an opsonin [7]. Peroxinectin is synthesized and stored within the granular and semi-granular hemocytes in an inactive form. It is released and activated concomitant with activation of the proPO activating system [6]. Peroxinectin can also trigger attachment, spreading, and degranulation of crayfish blood cells.

Exocytosis of amoebocytes of the Japanese horseshoe crab, *Tachypleus tridentatus*, by LPS induces the secretion of various soluble defense molecules, including serine protease zymogens, a clottable protein coagulogen, protease inhibitors, anti-microbial peptides, and lectins [8]. The LPS-induced exocytosis of the granular amoebocytes is mediated by a heterotrimeric GTP-binding protein and increasing the intracellular concentration of Mg^{2+} and Ca^{2+} will lead to a stimulation of the inositol-1, 4, 5-triphosphate-signaling pathway [9].

Lipopolysaccharides and the β-1, 3-glucan laminarin G could induce exocytosis of isolated semi-granular hemocytes from the crayfish, whereas the Ca^{2+} ionophore A23187 was found to strongly induce exocytosis both of semi-granular and granular cells [4]. LPS on the surfaces of invading bacteria induce degranulation of semi-granular cells, which thus release the proPO system into plasma. The system will be activated by the polysaccharides previously complexed with pattern recognition proteins and then causes an amplified secretion of the proPO system from both semi-granular and granular cells, since peroxinectin will be in its active form [10,11]. Also, exocytosis in crayfish hemocytes triggered by β-1, 3-glucans, LPS or peroxinectin has been shown to involve an intracellular signaling cascade with protein kinase C and tyrosine phosphorylation [4].

In this study, LPS–PGN, Ca^{2+} ionophore A23187, and peroxinectin were used to induce exocytosis of isolated granular crayfish hemocytes in vitro and to analyze which proteins are present and released from the vesicles.

2. Materials and methods

2.1. Animals

Freshwater crayfish, *P. leniusculus*, were from Berga kräftodling, Sweden, and kept in aquaria in aerated tap water at 10 °C. Only intermoult animals were used for the experiments.

2.2. Separation of hemocytes

The different hemocyte populations of *P. leniusculus* were separated and harvested by a density gradient centrifugation method described by Söderhäll and Smith [10]. Continuous density gradients of 70% Percoll in 0.15 M NaCl were centrifuged at 25,000*g* for 30 min at 4 °C. About 0.5 ml crayfish hemolymph was bled from the abdominal hemocoel through a needle (0.8 mm) into 1 ml of anti-coagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA, pH 4.6) and then immediately put on top of a preformed Percoll gradient. After centrifugation at 2900*g* for 18 min at 4 °C, granular cells (G-cells) were harvested with a Pasteur pipette and diluted with 0.15 M NaCl to 1:1.

2.3. Purification and immunoblotting of peroxinectin

Hemocyte lysate supernatant (HLS) was prepared by collecting hemolymph from 150 crayfish with 0.8×50 mm needles into 10 ml ice-cold buffer of 10 mM Na cacodylate, 0.25 M sucrose, and 100 mM $CaCl_2$, pH 7.0. The hemocytes were spun down at 800*g* for 10 min at 4 °C and the supernatant was removed. The resulting cell pellet was washed with the same buffer and homogenized with a glass

homogenizer in 12.5 ml cacodylate buffer containing 5 mM $CaCl_2$. The cell lysate was precipitated with ammonium sulfate (50% saturation) at 0 °C for 2 h and was collected by centrifugation at 10,000*g* for 15 min. The pellet was resuspended in 1.5 ml cacodylate buffer (10 mM Na cacodylate, pH 7.0) and then dialyzed against of the same buffer overnight. The dialyzed fraction was spun down at 5000*g* for 15 min. The supernatant was applied to a carboxymethyl-cellulose column (0.8×3.0 cm) (Sigma Chemical Co.) previously equilibrated with cacodylate buffer and then eluted with 0.25 M NaCl in cacodylate buffer. The fractions containing peroxinectin were pooled and 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.5 M NaCl were added. A Con A-Sepharose 4B (Pharmacia) (0.8×5.0) was equilibrated with 10 mM Na cacodylate, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.0, and the peroxinectin fractions were applied to this column, and then the column was washed with the same buffer until $A_{280}=0$ is achieved. The column was equilibrated for 2 h with 0.25 M α-methylmannoside in 10 mM Na cacodylate, 0.5 M NaCl (pH 7.0), and then washed with the same buffer until $A_{280}=0$. The peroxinectin was eluted by incubating the column overnight in 0.75 α-methylmannoside in 10 mM Na cacodylate, 0.5 M NaCl, pH 7.0. The fractions containing peroxinectin were pooled and stored at −20 °C. For immunoblotting, the purified peroxinectin was subjected to 15% SDS-PAGE under reducing conditions and then electrotransferred to nitrocellulose membranes in transfer buffer (25 mM Tris–HCl, 190 mM glycine, and 20% methanol) for 2 h at 280 mA on ice. The membrane was subsequently blocked in TTBS (0.1% Tween 20 mM Tris–HCl and 150 mM NaCl, pH 7.4) containing 3% BSA for 1 h and incubated with Ab TTBS containing 0.1% BSA overnight. An affinity-purified Ab (10 μg/ml) to peroxinectin was used for immunoblotting. The membrane was washed with TTBS once for 15 min and three times for 5 min each time. The anti-rabbit IgG peroxidase-conjugated IgG diluted 1/1000 with TTBS containing 0.1% BSA was incubated for 1 h and washed with TTBS for 15 min and three times for 5 min each time. The ECL Western blotting reagent kit (Amersham Pharmacia Biotech) was used for the detection and the resulting peroxinectin was used in the experiments.

2.4. Degranulation assay

Isolated suspended granular cells were treated with $CaCl_2$ 20 mM final concentration to allow the cells to attach to sterile well plates for 25 min at room temperature (20 °C). The resulting cell monolayers were washed with crayfish saline (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) [12] and then treated with triggers of degranulation: Ca^{2+} ionophore A23187 (Sigma Chemical Co.) (10 μM, diluted from a stock solution of 5 mM in DMSO containing 10 mM $CaCl_2$), lipopolysaccharides–peptidoglycan (LPS–PGN) (10 μg/ml) and peroxinectin (20 μg/ml) for 30 min to 1 h at 20 °C. Control monolayers were incubated with only CFS. For inhibition studies, monolayers of attached cells were pretreated with SITS (4-acetamido-4′-isothiocyanatostilbene-2, 2′-disulfonic acid disodium salt) at 20 °C for 30 min. The cells were then stimulated with LPS–PGN at a final concentration of 10 μg/ml in CFS at room temperature for 45 min. The exocytosed fluid was collected and precipitated with trichloroacetic acid (TCA) precipitation to recover proteins. The precipitated proteins were subjected to SDS-PAGE and stained with 0.2% Coomassie blue in 50% methanol.

2.5. Electrophoresis

SDS-PAGE was conducted by the method of Laemmli [13]. Samples were denatured by heating on a hot plate at 95 °C for 4 min in SDS-PAGE sample loading buffer with 0.1% DTT. The gels were stained with Coomassie blue and destained with destaining solution. A low molecular mass calibration kit for electrophoresis (Amersham Pharmacia Biotech, Arling Heights, IL) was used which contains: rabbit muscle phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa).

2.6. Determination of the amino acid sequence and mass spectrometry analysis

Nine protein bands were identified using an Applied Biosystem 476A automated amino acid

sequencer. To confirm amino acid sequences, mass spectrometry analysis, MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), was performed in a Q-tof tandem mass spectrometer (Micromass, Manchester, UK) equipped with nanospray interphase and using MassLynx program (Micromass, Manchester, UK) for interpretation of mass spectra.

2.7. cDNA cloning and sequencing

Partial cDNA sequences corresponding to the protein bands were obtained by random sequencing of a ZAP Express cDNA library constructed from mRNA of crayfish hemocytes. Degenerated primers were synthesized according to the amino acid sequences of proteins, and polymerase chain reaction (PCR) were followed by 94 °C for 2 min, and 35 cycles of 94 °C for 20 s, 45 °C for 20 s, and 70 °C for 1 min, followed by one 7 min extension period at 70 °C. The PCR products were purified by QIAquick-spin PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and subcloned into TOPO cloning vector (Invitrogen). The plasmids were released according to the instructions of the manufacturer (Sigma). The insert was digested out by the restriction enzyme *Eco*RI (Amersham Pharmacia Biotech) for 1 h at 37 °C and run on 1% agarose gel to confirm its size and finally sequenced with an Applied Biosystems PRISM dye terminator cycle sequencing ready reaction kit (Perkin–Elmer Life Sciences ABI 310). If the clones lacked the 5′ and 3′ region of the cDNA, this remaining region was amplified by 5′ RACE and 3′ RACE with a specific primer designed from the sequence, using hemocyte cDNA as template. Total RNA was isolated from hemocytes by a total RNA isolation kit (Sigma) following the manufacturer's instructions followed by treatment with DNase I. One microgram RNA was used to synthesize the first-strand cDNA (Thermoscript RT) (Invitrogen) and oligo dT primers according to the manufacturer's instruction. The cDNA was amplified in a 50 μl PCR reaction containing 2 μl cDNA, 1 μl of company supplied PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 μM of each primer and 2.5 unit of taq DNA polymerase (Invitrogen). The fragment was generated by PCR using a specific primer and an anchor primer (SMART IIA oligo). The PCR conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and one cycle of final extension at 72 °C for 7 min. The amplified products were separated on 1% agarose gel and visualized under UV illumination. The PCR fragments were done using the same protocol as above using QIAquick-spin PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and subcloned into TOPO cloning vector (Invitrogen).

2.8. Sequence analysis

The cDNA sequence was analyzed with the Mac Vector 4.1.4 software (Kodak Scientific Imaging Systems, New Haven, CT, USA). The nucleotide sequence and the deduced amino acid sequence were compared with the BLAST program (National Center Biotechnology International, Bethesda, MD, USA).

3. Results

3.1. Calcium ionophore A23187, LPS–PGN, and peroxinectin induce exocytosis

Degranulation occurred on the in vitro monolayers of separated granular cells of *P. leniusculus* when the cells were incubated with activators; the calcium ionophore A23187 (10 μM), LPS–PGN (10 μg/ml) and peroxinectin (20 μg/ml) (Table 1). All triggers caused degranulation but LPS–PGN caused the granular cells to round up and degranulate but not to undergo lysis (Fig. 1) while the control cells in CFS appeared to spread on the glass without degranulation (Fig. 2). These activators of degranulation induced the hemocytes to secrete their granular contents in a concentration-dependent manner. The secreted proteins were precipitated by TCA and then analyzed by 15% SDS-PAGE under reducing conditions. Many proteins were released by the calcium ionophore A23187 and nine of them, I_1–I_9 were subjected to further analysis. They had molecular masses of 134, 42, 38, 32, 28, 20, 16, 14, and 12 kDa in SDS-PAGE (Fig. 3). Six strong bands, L_1–L_6, were observed using LPS–PGN as a trigger with molecular masses of 134, 32, 28, 20, 16, and 14 kDa as shown in SDS-PAGE (Fig. 4). Seven strong bands, P_1–P_7, were obtained when peroxinectin was used as a trigger of

Table 1
Exocytosis of granular cells induced by the activators: A23187, LPS–PGN and peroxinectin

Treatment[a]	% Degranulated cells ± SD[b]	Number of experiments (*n*)
A23187 10 μM + $CaCl_2$ 10 mM	90 ± 12	5
LPS (10 μg/ml)	23 ± 8	5
Peroxinectin (20 μg/ml)	10 ± 4	6
Control (CFS)	0 ± 0	4
A23187 10 μM + $CaCl_2$ 10 mM + SITS 100 μM	4 ± 1	3
LPS (10 μg/ml) + SITS 100 μM	2 ± 1	3

n = number of experiments.

[a] Bands of separated granular cells from Percoll gradients were pooled and collected by centrifugation. The cell pellets were resuspended in 0.15 M NaCl and attached to coverslips as described in Section 2. The cell monolayers were incubated with A23187 10 μM and 10 mM $CaCl_2$ (final concentration) for 30 min at room temperature, or 10 μg/ml LPS–PGN for 40 min at room temperature, or peroxinectin 20 μg/ml for 1 h at room temperature. Control monolayers were kept in crayfish saline (CFS). In one experiment, the cell monolayers were preincubated with 100 μM SITS for 30 min at 20 °C before addition of A23187 10 μM + 10 mM $CaCl_2$ and LPS (20 μg/ml).

[b] At least 200 cells on each coverslip were counted under the phase contrast microscope; the percentage of lysed cells in these experiments was always less than 5%.

degranulation with masses of 134, 42, 32, 28, 20, 16, and 14 kDa, as shown in the SDS-PAGE (Fig. 5). The first band with a mass of 134 kDa was found to be the masquerade-like protein [14]. The second band, I_2, with a mass of 42 kDa is a masquerade-like serine protease. The third and fourth band with masses of 38 and 32 kDa (Fig. 3, I_3 and I_4) were found to contain glycine-rich sequences (GGGGFGGGGFGGG or LGGGGGGLGGGGFGGG). These glycine-rich

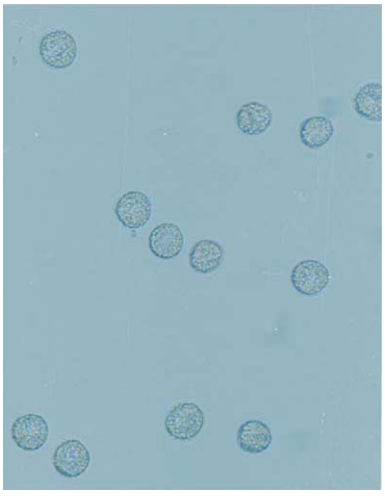

Fig. 1. Degranulated granular cells, incubated with LPS–PGN (10 μg/ml).

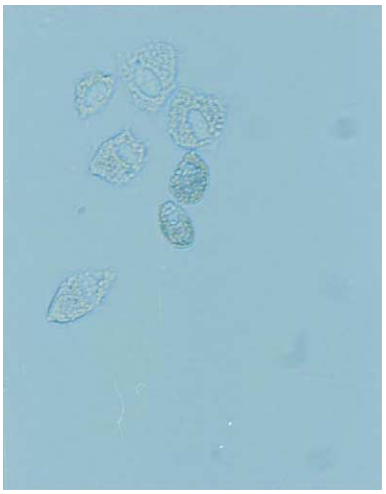

Fig. 2. Unaffected granular cells, control; incubated with CFS.

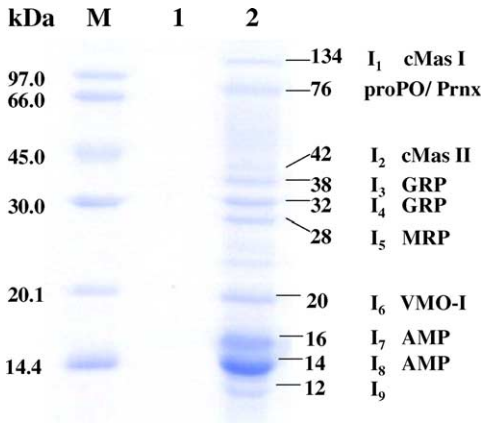

Fig. 3. Analysis of the exocytosed fluid from crayfish granular cells resulting after treatment with Ca^{2+} ionophore A23187. The exocytosed fluid was precipitated with trichloroacetic acid (TCA). The precipitated proteins were subjected to SDS-PAGE under reducing conditions and then stained with 0.2% Coomassie blue in 50% methanol. Lane M, size marker; lane 1, the precipitated proteins of the control cells after incubation with CFS; lane 2, 12 μg precipitated protein after treatment with the calcium ionophore A23187 for 30 min at room temperature. The first band with a molecular mass of 134 kDa was named band I_1 and 42, 38, 32, 28, 20, 16, 14, and 12 kDa were named I_2, I_3, I_4, I_5, I_6, I_7, I_8, and I_9, respectively.

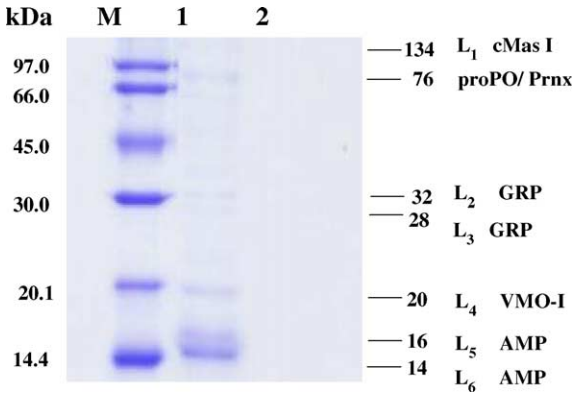

Fig. 4. Analysis of the exocytosed fluid from crayfish granular cells after LPS–PGN treatment. Proteins were recovered by trichloroacetic acid (TCA) precipitation of the exocytosed fluid. The precipitated proteins were subjected to SDS-PAGE under reducing conditions stained with 0.2% Coomassie blue in 50% methanol. Lane M, size marker; lane 1, 12 μg precipitated protein after treated with LPS–PGN for 40 min at room temperature; lane 2, the precipitated proteins from the control cells after incubation with CFS. The first band with a molecular mass of 134 kDa was named L_1 and 32, 28, 20, 16, and 14 kDa were named L_2, L_3, L_5, and L_6, respectively.

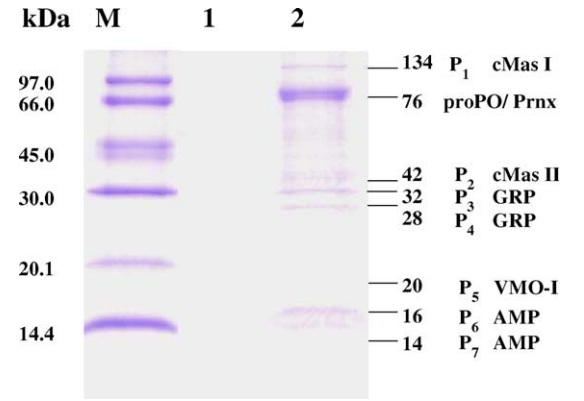

Fig. 5. Analysis of the exocytosed fluid from crayfish granular cells resulting after peroxinectin (20 μg/ml) treatment. Proteins were recovered by trichloroacetic acid (TCA) precipitation of the exocytosed fluid. The proteins precipitated were subjected to SDS-PAGE under reducing conditions and stained with 0.2% Coomassie blue in 50% methanol. Lane M, size marker; lane 1, control monolayers were incubated with only CFS; lane 2, 12 μg precipitated protein after treatment with peroxinectin for 1 h at room temperature. The first band with a molecular mass of 134 kDa was named P_1 and 42, 32, 28, 20, 16, and 14 kDa were named P_2, P_3, P_4, P_5, P_6, and P_7, respectively.

sequences were also found in the released band with a mass of 32 kDa ($I_4 = L_2 = P_3$) after LPS–PGN or peroxinectin treated cells. The fifth band, I_5, with a mass of 28 kDa was strong following calcium ionophore treatment and weaker when peroxinectin (P_4) was used. It is identical to the third band when LPS–PGN is used ($I_5 = P_4 = L_3$). The sixth band (I_6) with a mass of 20 kDa is the same as the fourth LPS–PGN induced band (L_4) and is identical to the fifth band when peroxinectin was used ($I_6 = L_4 = P_5$). The seventh band (I_7) with a mass of 16 kDa is the same as the fifth band (L_5) of LPS–PGN and the sixth band (P_6) of peroxinectin treatment ($I_7 = L_5 = P_6$), while the eighth band (I_8) with a mass of 14 kDa is the same as the seventh band (P_7) of peroxinectin treatment and the sixth band (L_6) of LPS–PGN ($I_8 = P_7 = L_6$). The last band, I_9, with a mass of 12 kDa was only detected after the calcium ionophore treatment. In summary, all proteins secreted as a result of peroxinectin and LPS–PGN treatment of the cells were detected after degranulation induced by the calcium ionophore A23187.

3.2. Determination of amino acid sequences

The partial amino acid sequences of the band with a molecular mass of 134 kDa showed that it is the masquerade-like protein (cMas I) [14], whereas the partial amino acid sequences of the protein with a mass of 42 kDa protein were determined as K/RFPVEDPYAGQLK, K/RDAFEGVGEFQR, K/RLVNLLTGGQCAONK, K/RLGQTFTLDR, K/RDVTLQNDVGLLNLQR, and PVQDPFV. Degenerate primers were designed based on those partial amino acid sequences and they were used for screening a hemocyte first-strand cDNA library. One of the clones derived from this hemocyte cDNA was identified as a masquerade-like serine proteinase (cMas II). To obtain a full sequence, gene specific primers were designed and were then used to screen a hemocyte cDNA by 5′ RACE and 3′ RACE. The obtained full-length cDNA sequence had an open reading frame of 1466 nucleotides corresponding to 535 amino acid residues with a predicted mass of 42 kDa and an isoelectric point of 6.4 (Fig. 6A). In Fig. 6B, it is shown that cMas II has a putative catalytic domain, E_{150} to I_{385}, characteristic of a trypsin-like serine proteinase. However, a serine

A

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CAGACTGCTACACCTTCCAGTTACTGTGTTTAGTGCTCTCCTAACATTACCATCTACACACTACGATGAGAGTAT     75
                                                                  M  R  V

GGGCGAGTGTATGCTTGGTGCTGGCGGTGACAGTGGAGAGTCAGAGGCTAGGAGTCACCACAAGGCTGGGTCTGT   150
W  A  S  V  C  L  V  L  A  V  T  V  E  S  Q  R  L  G  V  T  T  R  L  G  L

TGGGCCCAGAGATCGGCTTGGATCCCGTTCCCGGCAGCAACTTCAACCCCCCACCCAGGGACGCTGGGATCACCA   225
L  G  P  E  I  G  L  D  P  V  P  G  S  N  F  N  P  P  P  R  D  A  G  I  T

GGTGTGTCTGCCTCCCCGTTAACCAGGTGTGTCCCGAAGGCCAAGCTACTCCTCCACAGAGGCCTGAAGGGGTAG   300
R  C  V  C  L  P  V  N  Q  V  C  P  E  G  Q  A  T  P  P  Q  R  P  E  G  V

CAATCAACCATGGAGCTGGTCAGATCGACGTCCGCATAGTTAACTTGCTTACAGGAGGGCAGTGTCCGGGTCAGA   375
A  I  N  H  G  A  G  Q  I  D  V  R  I  V  N  L  L  T  G  G  Q  C  P  G  Q

AGATGTGCTGCCCTGGAGGTGAACTCTCCACAGGACAAGGGACGAACCCTGTACTTCCCAACAAATTGCCGATCA   450
K  M  C  C  P  G  G  E  L  S  T  G  Q  G  T  N  P  V  L  P  N  K  L  P  I

ATACTGGTGGCTGCGGCTTCCAGAATCCTTTACCTGTACCCAACCAACCAGCCAAGTTTGCCGAGGCAGAGTTCG   525
N  T  G  G  C  G  F  Q  N  P  L  P  V  P  N  Q  P  A  K  F  A  E  A  E  F

GAGAGTATCCATGGATGGCGGTGGTGCTGGACAATGGTAACAACTACAAGGGTGGTGGAGTCCTCATCAGCGAGA   600
G  E  Y  P  W  M  A  V  V  L  D  N  G  N  N  Y  K  G  G  G  V  L  I  S  E

ACTGGGTGCTCACCGCCGCGCACAAGGTCAACAATGAGAGGAACCTGAAAGTGCGGTTGGGCGAGCACGATGTCA   675
N  W  V  L  T  A  A  H  K  V  N  N  E  R  N  L  K  V  R  L  G  E  H  D  V

CTAAGCCAAAGGACCACCCAAATTTTGATCACATCGAGATACCAGTCGGAAGAATCATCATTCACCCAGAGCTCA   750
T  K  P  K  D  H  P  N  F  D  H  I  E  I  P  V  G  R  I  I  I  H  P  E  L

AAGTTGACACCTTACAGAACGACGTGGGCCTCCTGAACCTCCAGAGACCGGTCAACACAAACAGGTTCCCACACA   825
K  V  D  T  L  Q  N  D  V  G  L  L  N  L  Q  R  P  V  N  T  N  R  F  P  H

TCGGGACAGCCTGTCTCCCTCGCCAGGGCCAGATCTTCGCTGGCGAAAACCAGTGCTGGGTGACCGGGTTCGGGA   900
I  G  T  A  C  L  P  R  Q  G  Q  I  F  A  G  E  N  Q  C  W  V  T  G  F  G

AGGACGCCTTCGAAGGTGTGGGAGAGTTCCAGCGCATCCTGAAGGAGGTGGACGTGCCCGTACAGGACCCCTTCG   975
K  D  A  F  E  G  V  G  E  F  Q  R  I  L  K  E  V  D  V  P  V  Q  D  P  F

TNTGCCAGGAAAGGCTCAGGAGCACTCGCCTCGGGCAGACATTCACTCTAGATAGAAATTCATTCTTGTGCGCTG  1050
V  C  Q  E  R  L  R  S  T  R  L  G  Q  T  F  T  L  D  R  N  S  F  L  C  A

GAGGTATCGAAGGGAAGGACGCCTGCACGGGTGACGGAGGGGCCCCCTTGGTGTGTAGGCCGGAGAGAGGGCAGT  1125
G  G  I  E  G  K  D  A  C  T  G  D  G  G  A  P  L  V  C  R  P  E  R  G  Q

GGACAGTAGCTGGTCTGGTCGCATGGGGTATTGGCTGCGCCACCAGCGAAGTCCCAGGCGTCTACGTCAACATCG  1200
W  T  V  A  G  L  V  A  W  G  I  G  C  A  T  S  E  V  P  G  V  Y  V  N  I

CCTCCTACGCTGACTTCATCCGTCGGTACGTCAGGTAAGACATACTCGCCTCTCCACCAGCTCAGAGTTAAGACA  1275
A  S  Y  A  D  F  I  R  R  Y  V  R  *

CTGCTAACATGTGGTATTCGTTATAAATTTTACAGTAAATCCTGGCAATTCTTGTGTATAGTAATAAGACGAACT  1350

TAATTTGTTCAACAGATTTTATTGATTTGTATTACTTTTCATAACTCTGACCCCCTGTTAAAAAGTGGGAAACAG  1425

TCGCCAGCAAAGCGGGCAGTAAATAAAAAAAAAAAAAAAAA                                    1466
```

Fig. 6. (A) Nucleotide sequence of the masquerade-like serine proteinase (cMas II) and its deduced amino acid sequence. Bold amino acids are start codon ATG and stop codon TAA. Circles indicate the residues corresponding to the catalytic triad. Underlined are the deduced amino acid sequences, which match the MALDI-TOF sequences. (B) The partial amino acid sequence alignment of the masquerade-like serine proteinase from the crayfish, *Pacifastacus leniusculus* (cMas II) with other mas-like proteins: *Tenebrio* PPAF (*Tm PPAF*), *Bombyx* masquerade-like serine proteinase homolog (*Bm mas-h*), *Holotrichia* PPAF-2 (*Hd PPAF-2*), *Manduca* serine proteinase-like protein-1 (*Ms SPLP-1*), *Tachypleus tridentatus* big defensin (*Tt FD*), *P. leniusculus* mas-like protein (*Pl mas*) and *Drosophila melanogaster* mas (*Dm mas*). The numbers show the order of amino acid sequence residues from each protein. The conserved residues of the catalytic triad are indicated by asterisks. The alignment was performed by using ClustalW.

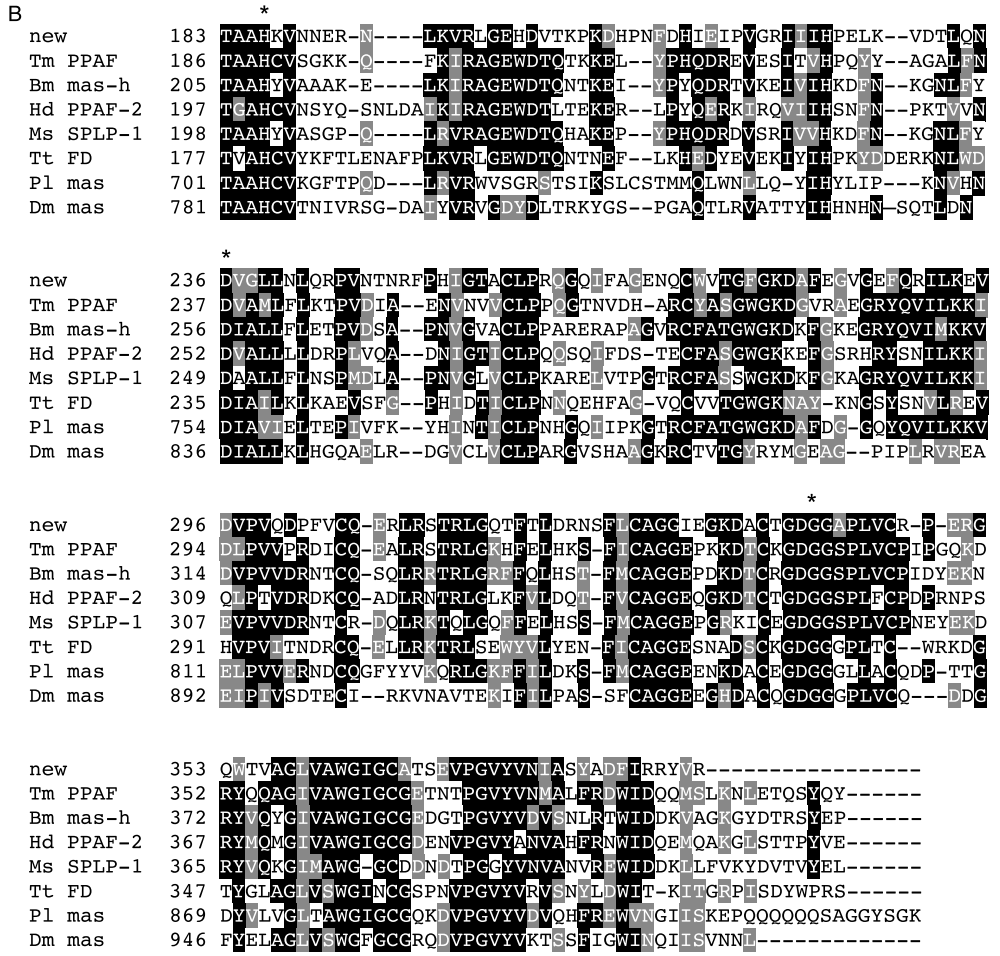

Fig. 6 (*continued*)

residue in the catalytic site is replaced by a glycine residue, and it belongs to the family of masquerade-like proteins presumably without any proteinase activity.

Partial internal amino acid sequences of the protein with a mass of 38 kDa were LGGGGGGGLGGGG LGGGFGGGK, K/RDYSNPCFLALAACR, K/RDL GFTEQQFLGK and K/RQFWTLQLN and the band with a mass of 32 kDa sequences had the following partial amino acid sequences SGGGFGGG GGGFGGSSGDK, LGGGGGGLGGGGAVGGGY GGGK and GGGGGGLGGGGLGGGYGGGK and show some similarities with glycine-rich anti-microbial peptides from the spider *Acanthoscurria gomesiana* [15]. No full cDNA-clones were obtained for these proteins.

The internal amino acid residues with the sequences K/RYVWQQANSYCK, K/RHSLPY LWLMDR, and ESPTENQFLSTLLDK of the 28 kDa band were used to design and synthesize degenerate primers. A corresponding cDNA was found to code for the complete amino acid sequence of a mannose receptor protein. It includes 1398 nucleotides that encode an open reading frame of 273 amino acids. The amino acid sequence from Q_{142} to K_{267} corresponds exactly to the deduced amino acid sequence of the mannose receptor from house mouse (*Mus musculus*) (accession # NP 032651) [16].

A calculated molecular mass of the deduced sequence of the mannose receptor protein is 28.3 kDa with an isoelectric point of 8.74. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 7.

For the protein with a mass of 20 kDa, we obtained three partial amino acid residues sequences SDDTSLDGLK, DVLFLCCR, and FLVDNLA. These sequences were used to design and synthesize

```
ATCACAACACCGCATTTCAAGATGAAGTGTGTGTTGCTGGTCCTGAGCGTGGTAGTGGCG      60
                      M  K  C  V  L  L  V  L  S  V  V  V  A

GTGGCCCAGAGTCAGGTCCCCTACGGTAGCTTCGGGGGAGGCGGCGGCTTCCCTGGGAGA     120
 V  A  Q  S  Q  V  P  Y  G  S  F  G  G  G  G  G  F  P  G  R

CCCGGTATTCACCCCGGGAGACATGGCGGCGGGGGACAGATTCAGTTCCCCGGTTCAAGA     180
 P  G  I  H  P  G  R  H  G  G  G  G  Q  I  Q  F  P  G  S  R

CCCGGGCTCCATGGGAAGCCGGGAGGGATTAGTGGATTTGGTGGTGCAGGGGGAGTCGGT     240
 P  G  L  H  G  K  P  G  G  I  S  G  F  G  G  A  G  G  V  G

GGATTTGGCGGGGCAGGAGGAGTTGGTGGTGCAGGAGGATTTGGTGGTGCGGGAGGAGTC     300
 G  F  G  G  A  G  G  V  G  G  A  G  G  F  G  G  A  G  G  V

GGTGGATTGGTTGGTACTGGAGGCGGTGGACAGGCACAGGAGCAGCCTCTCTCAACCAAG     360
 G  G  L  V  G  T  G  G  G  G  Q  A  Q  E  Q  P  L  S  T  K

TTCTATCCGGCGTACATCAGTCCCCAGGTTCACGTATCTCTGGGTGGAAGCAACTACCAC     420
 F  Y  P  A  Y  I  S  P  Q  V  H  V  S  L  G  G  S  N  Y  H

TTCTCCTGGTGCGTTGACGGTGGGCAGAAGTACGTGTGGCAGCAAGCCAACAGTTACTGC     480
 F  S  W  C  V  D  G  G  Q  K  Y  V  W  Q  Q  A  N  S  Y  C

AAGCAGCTGGGACCTGGGTGGGGCGCTGTGAGCATAGAGTCCCCGACTGAGAACCAGTTC     540
 K  Q  L  G  P  G  W  G  A  V  S  I  E  S  P  T  E  N  Q  F

ATCTCCACCATCATCGACAAGCACAGCCTACCATACATCTGGACCAGCGGGAACCGTTTG     600
 I  S  T  I  I  D  K  H  S  L  P  Y  I  W  T  S  G  N  R  L

AGTGGTGGTCTCAACGACTGGAGGTGGGGCACAGGCCAGCCTCTCAAATATGAAAACTGG     660
 S  G  G  L  N  D  W  R  W  G  T  G  Q  P  L  K  Y  E  N  W

GCTCGCACTGGATTCATTCCCGGCAATCCTCAGCCCGACAACCAGGAGGACAACAATGAG     720
 A  R  T  G  F  I  P  G  N  P  Q  P  D  N  Q  E  D  N  N  E

CAATGCCTCTCAGTGCTCAACCGGTTCTACCCCAACGATGGCATTACCTGGCATGACGTC     780
 Q  C  L  S  V  L  N  R  F  Y  P  N  D  G  I  T  W  H  D  V

GGTTGCCACCACGTCAAGCCTACCATCTGCGAGTATAGCAAAGTCCAGAGCTACGCTGGA     840
 G  C  H  H  V  K  P  T  I  C  E  Y  S  K  V  Q  S  Y  A  G

TAAACCTGCCGAGTGGTATAACTTATTTGAATGACAAATCTTTATTCCTATTTTTTAGCA     900
 *

GTCTGTGCAGTCTTACAGAGAACATTAAAGTTCGAGTTTGCTTAAAATCACAAGTTACTC     960
CTTATAACAGAAAACGGATATTAAATTCTTTCACATGCCGTTCTCTCTCATTGGTGCTGT    1020
AAATCTGTAGAAGACGATATGAAAAAATAATCAGGGATATATATATATATACACCGAGAG    1080
GTGTGCCCCGTCTCTCGTTGTATATATACAGAGTTTACTTTGGTCCTGGACTATGATCAG    1140
AACTAAAGTGTACTTGTTGGTTGGTCAGTAAGCTCCTGGGGTGGCCATAATAACGCTCCT    1200
GCGGTGGCCATAATAACGCTCCTGGGGTTAGAAAGCCTTTGGACATTCGAAAAGCTTCCA    1260
GGATTGAATTATGTGTTTCGTGATAGAAAAATGGCATTATCTTTTTCTGAATTTCTCTTA    1320
ATTTACCCATTAATTAATTTAAGTCCTTCCTGTTATCTTGAATTGTAATAAAAAAAATAT    1380
TCTTCAAAAAAAAAAAAA                                              1398
```

Fig. 7. Nucleotide sequence of the mannose receptor protein and its deduced amino acid sequence. Bold–italics are the deduced amino acid sequences, which match the MALDI-TOF sequences. The bold–underline area corresponds to the sequence used for design of the primers for 5′-, and 3′ RACE. The polyadenylation signal is underlined (AATAAA).

degenerate primers. Several positive clones were sequenced and were found to encode a protein similar to a vitelline membrane outer layer protein I (VMO-I). The amino acid residues from S_{20} to R_{184} correspond to the deduced amino acid of a VMO-I homologue from *Gallus gallus* (chicken) (accession # BAA 05086) [17], *Cyprinus carpio* (common carp) (accession # AAD 23572) [18], and also from the crayfish hemocyte EST-library (CF # 542389). The mature protein consists of 1368 nucleotides that encode an open reading frame of 185 amino acids with a calculated molecular mass of 20 kDa and an isoelectric point of 4.44. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 8.

The 16 kDa protein ($I_8 = L_6 = P_7$) had amino acid sequences, YLPDVHQLLCR, LPGCVNTCQK,

```
ACAGATTTGTCTTCGCGGTGGTCCAGACTAAGTCTTCAGACGAGGTTCTAGCGAGGATGA      60
                                                            M

CGAACACTTTCATAATCCTTCTCTGCGTCGCAGGTGCTGTGCTGGGGAAGGACTCCACCA     120
T  N  T  F  I  I  L  L  C  V  A  G  A  V  L  G  K  D  S  T

TCCGGTCTAACAATGGAGGGCCTTGGGGTGACTGGGGGTCAGAATCTCACTGTCCCCCTA     180
I  R  S  N  N  G  G  P  W  G  D  W  G  S  E  S  H  C  P  P

ACAGCTTTGCCACCGGCTTTGCTATTAAGGTGGAGCGACCAGTTGGCGATTCGGACGACA     240
N  S  F  A  T  G  F  A  I  K  V  E  R  P  V  G  D  S  D  D

CGTCCATAAACGGGATCAAGTTGTTCTGCACGTCTGGCAACGACGGCTCCGAGACGGAAG     300
T  S  I  N  G  I  K  L  F  C  T  S  G  N  D  G  S  E  T  E

TCACCTCCAACCAGCAGCAGTGGGGCAGCTGGACCGATAGGCGTCAATGCCCCCACGGTC     360
V  T  S  N  Q  Q  Q  W  G  S  W  T  D  R  R  Q  C  P  H  G

GCTTGACTAGCATGAGGCTGCGAGTTGAGGGGAGGCAAGGATCCGGTGATGACACGGCCG     420
R  L  T  S  M  R  L  R  V  E  G  R  Q  G  S  G  D  D  T  A

CCAACAACCTGGACATGCGCTGCCAGAACGGTCAAGAACTTGGCGGAGGAGGCAACAACT     480
A  N  N  L  D  M  R  C  Q  N  G  Q  E  L  G  G  G  G  N  N

GGGGCGACTGGAGCCCGTGGGAGACCTGCCAACTTGGCCAAGCGATCTGTGGGCTGCAGA     540
W  G  D  W  S  P  W  E  T  C  Q  L  G  Q  A  I  C  G  L  Q

CCCGGGTTGAAGGCACTCAAGCAGGTGACGACACAGCTCTCAACGATGTCATATTCTTAT     600
T  R  V  E  G  T  Q  A  G  D  D  T  A  L  N  D  V  I  F  L

GCTGCAGGCAGTAGGCCAGAGATAAAACCAAACATCCTGGTCATAACTTCCAAAAGGGCT     660
C  C  R  Q  *

TCAAGAAGTATATTGATATATGACATTACGAGGTTCCCTATTGTATGATTTAGTTCTTGT     720
TCATTCGATATTTTTACAGTTTTTAGGCTTCCAAGAATGATTACCATGGCTTCATTTTGT     780
ATGCTTTATTCATGTTGATTATACAGCATTTTCCCAATGTACACATCAATAAAGACGGCA     840
CCCACATACAATGACTTAGTGTCCACTATCAAGAGATGCAATTATATGATCATTTCCGGA     900
ATCTTTTGTTGTAGGAAGATACGGCAGAATTTTGAGCTTTGAATGTTTTGTTTATTGTGG     960
TTTACTTATGGAGAACATTGCATACGCAGCTGAATTGTTGTGTAGGGATCTCCAAAAATG    1020
CGAATAAAGACATCTGCAACCATTATAGGGAAAGTAATTTTAGATGAATTGGTAAATGTT    1080
TCAGAGGCACAAGTGTAACATATCAAATACATCTTGTACTAGAAAGAAACCCTTGCAATC    1140
CCTTTTGAATGATATATAGCACTGGATTTTATCATTATCAAATAAAAGTACACAAAATGT    1200
AAAGCAGAAATGTGGAATATAAGGTTTTAGAAAGACACCTTCAAACTTTATTATTAAAGA    1260
GAGCCTCATGCTAGGCTCTGAATAAACTTATCCAGATAAAATGTTTTTTGTGCCAAATCT    1320
TGACATGAAGCATCAACTGACACGAACTTCTGTTGTTGACAAAATAAATATCCTGCTTTA    1380
AAAAAAAAAAAAAAAAAAAA                                            1400
```

Fig. 8. The nucleotide and deduced amino acid sequence of the VMO-I protein. The number of nucleotides is shown in the right column. The polyadenylation signal (AATAAA) is underlined. Bold–italics are the deduced amino acid sequences that match the MALDI-TOF analyzed sequences.

```
ATGCTGCGTGTACTGATGATGTCGCTGCTGGTGGTGGCGGCGCTCGGCCACATCTCCCCG  60
 M  L  R  V  L  M  M  S  L  L  V  V  A  A  L  G  H  I  S  P   20

CCCCGGCCGGAGGGCTGCAACTACTACTGCAAGAAGCCTGAAGGTCCTAACAAGGGCTCT  120
 P  R  P  E  G  C  N  Y  Y  C  K  K  P  E  G  P  N  K  G  S   40

AACTACTGCTGCGGCCCGGAGTACATCCCGCTGAAGCGGGAAGAGAAGCACGCTGGTAAT  180
 N  Y  C  C  G  P  E  Y  I  P  L  K  R  E  E  K  H  A  G  N   60

TGCCCGCCTCCTCTCAAGGAATGCACAAGGTTCCCAAGACCACCTCAGGTGTGCCCCCAT  240
 C  P  P  P  L  K  E  C  T  R  F  P  R  P  P  Q  V  C  P  H   80

GATGGACATTGTCCCTACAACCAGAAGTGTTGCTTCGACACCTGTCTCGACATCCACACC  300
 D  G  H  C  P  Y  N  Q  K  C  C  F  D  T  C  L  D  I  H  T  100

TGCAAGCCAGCTCACTTCTATATTAACTAG                                 330
 C  K  P  A  H  F  Y  I  N                                     109
```

Fig. 9. The nucleotide and deduced amino acid sequence of the 16 kDa protein show that the nucleotides perfectly match a putative anti-microbial peptide found in a crayfish hemocyte EST-library (accession # CF542515). The numbers of nucleotides are shown in the right column.

NYPNHLNCQDDED, and K/RGSNYCCGPEYL PLKR that perfectly match a putative anti-microbial peptide found in the crayfish hemocyte EST-library (accession # CF542515, Fig. 9). This peptide shows some similarities with a putative carcinin-like anti-bacterial protein from *Homarus gammarus* (accession # CAH10349). Similarly, the 14 kDa ($I_9 = L_7 = P_8$) band turned out to match another putative anti-microbial peptide from the crayfish hemocyte EST-library (accession # CF542483, Fig. 10), that shows high similarity

```
GGCACGAGGCATCAGCTCACCTGCGCCTCCAGCACTTCCTCTCACTAAACATGCTGCGTG   60
                                                    M  L  R

TACTGATGGTGTCGCTGCTGGTGGTGGCGGCGCTCGGCCACATCTCCCCGCCCCGGCCGG  120
V  L  M  V  S  L  L  V  V  A  A  L  G  H  I  S  P  P  R  P

AGGGCTGCAACTACTACTGCAAGAAGCCTGAAGGTCCTAACAAGGGCTCTAACTACTGCT  180
E  G  C  N  Y  Y  C  K  K  P  E  G  P  N  K  G  S  N  Y  C

GCGGCCCGGAGTACATCCCGCTGAAGAGGGAAGAGAAGCACGCTGGTAATTGCCCGCCTC  240
C  G  P  E  Y  I  P  L  K  R  E  E  K  H  A  G  N  C  P  P

CTCTCAAGGAATGCACAAGGTTCCCAAGACCACCTCAGGTGTGCCCCCATGATGGACATT  300
P  L  K  E  C  T  R  F  P  R  P  P  Q  V  C  P  H  D  G  H

GCCCCTACAACCAGAAGTGTTGCTTCGACACCTGTCTCGACATCCACACCTGCAAGCCAG  360
C  P  Y  N  Q  K  C  C  F  D  T  C  L  D  I  H  T  C  K  P

CTCACTTCTATATTAACTAGCTTGAGCGGGCGAGAGCGTACCCGCAGCTCGACTGTGTCC  420
A  H  F  Y  I  N  *  L  E  R  A  R  A  Y  P  Q  L  D  C  V

CTTCAAGACGGAGCCACACTTCGCTGAACTGAACTACCGTGCTATGCTTGTTTAGTGGCT  480
P  S  R  R  S  H  T  S  L  N  *  T  T  V  L  C  L  F  S  G

GACTTGCTTGTTATGCTCACTTGTCTTTCTTATTGTTTATTTAAAAGGAACATCGGTTTC  540
*  L  A  C  Y  A  H  L  S  F  L  L  F  I  *  K  E  H  R  F

ATGTATAAATGAGAGGAGAATTGTTTATTCAATAAAAAAATACTTTGTTAAAAAAAAAAA  600
H  V  *  M  R  G  E  L  F  I  Q  *  K  N  T  L  L  K  K  K

AAAAAA                                                         606
```

Fig. 10. The nucleotide and deduced amino acid sequence of the 14 kDa protein. The numbers of nucleotides are shown in the right column. The nucleotide sequence of the 14 kDa ($I_9 = L_7 = P_8$) band matches a putative anti-microbial peptide from the crayfish hemocyte EST-library (accession # CF542483), that shows high similarity (45% identity and 53% similarity) with a carcinin-like protein from *Carcinus maenas* (accession # CAH25401).

Table 2
Abundant proteins released by exocytosis from crayfish granular cells

Protein	Abbreviation	Mass (kDa)	Identification	Treatment (A23187, LPS–PGN, Prnx)	References
Crayfish masquerade-like protein I	cMas I	134/129	MALDI-TOF	All	[14]
Pro-phenoloxidase/peroxinectin	ProPO/Prnx	76	MALDI-TOF	All	[24]
Crayfish masquerade-like protein II	cMas II	42	Cloning	Ca^{2+}, Prnx	This paper
Mannose receptor protein	MRP	28	Cloning	All	This paper
Vitelline membrane outer layer protein I	VMO-I	20	Cloning	All	This paper
Anti-microbial peptide	AMP-16	16	Cloning	All	This paper
Anti-microbial peptide	AMP-14	14	MALDI-TOF	All	GenBank accession # CF542483

(45% identity and 53% similarity) with another carcinin-like protein from *Carcinus maenas* (accession # CAH25401) (Table 2).

4. Discussion

Invertebrate animals, such as the crayfish, *P. leniusculus*, mount extremely efficient cellular and humoral innate immune response against invading pathogens and the circulating hemocytes are important in this defence [19]. The semi-granular hemocytes display limited phagocytotic ability and are involved in encapsulation reactions [10,19]. The release of proteins from cellular vesicles in response to various stimuli is a fundamental process [20]. The exocytosis of granular contents from hemocytes into the external milieu in response to endotoxin, peptidoglycans, and β-1, 3-glucans confers humoral immunity to invertebrates [19]. For example, the proPO-activating system and anti-bacterial proteins and peptides are released into the plasma [10,19]. In this study, we show that the Ca^{2+} ionophore A23187, LPS–PGN, and peroxinectin were able to induce degranulation in vitro of isolated granular cells from crayfish and as a result nine, six, and seven proteins were exocytosed, respectively. The identity of the bands were analyzed by MALDI-TOF, or sequenced and cloned. Six identical proteins, $I_{1,4,5,6,7,8}$ and $L_{1,2,3,4,5,6}$, and $P_{1,3,4,5,6,7}$ were found to be released by the three exocytosis triggers. One protein was released upon Ca^{2+} ionophore A23187 and peroxinectin (I_3 and P_2) treatment whereas bands I_4 and I_9 were strongly released only from Ca^2 ionophore A23187.

The cMas I has been isolated, purified, and characterized from hemocytes of the crayfish by Lee and Söderhäll [14]. This protein is present in crayfish hemocytes as a heterodimer composed of two subunits with molecular masses of 134 and 129 kDa. The cMas I is functionally similar to human azurocidin, which is a multifunctional protein with anti-microbial activity against both Gram-negative, Gram-positive bacteria and *Candida albicans*, as well as it exhibits both LPS and heparin binding activity [14]. Several serine proteinase homologue proteins have been characterized from different animals and the inactive serine proteinase-like domains show significant functions, for example cell adhesion activity [21], LPS binding activity [22], and anti-microbial activity [23]. The cMas I protein plays a key role in innate immunity as a pattern recognition protein that recognizes Gram-negative bacteria and yeast and is involved in the clearance of microorganisms from the hemolymph [14].

We also observed a significant band released with a mass of about 76 kDa. It is supposed to be peroxinectin [24] and/or pro-phenoloxidase [1,3] and those bands were not analyzed. Previously we have shown that the pro-phenoloxidase activating system is released from hemocytes by the Ca^2 ionophore A23187 [4] and that components of the system are present in their vesicles [25].

The protein with a mass of 42 kDa is a masquerade-like serine proteinase which contains a non-catalytic serine proteinase domain from E_{150} to I_{385}.

This protein, cMas II, is similar to several masquerade-like serine proteinases in insects such as that of *Tenebrio* pro-phenoloxidase activating factor [26], *Bombyx* masquerade-like serine proteinase homologue (AAN # 77090), *Holotrichia* pro-phenoloxidase activating factor-2 (PPAF-2) [27], *Manduca* serine proteinase-like protein-1 [28] and *Anopheles* serine protease like protein Agmas1 (CAB # 93496). It is also similar to *Tachypleus* factor-D involved in the clotting system in horseshoe crab. The masquerade-like serine proteinase homologues from *Tenebrio* and *Holotrichia* are involved in the proPO activating cascade and are therefore named pro-phenoloxidase activating factors (PPAFs). These PPAFs have some common characteristics in structure and function. They possess a carboxy-terminal serine proteinase domain and a clip domain in the amino terminus and they are involved in cleaving proPO [26,29]. In *Holotrichia*, the serine proteinase PPAF-I in the absence of a protein cofactor of PPAF-II converts the 79 kDa proPO to an intermediate form with a mass of 76 kDa, without exhibiting PO activity. However, in the presence of PPAF-II and PPAF-I, the 79 kDa proPO is converted to active PO with a mass of 60 kDa [29]. In crayfish, a pro-phenoloxidase activating enzyme [30] can cleave proPO by itself without interaction of a masquerade-like protein. Here, we report a new masquerade-like protein more similar to insect PPAFs and this cMas II protein may be required in the upper steps of the proPO-cascade.

The two proteins with masses of 38 and 32 kDa contain high glycine-rich sequences similar to a novel glycine-rich anti-microbial peptide constitutively expressed in the hemocytes of the spider, *A. gomesiana* [15]. Acanthoscurrins are linear cationic peptides with a high glycine (72–73%) amino acid composition and presence of the three-fold repeats of 26 amino acids (GGGLGGGGLGGGGLGGG KGLGGGGLG) [15].

The protein with a mass of 28 kDa show similarities with a mannose receptor protein from *M. musculus* (house mouse) [16] which mediates macrophage phagocytosis of bacteria, fungi, and some enveloped viruses in the vertebrate innate immune response [31]. Mannose binding proteins (MBP), or mannose binding lectin (MBL) are C-type lectins, which bind to mannose or N-acetylglucosamine (GlcNAc) on the surfaces of pathogens in a calcium-dependent manner [32]. MBP contains collagen-like regions and the Ca^{2+}-dependent carbohydrate recognition domain (CRD), and acts as an opsonin and also activates the complement system by forming a complex with a novel serine protease termed MBL-associated serine protease (MASP) in serum [33].

The ~20 kDa protein, VMO-I, was shown to be similar to a vitelline membrane outer layer protein I (VMO-I) from common carp (*C. carpio*) [18] and chicken (*G. gallus*) [17]. VMO-I is a basic protein composed of 163 amino acids, with a molecular mass of ~18 kDa in quail and other poultry eggs [34,35], whereas the mature protein of an outer layer protein of carp fertilization envelope is 21 kDa and has 58% identity to the outer layer protein of chick vitelline membrane [18]. In crustaceans, VMO-I proteins are known to be synthesized in females by extraovarian tissue and then transported via the hemolymph to the developing oocytes [36]. The major role of the vitelline membrane is to prevent the mixing of the yolk and albumen and also act as an important anti-microbial barrier, as indicated by the high content of lysozyme in the outer layer [37]. Sugihara et al. [38] demonstrated the presence of a substance with an ovomucin-like property, viral anti-hemagglutinin activity, in the vitelline membrane. In crayfish, this protein is stored in the granules of the granular hemocytes and is released by exocytosis. However, the specific role of this protein in crayfish immune defense still remains to be elucidated.

Both proteins with masses of 16 and 14 kDa are anti-microbial peptides. Anti-microbial peptides are known to be involved in the innate immune response of vertebrates and invertebrates as well as plants [39,40]. In horseshoe crab, anti-microbial peptides are mainly synthesized in hemocytes, stored in the granules and released into the hemolymph upon microbial stimulation [41]. Anti-microbial peptides from crustaceans have previously been isolated from the hemocytes of the green crab *C. maenas* [42], blue crab *Callinectes sapidus* [43], freshwater crayfish, *P. leniusculus* [44], and the shrimp *Penaeus vannamei* [45].

Exocytosis of granular cells in vitro could be evoked by the Ca^{2+} ionophore A23187, LPS–PGN, and also peroxinectin. LPS–PGN is a component of bacterial cell walls, which was found to induce exocytosis in vitro of granular hemocytes from horseshoe crab. It has been reported to occur through

a G protein-mediating pathway that activates a phospholipase C on the hemocyte surface, leading to the LPS-induced exocytosis [46]. Peroxinectin is a cell adhesive factor and also acts as a degranulation factor [6,7] and this is brought about by triggering an intracellular signaling pathway involving protein kinase C [4]. An interesting finding was that a majority of the proteins in the vesicles is anti-microbial peptides and this is logic, since microorganisms release LPS–PGN or β-1, 3-glucans which can cause exocytosis of arthropod hemocytes and hence these microorganisms eliciting this release will be killed in proximity to the hemocytes. Further, two masquerade-like proteins, one of which functioning as an opsonin, and the other may be involved in the proPO-system are also abundant and released from the hemocytes upon microbial challenge.

Acknowledgements

This work has been financed by Swedish Science Research Council and Formas to Kenneth Söderhäll and S.S. was supported by a scholarship from Faculty of Agriculture, KKU and Center for Agricultural Biotechnology, KU, Thailand.

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Appendix B

White Spot Syndrome Virus (WSSV) Interaction with Crayfish Hemocytes

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Fish & Shellfish Immunology xx (2006) 1–10

Fish & Shellfish Immunology

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White spot syndrome virus (WSSV) interaction with crayfish haemocytes

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Received 3 May 2005; revised 15 August 2005; accepted 5 September 2005

Abstract

WSSV particles were detected in separated granular cells (GCs) and semigranular cells (SGCs) by in situ hybridisation from WSSV-infected crayfish and the prevalence of WSSV-infected GCs was 5%, whereas it was 22% in SGCs. This indicates that SGCs are more susceptible to WSSV and that this virus replicated more rapidly in SGCs than in GCs and as a result the number of SGCs gradually decreased from the blood circulation.

The effect of haemocyte lysate supernatant (HLS), containing the degranulation factor (peroxinectin), phorbol 12-myristate 13-acetate (PMA), the Ca^{2+} ionophore A23187 on GCs from WSSV-infected and sham-injected crayfish was studied. The results showed that the percentage of degranulated GCs of WSSV-infected crayfish treated with HLS or PMA was significantly lower than that in the control, whereas no significant difference was observed when treated with the Ca^{2+} ionophore. It was previously shown that peroxinectin and PMA have a degranulation effect via intracellular signalling involving protein kinase C (PKC), whereas the Ca^{2+} ionophore uses an alternative pathway. HLS treatment of GCs and SGCs from WSSV-infected crayfish results in three different morphological types: non-spread, spread and degranulated cells. The non-spread cell group from both GCs and SGCs after treatment with HLS had more WSSV positive cells than degranulated cells, when detected by in situ hybridisation. Taken together, it is reasonable to speculate that the PKC pathway might be affected during WSSV infection.

Another interesting phenomenon was that GCs from non-infected crayfish exhibited melanisation, when incubated in L-15 medium, while no melanisation was found in GCs of WSSV-infected crayfish. However, the phenoloxidase activities of both sham- and WSSV-injected crayfish in HLS were the same as well as proPO expression as detected by RT-PCR. This suggests that the WSSV inhibits the proPO system upstream of phenoloxidase or simply consumes the native substrate for the enzyme so that no activity is shown. The percentage of apoptotic haemocytes in WSSV-infected crayfish was very low, but it was significantly higher than that in the sham-injected crayfish on day 3 or 5 post-infection. The TEM observation in haematopoietic cells (hpt cells) suggests that WSSV infect specific cell types in haematopoietic tissue and non-granular hpt cells seem more favourable to WSSV infection.

Keywords: White spot syndrome virus (WSSV); Haemocyte lysate supernatant (HLS); Ca^{2+} ionophore A23187; Phorbol 12-myristate 13-acetate (PMA); Crayfish haemocytes; Haematopoietic tissue

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doi:10.1016/j.fsi.2005.09.002

1. Introduction

The goal of all viruses is to infect target cells, replicate large number of progeny virions, and spread their progeny to new host target cells. Viruses encode highly efficient proteins to optimise such replication. However, the target organism possesses both systemic and cell-based defences to limit virus infection, including immune and inflammatory processes and the execution or suicide of infected cells [1].

In arthropods, the defence against invasive microorganisms relies on innate immunity and is carried out mainly through the phagocytic, encapsulating and agglutinating activities of the circulating haemocytes as well as by antimicrobial factors in haemolymph [2]. The prophenoloxidase (proPO) activating system present in the blood cells is an important innate immune reaction in arthropods [3]. The proPO system resides in the haemocytes in crustaceans and in most insects, and comprises several serine proteinases and prophenoloxidase, and is specifically released from the haemocytes and activated by non-self molecules. The proPO system participates in host defence in arthropods by enhancing phagocytosis, initiating nodule or capsule formation, mediating coagulation and producing fungistatic substances [4].

Apoptosis or programmed cell death is a genetically controlled process involved in the regulation of homeostasis, tissue development, and the immune system by eliminating cells that are no longer useful. Apoptosis also functions by eliminating aberrant DNA created by DNA damage or from viral pathogens. Cell death by apoptosis is characterised by chromatin condensation, DNA fragmentation to nucleosome-sized pieces, membrane blebbing, cell shrinkage, and compartmentalisation of the dead cells into membrane-enclosed vesicles or apoptotic bodies [1].

Apoptosis occurs in most animal tissue to eliminate damaged or harmful cells and has been proposed as the main anti-viral mechanism in invertebrates [5,6]. It has been suggested that apoptosis is involved in the pathogenesis of WSSV infection and that it may be the cause of death in shrimp with lethal viral infections [7,8]. In another study of *Penaeus monodon* infected by yellow head virus (YHV), the infection caused high number of apoptotic cells and apoptosis was also suggested as the primary cause of death [9]. In contrast, the studies by Granja et al. [10] suggest that hyperthermia might facilitate apoptosis in WSSV-infected *Litopenaeus vannamei* and might be one of the mechanisms responsible for increased survival of infected shrimp maintained at 32 °C. However, some viruses appear to encode products that actively induce apoptosis as part of an exit strategy to enhance virus spread. In these cases a delicate balancing act between inhibition and induction of apoptosis is performed by the combinations of viral products [1].

The present study aims to investigate the effect of white spot syndrome virus (WSSV) in general on circulating haemocytes of the freshwater crayfish, *Pacifastacus leniusculus*.

2. Materials and methods

2.1. Experimental animals

Freshwater crayfish *P. leniusculus* were purchased from Berga kräftodling, Södermanland, Sweden. The crayfish were held in tanks with running aerated water at 16 °C. Only intermolt male crayfish were used for the experiments.

The WSSV stock was prepared as described by Jiravanichpaisal et al. [14]. For injection experiments, an amount of 200 μl from 1:3 dilution of WSSV stock solution was injected via the base of the fourth walking leg of crayfish. Injected crayfish were maintained at room temperature, 22 ± 2 °C until the termination of the experiment e.g. when they showed the characteristics of a weak response to the WSSV infection.

2.2. Preparation of haemocyte lysate supernatant (HLS)

Haemolymph from crayfish was collected with 1.2 mm needles in ice-cold buffer of 10 mM Na cacodylate, 0.25 M sucrose and 100 mM $CaCl_2$, pH 7.0. Haemocytes were spun down at 800 g for 10 min (4 °C). The cell pellet was washed with the same buffer and homogenised with a glass piston homogeniser in 1–3 ml of crayfish saline (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) [13] for degranulation experiment or in 10 mM Na cacodylate buffer, pH 7.0 containing 100 mM $CaCl_2$, to keep the enzymes of the proPO system in their inactivate

form for detecting of PO-activity. These homogenates were then centrifuged at 70,000 g for 20 min at 4 °C and the resulting HLS was used in the experiments. Phenoloxidase activity was determined as previously described in [15].

2.3. Preparation of monolayers of haemocytes

Granular cells from WSSV infected (moribund stage) and normal crayfish were separated by a density gradient centrifugation method described by Söderhäll and Smith [11]. Monolayers of pure granular cells were prepared on clean, pyrogen-free glass coverslips. Briefly, isolated GCs were diluted 1:2 with 0.15 M NaCl and then treated with $CaCl_2$ (20 mM final concentration) to allow the cells to attach to sterile coverslips for 30 min at room temperature.

2.4. Experiments with cell monolayers

2.4.1. Effect of HLS on degranulation of granular cells in vitro

The effect of HLS on crayfish haemocytes in vitro was examined by overlaying freshly prepared crayfish GC monolayers with 100 μl of crayfish HLS (2 mg protein/ml) containing the degranulation factor, peroxinectin, at different dilutions [12]. The cell monolayers were then incubated for 40 min at 20 °C, washed gently with CFS [13], and then fixed in 10% formalin in CFS. Control monolayers were incubated with CFS. After fixation, the cells were observed by use of phase contrast microscope and the percentage of degranulated, spread or non-spread (unaffected) cells was determined for at least 200 cells on each coverslip.

2.4.2. Effect of HLS on SGCs and GCs

The SGCs and GCs were separated and treated as in Section 2.4.1. After fixation, the cells were observed under the microscope, and the presence of WSSV was detected by in situ hybridisation using a digoxigenin labelled WSSV specific probe as described earlier [14].

2.4.3. Degranulation of granular cells

The GC monolayers were washed three times with CFS and then treated with the Ca^{2+} ionophore A23187 (Sigma) at a final concentration of 10 μM in CFS and 10 mM $CaCl_2$ (final concentration) for 30 min or phorbol 12-myristate 13-acetate (PMA) at a final concentration of 4 μM in CFS for 30 min. The ionophore was diluted from a stock solution of 5 mM in dimethyl sulphoxide (DMSO) as described in Johansson and Söderhäll [15] and the same amount of DMSO was always present in the controls. The cells were fixed and counted as above.

2.4.4. Melanisation of granular cells

Granular cells monolayers were incubated with modified Leibovitz's L-15 medium (L-15 M81) at room temperature for at least 16 h, then observed and the percentages of melanised cells were determined under the microscope.

2.5. RNA isolation and RT-PCR

After isolated GCs from WSSV-infected and sham-injected crayfish were allowed to attach to the well at room temperature for 30 min, the cells were washed with CFS three times. Total RNA was isolated by using total RNA isolation kit (Sigma) according to manufacture's instructions. Total RNA (100 ng) from each treatment was analysed by RT-PCR using Thermoscript (Invitrogen) and Oligo (dT) primer according to the manufacturer's instructions. The following primers were used for the final PCR step: proPO 5′-AGT GAA CAG GAC TCC ACC TAC TGC-3′ and 5′-ACT GAT GTC TAT GAA ATC CAG CCC-3′ corresponding to residues 622+/1045− (GenBank accession no. X83494), and a control RT-PCR analysis of expression of the housekeeping gene small ribosomal protein was also undertaken 40S forward and reverse, 5′-CCA GGA CCC CCA AAC TTC TTA G-3′ and 5′-GAA AAC TGC CAC AGC CGT TG-3′, respectively. The products were then analysed on an agarose gel, stained with ethidium bromide, and visualised by ultraviolet light. The expected size of proPO and 40S were 423 nt and 359 nt, respectively.

2.6. Cell death assay

Haemolymph from WSSV infected crayfish 3 and 5 days post-infection was collected with 1.2 mm needle in ice-cold anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 4.6). The cells were spun down and resuspended in CFS. The cell suspension was smeared on the glass slides and allowed to dry at room temperature. Air dried cell samples were fixed with freshly prepared paraformaldehyde (4% in CPBS, pH 7.4) for 1 h at room temperature. For detection of apoptotic cells a direct TUNEL assay was performed using the in situ cell death detection kit, Fluorescein (Roche) according to the manufacturer's instructions.

2.7. In situ hybridisation to monitor WSSV infected haemocytes

Some samples of fixed haemocytes on coverslip from (Sections 2.4.1 and 2.4.2) were subjected to in situ hybridisation to determine percentage of WSSV infection. A digoxigenin (DIG)-labelled WSSV-specific probe was prepared. Hybridisation and staining procedure were as previously described by Jiravanichpaisal et al. [14] except that the concentration of Proteinase K (Roche) was decreased to 10 μg/ml.

2.8. Statistical analysis

The data of mortality were analysed using Student's *t*-test; meanwhile the comparison of multiple groups in the haemocyte count experiments was performed by one-way analysis of variance. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

Jiravanichpaisal et al. [14] have demonstrated that WSSV had a significant effect on the proportion of different haemocyte types in crayfish, *P. leniusculus*. The number of GCs was significantly higher in WSSV-infected crayfish compared with sham-injected and non-injected animals. In the current study, WSSV particles were detected by in situ hybridisation in separated GCs and SGCs. The prevalence of WSSV-infected cells from moribund crayfish was 22% for SGCs while only 5% of the GCs contained the virus (Fig. 1a). This suggests that SGCs are more susceptible to WSSV and that this virus replicated more rapidly in SGCs than in GCs and then SGCs gradually decreased from the blood circulation. The susceptibility of SGCs to WSSV was also observed in the shrimp, *Penaeus merguiensis*, during WSSV infection. The results showed that the SGCs were highly susceptible to WSSV infection, had higher virus load and infection rate compared to GCs. The percentage of infected SGCs increased rapidly from 21% on day 2 to 65% on day 3, while the increase in percentage of the infected GCs was 7% and 23%, respectively [16]. In other studies, the total numbers of circulating haemocytes in shrimp were dramatically decreased after being infected with WSSV [17–19]. Söderhäll et al. [20] proposed that SGCs are the first haemocyte type to react to foreign particles in vivo and that they respond by degranulation and thus release of the proPO system into the plasma. The system will be activated and trigger more degranulation by peroxinectin, which then causes an amplified secretion of the proPO system from both SGCs and GCs.

In order to understand more of the virus-haemocyte interaction, the effect of haemocyte lysate supernatant (HLS), containing the degranulation factor (peroxinectin), on isolated granular cells was studied. As shown in Fig. 2, the percentage of HLS-degranulated cells of WSSV-infected crayfish was significantly lower ($p < 0.05$) than those of sham-injected crayfish. In addition, the Ca^{2+} ionophore A23187 and the PKC-activating PMA was also used as secretagogues [15]. As a result, PMA in a dose-dependent manner affected the degranulation of GCs from WSSV-infected crayfish as shown in Fig. 3. Similar to the result of HLS treatment, the percentage of PMA-degranulated cells from WSSV-infected crayfish was significantly lower ($p < 0.05$) than those from the sham-injected crayfish. There was no significant difference in the percentage of cell degranulation when treated with both Ca^{2+} ionophore A23187 and CFS in both sham- and WSSV-infected crayfish (Fig. 2). The active 76-kDa protein, peroxinectin in HLS, which mediates attachment and spreading of the haemocytes, also triggers degranulation (regulated exocytosis) of GCs [21]. Peroxinectin can bind to its receptor superoxide dismutase [22] on the haemocyte surface, but little is known how binding of the peroxinectin ligands leads to the biological effects and cellular activities of degranulation,

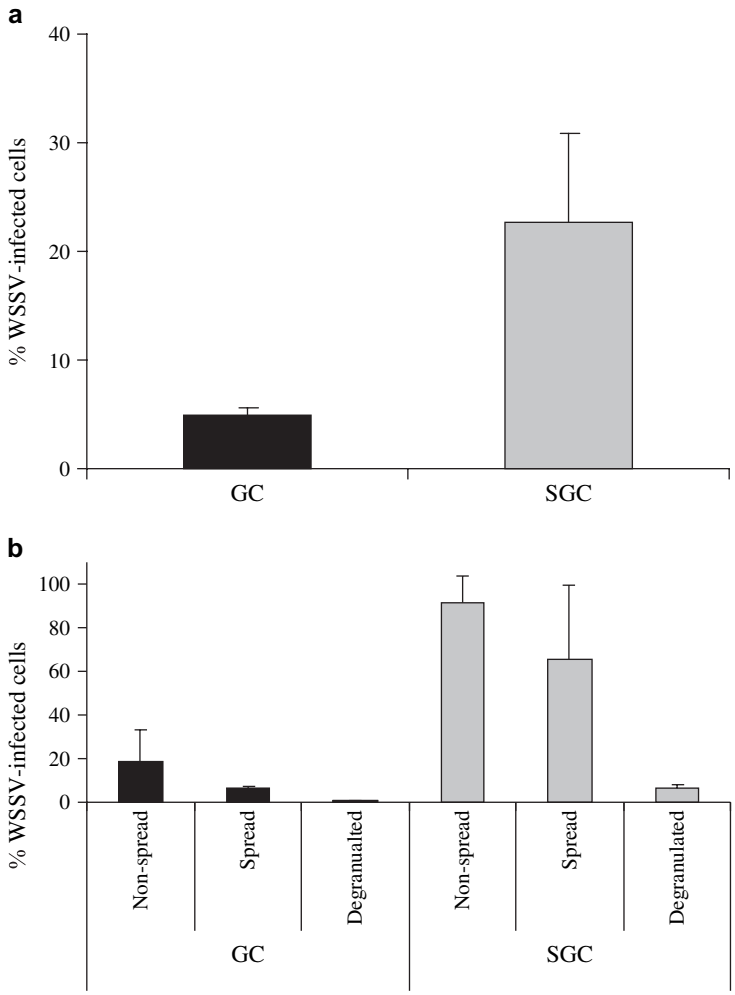

Fig. 1. (a) The percentage of WSSV-infected cells from moribund crayfish detected by in situ hybridisation of SGCs and GCs. (b) Percentage of WSSV-infected cells in non-spread, spread and degranulated cells after treatment with HLS (GCs = black columns; SGCs = grey columns).

cell spreading, and phagocytosis in arthropod blood cells. However, it was shown that binding of peroxinectin triggers cellular responses via a pathway that includes protein kinase C (PKC) activation and elevated protein tyrosine phosphorylation of a cellular protein of ~80 kDa [23]. PKC is a family of enzymes that are physiologically activated by 1,2-diacylglycerol (DAG) or its surrogate, phorbol 12-myristate 13-acetate (PMA) and other lipids. It was shown that PMA alone caused a dose-dependent degranulation [23]. The Ca^{2+} ionophore A23187 has been widely used to induce degranulation in amoebocytes of *Limulus polyphemus* and crayfish haemocytes and proteins released are for example protease inhibitors [24], the proPO system [15] and other proteins [25]. HLS treatment of GCs and SGCs from WSSV-infected crayfish resulted in three different cell-reactions: non-spread, spread and degranulated cells and these different ''cell-reactions'' were investigated for presence of virus infection by in situ hybridisation. The non-spread cell group from both GCs and SGCs had more WSSV positive cells than the degranulated cells (Figs. 1b and 4). Based on these results, it was reasonable to assume that the PKC pathway might be somehow affected by WSSV during its replication inside the cells.

Isolated GCs from sham-injected and WSSV-injected crayfish were incubated in modified L-15 medium at room temperature for at least 16 h and the GCs from sham-injected crayfish melanised after degranulation, while no melanisation was found in cells from WSSV infected crayfish regardless of whether the cells were or were not degranulated as shown in Fig. 5. So far little is known about the interactions of pathogenic viruses with the immune system of other

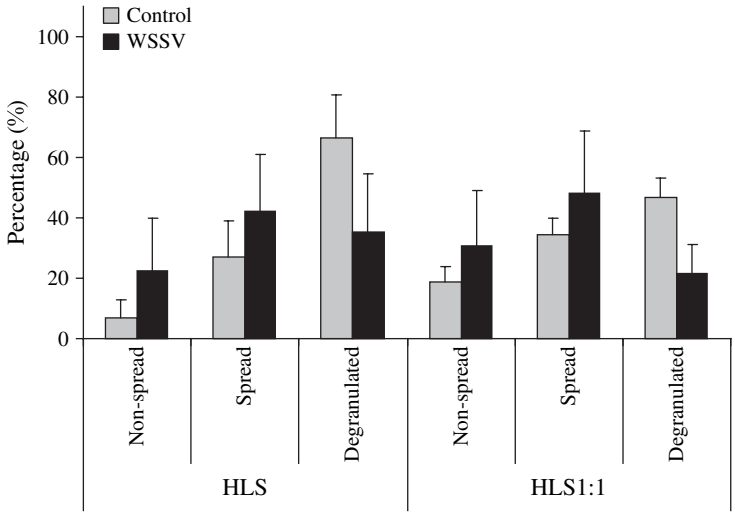

Fig. 2. Percentage of degranulated, spread and non-spread GCs from WSSV-infected and sham-injected crayfish after treatment with HLS (WSSV = black columns; control = grey columns).

invertebrates except for one study of polydnaviruses (PDVs) in insect. In many species parasitised by braconid and ichneumonid wasps, host immunosuppression appears to be mediated by PDVs injected by the female parasitoid into the host haemocoel. The parasitoids likely exploit other mechanisms of immunoevasion via antigen masking, antigen mimicry, or production of active inhibitors of the haemocyte-mediated encapsulation response as well as to inhibit the melanisation reaction directly [26]. However, the phenoloxidase activities in haemocyte lysate supernatant of both sham-injected and WSSV-injected crayfish were the same as well as proPO expression as detected by RT-PCR

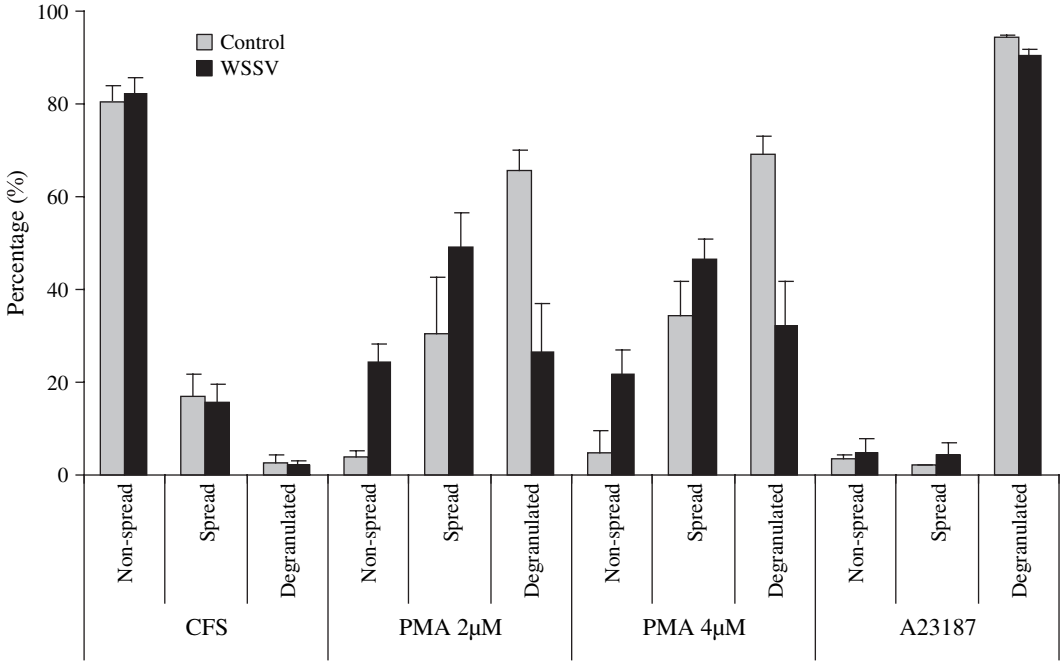

Fig. 3. Percentage of degranulated, spread and non-spread GCs of WSSV-infected and sham-infected crayfish after treatment with PMA (2 μM and 4 μM), the Ca^{2+} ionophore A23187 and CFS as control.

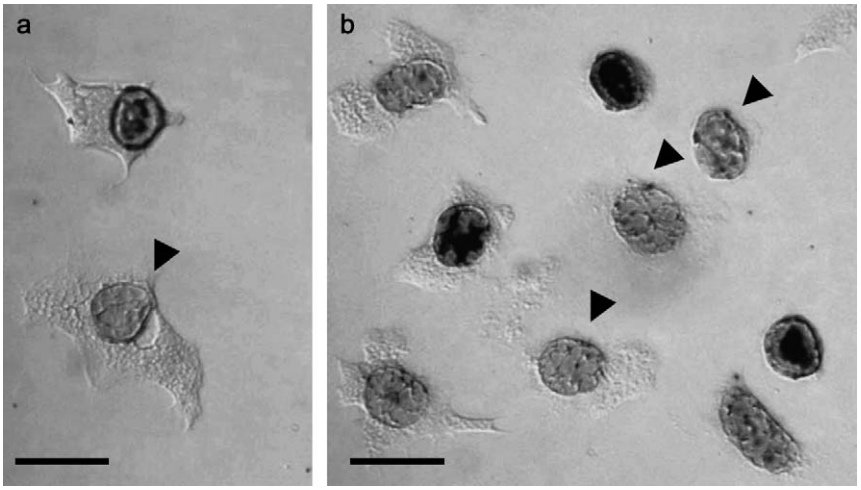

Fig. 4. The presence of WSSV in HLS-treated haemocytes as detected by in situ hybridisation. (a) GCs: WSSV is present in non-spread cells (dark nuclei) but absent in degranulated cells (arrows). (b) SGCs: WSSV is present in non-spread cells (dark nuclei) but absent in completely degranulated cells (arrows). Scale bars = 10 μM.

(Fig. 6). This suggests that the observed inhibition of melanisation in cells is affected upstream of phenoloxidase or alternatively by depletion of the native substrate for PO.

Successful viral replication requires not only the efficient production and spread of progeny, but also evasion of host defence mechanisms that could limit replication by killing infected cells. In addition to inducing immune and inflammatory responses, infection by most viruses triggers apoptosis or programmed cell death of the infected cell. Some viruses seem to use apoptosis as a mechanism of cell killing and enhanced virus spread. In both cases, successful replication relies on the ability of certain viral products to block or delay apoptosis until sufficient progenies have been produced.

The hallmark characteristic of apoptotic cells was described by Roulston et al. [1] that the membrane of an apoptotic cell actively blebs, but remains intact, ultimately blebbing the cell apart into membrane-bound apoptotic bodies that contain cytoplasmic and/or nuclear material. According to this description, many small apoptotic bodies were found scattered among the haemocytes from WSSV-infected crayfish. These small apoptotic bodies varied in size and contained nuclear material confirmed by propidium iodide staining. Moreover, WSSV-DNA was detected in these

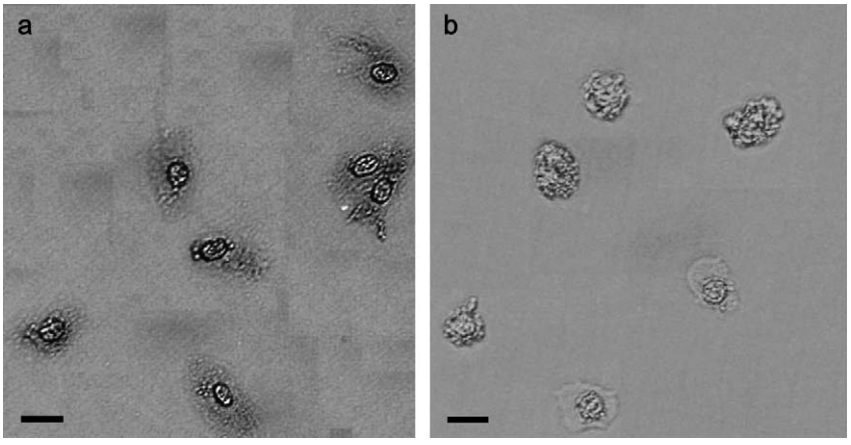

Fig. 5. Melanisation in isolated GCs after incubation in L-15 medium at room temperature for at least 16 h: (a) from sham-injected crayfish and (b) from WSSV-injected crayfish. Scale bars = 10 μM.

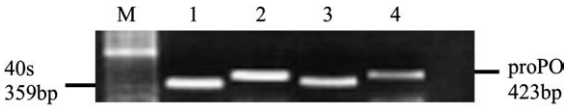

Fig. 6. Expression of proPO and a 40S ribosomal protein analysed by RT-PCR from non-infected and WSSV-infected crayfish.

bodies by in situ hybridisation as shown in Fig. 7. The presence of apoptotic cells in circulating haemocytes was also detected by TUNEL assay in sham-injected and WSSV-injected crayfish. Surprisingly, the percentage of apoptotic cells was low in both groups (Fig. 8). In infected crayfish, however, about 1.5% and 1% of the haemocytes were apoptotic at day 3 and day 5 post-injections, respectively, and significantly higher than that in the control crayfish, which were about 0.15% and 0.1%, respectively, at day 3 and day 5.

A low percentage of apoptosis was also found in the lymphoid organ of *Penaues japonicus* following WSSV-infection, and the percentages of apoptosis from infected-shrimp with high dose of WSSV were 2.9% and 1.6% on day 3 and day 5, respectively, whereas with a low dose were 1.7% and 0.4% on day 3 and day 5, respectively [27] and around 3% in the haematopoietic tissue of *P. monodon* at 36 h post-infection [19]. However, as detected by DAPI-staining, the percent of apoptotic haemocytes was low at 24 h post-infection but reached about 20% at 60 h [19]. Anggraeni and Owens [28] considered apoptosis as the defence responsible for eliminating the virus, whereas Khanobdee et al. [9] suggested that progressive occurrence of apoptosis may be the cause of death in *P. monodon* infected with yellow head virus (YHV). Wongpraset et al. [19] clearly demonstrated that apoptosis occurs following WSSV infection in *P. monodon*, but the importance of this for the mortality needs further investigation.

Since the prevalence of apoptotic cells was very low, it may indicate that WSSV may induce anti-apoptotic mechanisms. In baculovirus, P35 and IAPs (inhibitors of apoptosis proteins) proteins were found to be involved in inhibition of host cell apoptosis. The function of IAPs in insect and vertebrates is not only to inhibit apoptosis during development but also to prevent a defensive apoptotic response of the host cells. IAPs have been cloned and characterised in silkworm, *Bombyx mori* [29], and very recently, the ORF 390 of WSSV genome was identified as a novel anti-apoptotic gene [30]. They also confirmed that the WSSV could infect shrimp primary cells derived from lymphoid tissue and block the cell apoptosis induced by the actinomycin D, in turn; the cells infected with virus prior to actinomycin D treatment did not show characteristics of apoptosis.

Finally, WSSV-infected cells in haematopoietic tissues from moribund WSSV-infected crayfish were also observed by transmission electron microscope (TEM). As shown in Fig. 9a an infected cell with enlarged nuclei containing massive WSSV particles had no fine granules, whereas the cells with presence of fine granules adjacent to the infected cells did not contain WSSV. This suggests that WSSV infects specific cell types in the haematopoietic tissue. A cluster

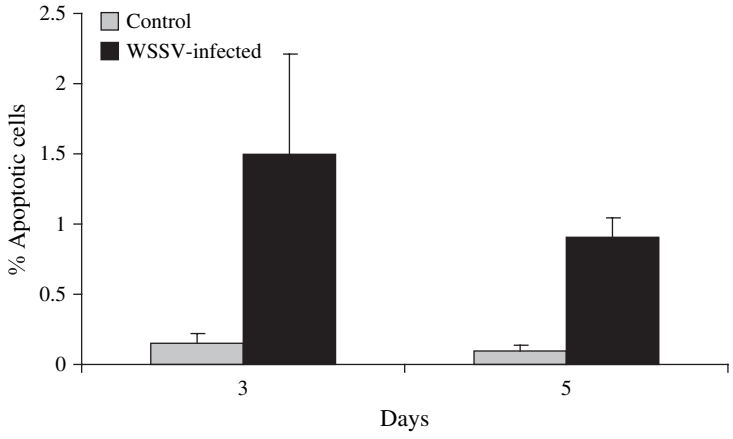

Fig. 7. Percentage of apoptotic cells among circulating haemocytes analysed by TUNEL assay at day 3 and day 5 post-injection of WSSV. Scale bars = 10 μM.

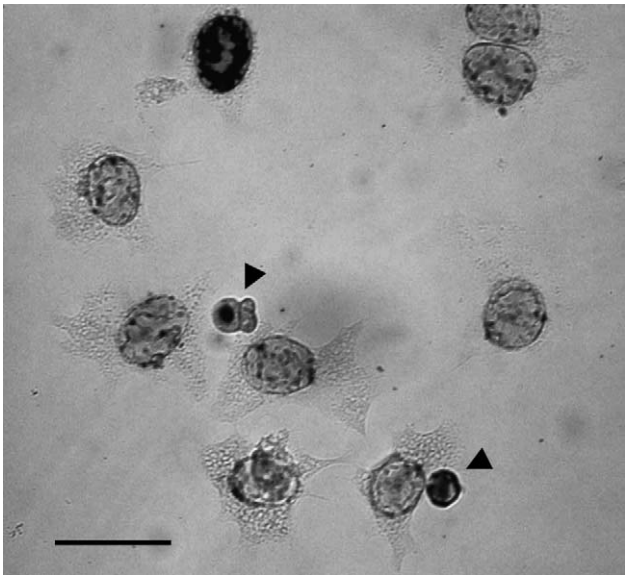

Fig. 8. The presence of WSSV in haemocytes (black nucleus) and small apoptotic bodies (arrows) as detected by in situ hybridisation. Scale bars = 10 μM.

of intact WSSV virions was found outside the cells as shown in Fig. 9b. In shrimp, intact virus particles were found in the cytoplasmic vacuoles of GCs but not in nuclei [16] possibly due to virus infection/replication, phagocytosis or endocytosis. We also found intact WSSV in vacuoles of haematopoietic cells with heavy infection.

In conclusion, these studies suggest that the SGCs are more susceptible to WSSV infection. WSSV infected GCs were resistant to degranulation induced by PMA or HLS, but not by the Ca^{2+} ionophore A23187. Therefore, the PKC pathway might be somehow affected by WSSV during its replication inside the cells. It was also found that WSSV inhibits the proPO system in haemocytes. However, the mechanism of this phenomenon has still to be elucidated.

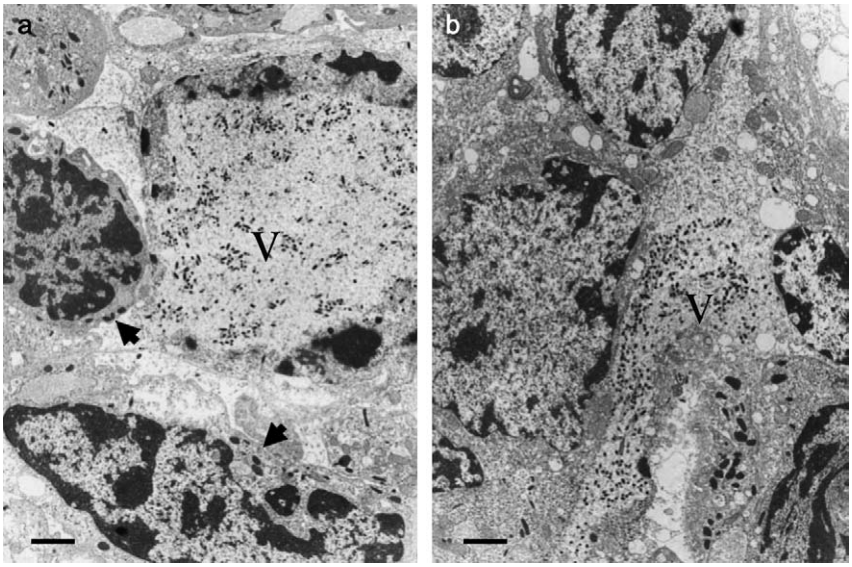

Fig. 9. Electron micrographs of WSSV-infected haematopoietic tissue. (a) Mature WSSV virions (V) in the enlarged nucleus of a heavily infected haematopoietic cell. No fine granules were found in the cytoplasm, whereas adjacent cells with define granules (arrows) were not infected. (b) Massive mature WSSV virions (V) were found outside the nucleus. Scale bars = 500 nM.

Acknowledgements

This work has been supported by the Swedish Research Council and FORMAS to KS and Carl Trygger Foundation to IS.

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