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Hemolymph proteins in marine crustaceans

W Sylvester Fredrick*, S Ravichandran

CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, Tamil Nadu, India

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ABSTRACT

This study is done with the aim to bring together the various antimicrobial peptides that are present in the crustacean hemolymph and their sources along with its characteristics. Invertebrates lack immune systems that involve antigen-antibody reactions and do not have an immune memory, therefore most invertebrate species show no evidence of acquired immunity. Crustaceans possess an open circulatory system, where nutrients, oxygen, hormones, and cells are distributed in the hemolymph. They lack adaptive immune system and rely exclusively on their innate immune mechanisms that include both cellular and humoral responses. Antimicrobial peptides and proteins form an important means of host defense in eukaryotes. In addition to their role as endogenous antibiotics, antimicrobial peptides have functions in inflammation, wound repair and regulation of the adaptive immune system. Over the past several years, many antimicrobial peptides have been found and characterized in crabs.

1. Introduction

Crustaceans form a very large group of arthropods, usually treated as a subphylum, which includes familiar animals such as crabs, lobsters, crayfish, shrimp, krill and barnacles. More than 10 million tons of crustaceans are produced by fishery or farming for human consumption, the majority of it being shrimps and prawns. Hemolymph, is a fluid in the circulatory system of some arthropods. Hemolymph fills all of the interior (the hemocoel) of the animal's body and surrounds all cells. It contains hemocyanin, a copper-based protein which turns blue in color when oxygenated, instead of the iron-based hemoglobin in red blood cells found in vertebrates, thus giving hemolymph a blue-green color rather than the red color of vertebrate blood.

Naturally occuring agglutinating materials from the hemolymph of numerous invertebrates have often been reported. It seems quite clear that these proteins are not immunoglobulins, although in their biological activity, they may resemble vertebrate antibodies. Although the details of some of these chemical interactions are increasingly available, the basic biological usefulness of the materials stays unknown. Studies on the immunity of invertebrates have been focussed on identifying defence mechanisms and biochemical pathways activated during an infection, and on identifying cell-free hemolymph and cellular factors involved in the destruction of pathogens, regulation, and damage repair. Crustaceans possess an open circulatory system, where nutrients, oxygen, hormones, and cells are distributed in the hemolymph[1]. Crustaceans lack adaptive immune system and they rely exclusively on their innate immune mechanisms that include both cellular and humoral responses[2].

Research in immunology of commercially important marine invertebrates is currently related to infectious pathology but is progressively drawing nearer to genetics, on the one hand to characterize the genes of defence response effecters, and on the other hand to select pathogen-resistant strains, either by quantitative genetics or by genetic transformation[3].

2. Antimicrobial peptides and proteins

Antimicrobial peptides are important members of the host defense system. They have a broad ability to kill microbes. Large antimicrobial proteins (>100 a.a.), are often lytic, nutrient-binding proteins or specifically target microbial macromolecules. Small antimicrobial peptides act by disrupting the structure or function of microbial cell

*Corresponding author: W Sylvester Fredrick, Ph.D, Research Scholar, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University Parangipettai-608502, Tamil Nadu, India.
E-mail: sylvesterfr@gmail.com
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membranes. A multitude of antimicrobial peptides have been found in the epithelial layers, phagocytes and body fluids of multicellular animals including man. In addition to their role as endogenous antibiotics, antimicrobial peptides have functions in inflammation, wound repair and regulation of the adaptive immune system. The various antimicrobial peptides and their sources are tabulated in Table 1.

Antibacterial peptides can also be induced in epidermal cells in response to wounding or infection in the cuticles[4]. The whole process of synthesizing antibacterial proteins may take few minutes or hours after the challenge and these are secreted into hemolymph when invertebrates are in acute phase. Most of these proteins are small cationic molecules exhibiting a broad spectrum of activity against gram-positive and/or gram-negative bacteria. There are some antibacterial proteins that are not inducible such as lysozyme[5] and andropin[6]. Over the past several years, many antimicrobial peptides have been found and characterized in crab species. The first antimicrobial peptide characterized was a proline peptide of 6.5 kDa from the hemocytes of the shore crab *Carcinus maenas*[7]. The antimicrobial peptide callinectin is a cationic antimicrobial peptide of 3.7 kDa isolated from the blue crab *Callinectes sapidus*[8]. Recently, scygonadin, an anionic antimicrobial peptide has been isolated from seminal plasma of the mud crab *Scylla serrat*[9]. More recently, antilipopolysaccharide factors (ALFs), originally identified from the amoebocyte of the horseshoe crab *Limulus polyphemus*[10,11] have been identified from the hemocytes of several species of decapods including *Fenneropenaeus chinensis*[1], *Marsupenaeus japonicas*[12], *Penaeus monodon*[13], *Litopenaeus setiferus*[14], *Pacifastacus leniusculus*[15], *Carcinus maenas*, *Callinectes sapidus*[16], *Eriocheir sinensis*[17] and *Scylla paramamosain*[18].

2.1. Lectin

Lectins are naturally occurring proteins or glycoproteins which bind selectively and noncovalently to carbohydrate residues. The main characteristic of this class of protein is their ability to interact specifically with carbohydrates and combine with glyco-components of the cell surface[19]. Lectins from the hemolymph of invertebrates, including crustaceans, have been regarded as potential molecules involved in immune recognition and microorganism phagocytosis through opsonisation. These proteins, considered as functional precursors of antibodies, constitute a group of proteins generically denominated lectins. Lectins in cell-free hemolymph have been identified in almost all crustaceans. Moreover, lectins with structural characteristics and identical specificity to the cell-free hemolymph lectins, have been identified in the hemocyte membrane and in cytoplasmic granules[20]. In crustaceans, sialic acid-specific lectin of Indian horseshoe crab, *Carcinoscorpius rotunda cauda*, could agglutinate many types of bacteria and a lectin identified from the hemolymph of the blue crab *Callinectes sapidus* has also been found to possess agglutinating activity against several serotypes of *Vibrio* spp.[21-23]. The relevance of these lectins in the host defense system relies on the observation that sialic acid, an important constituent of many glycoconjugates, is present on different cell surfaces[24,25]. The selective binding of hemolymph lectin to some of the shrimp pathogenic bacteria suggests that the lectin plays a role in the defense against these pathogenic bacteria.

2.2. Callinectin

The blue crab, *Callinectes sapidus*, is a decapod crustacean of the brachyuran family Portunidae. Callinectin has broad-spectrum of antibacterial activity in its hemolymph that constitutes part of its nonspecific defences. Callinectin from blue crab hemocytes was purified using its basic (cationic) nature. Purified callinectin was active against *Escherichia coli* D31. Blue crab hemolymph has potent, broad-spectrum, antibacterial activity against many gram-negative organisms, including *Vibrios* and aeromonads. Callinectin's predominance as an antibacterial factor in blue crab hemocytes suggests that it plays a major role in blue crab immunity. The antibacterial activity of blue crab hemolymph is severely depressed in polluted waters[26]. Thus, callinectin

Table 1
Various antimicrobial peptides and its sources.

S.No	Protein	Molecular weight (kDa)	Species
1	Callinectin	3.7	*Callinectes sapidus*
2	Scygonadin	43.0	*Scylla serrata*
3	ALFs	–	*Limulus polyphemus*
4	Lectin	–	*Carcinoscorpus rotunda*, *Callinectes sapidus*
5	Apoprotein	–	Shrimp
6	Cryptocyanin	–	*Cancer magister*
7	Phenol oxidase	300.0	*Panulirus argus*
8	Crustins	7.0-14.0	*Hyas araneus*
9	Scyllin	4000.0	*Scylla serrata*
10	Artemocyanin	–	*Streptocephalus*, *Leptestheria*
11	Penaeidin	5.5-6.6	*Litopenaeus vannamei*
12	Clottable protein (CP)	400.0	*Penaeus vannamei*
13	Pernin	60.0	Mytilidae
14	Glycoproteins	–	*Tigriopus japonicus* and *Coullana* spp.

may provide a useful biomarker for health assessment of coastal ecosystems[27].

2.3. Lipoprotein

Lipids are a major source of energy in marine invertebrates, including shrimp. Furthermore, they are involved in several essential processes for their growth, molting and reproduction serving as energy storage. Lipids are also found in the hemolymph as water-soluble molecules formed by apoproteins and lipid moieties constituting the lipoproteins (LPs). LPs transport lipids from sites of absorption, storage or synthesis to sites of utilization. Due to their hydrophobic nature, lipids are transported in the hemolymph of shrimp by protein-lipid-complexes named lipoproteins. Since cholesterol (Ch) and polyunsaturated lipids must be provided by the diet, and they are stored mainly in the hepatopancreas, a special vehicle is necessary for their mobilization to other tissues. Two types of hemolymph lipoproteins have been isolated from penaeid shrimp. Non sex-specific lipoproteins are present in males and females and female-specific lipoproteins (LPII or Vg) occur mainly in mature females undergoing ovarian maturation. These lipoproteins are of the high density and very high density types. Their lipids are predominantly phospholipids, but sterols, diacylglycerols, triacylglycerols, and hydrocarbons have also been found. The apolipoproteins are high molecular mass polypeptides. The LPI generally contains a fewer number of apoproteins or subunits than the LPII or Vg[28].

2.4. Cryptocyanin

Cryptocyanin, a copper-free hexameric protein in crab (*Cancer magister*) hemolymph, has been characterized and the amino acid sequence has been deduced from its cDNA. It is markedly similar in sequence, size, and structure to hemocyanin, the copper-containing oxygen transport protein found in many arthropods. Cryptocyanin does not bind oxygen, however, and lacks three of the six highly conserved copper-binding histidine residues of hemocyanin. Cryptocyanin has no phenoloxidase activity, although a phenoloxidase is present in the hemolymph. The concentration of cryptocyanin in the hemolymph is closely coordinated with the molt cycle and reaches levels higher than hemocyanin during premolt. Cryptocyanin resembles insect hexamerins in the lack of copper, molt cycle patterns of biosynthesis, and potential contributions to the new exoskeleton. Phylogenetic analysis of sequence similarities between cryptocyanin and other members of the hemocyanin gene family shows that cryptocyanin is closely associated with crustacean hemocyanins and suggests that cryptocyanin arose as a result of a hemocyanin gene duplication. The presence of both hemocyanin and cryptocyanin in one animal provides an example of how insect hexamerins might have evolved from hemocyanin. Cryptocyanin may provide important molecular data to further investigate evolutionary relationships among all molting animals.

2.5. Prophenol oxidase

Phenol oxidase, a copper-containing enzyme, is widely distributed not only in animals but also in plants and fungi, which is responsible for initiating the biosynthesis of melanin. Activation of prophenol oxidase in arthropods is important in host defense. The prophenol oxidase activating system plays a major role in the defense mechanism of arthropods. The phenol oxidase activity and its location in the hemolymph of the spiny lobster *Panulirus argus* are presented. Phenol oxidase activity was observed in the hemocyte lysate supernatant (HLS) and plasma after their incubation with trypsin. Higher amounts of trypsin were required to activate the HLS prophenol oxidase, due to the presence of a trypsin inhibitor in this fraction. Activation of prophenol oxidase was found when HLS was incubated with calcium, with an optimal pH between 7.5 and 8. This spontaneous activity is due to the prophenol oxidase activating enzyme, a serine proteinase that activates the prophenol oxidase once calcium ions were available. Sodium dodecyl sulfonate (SDS) was able to induce phenol oxidase activity in plasma and hemocyte fractions. It is suggested that phenol oxidase activity found in plasma is produced by hemocyanin[29]. A major innate defense system in invertebrates is the melanization of pathogens and damaged tissues. This important process is controlled by the enzyme phenol oxidase that in turn is regulated in a highly elaborate manner for avoiding unnecessary production of highly toxic and reactive compounds. Recent progress, especially in arthropods, in the elucidation of mechanisms controlling the activation of zymogenic prophenol oxidase into active phenol oxidase by a cascade of serine proteinases and other factors is reviewed. The prophenol oxidase-activating system (prophenol oxidase system) is triggered by the presence of minute amounts of compounds of microbial origins, such as beta-1,3-glucans, lipopolysaccharides, and peptidoglycans, which ensures that the system will become active in the presence of potential pathogens. The presence of specific proteinase inhibitors prevents superfluous activation. Concomitant with prophenol oxidase activation, many other immune reactions will be produced, such as the generation of factors with anti-microbial, cytotoxic, opsonic, or encapsulation-promoting activities[30,31].

2.6. Crustin

Crustins are antibacterial proteins of 7-14 kDa with a characteristic four-disulphide core-containing whey acidic protein (WAP) domain, expressed by the circulating haemocytes of crustaceans[5]. Many immune proteins are released from crustacean haemocytes by exocytosis so it is plausible that crustins might be liberated into the body fluids in the same way. The arrangements of these are largely, but not entirely, conserved within taxonomic sub-groups of arrangements within the decapoda. At least three main subgroups appear to exist and we propose that they should be designated types I-III. Crustins are widely regarded as

antimicrobial effectors, yet there have been surprisingly few studies of their antibacterial properties *in vitro*. The crustins purified from the spider crab, *Hyas araneus*, also seem to kill gram-positive bacteria, most strongly showing an MIC value of 3 mM against *Corynebacterium glutamicum*[32]. One of the *Penaeus monodon* crustins (D766060), expressed as a recombinant protein, similarly shows activity only against gram-positive bacteria with particularly strong activity against *Staphylococcus aureus* and *Streptococcus iniae* but has no effect on *Aerococcus viridans* or *Micrococcus luteus*. Unusually, however, another recombinant type II crustin (EF654658) from the same species has recently been reported to show strong bactericidal activity not only against gram-positive bacteria, such as *Aerococcus viridans* var homari, but also against *Escherichia coli* 363 and the pathogen, *Vibrio harveyi*, both gram-negative bacteria. As, yet no studies have been made of the spectra of antibacterial activities of the type III molecules and this is urgently required to confirm their status as members of the crustin family. It would be very interesting to see if they too kill gram-positives and have any effects at all on gram negatives. Crustins have antibacterial properties, they must contribute to a greater or lesser extent in defence against bacterial infections, at least in decapods. However, the variable pattern in their expression after bacterial challenge is unlike than known for other arthropod antimicrobial peptides[33].

2.7. Scyllin

Interaction of plant and/or invertebrate lectins with mammalian cells and different microorganisms is well known. Scyllin is a low molecular weight (MW 4 000) lectin from the edible crab *Scylla serrata* hemolymph. It is purified by GalNAc-Sepharon affinity column followed by Mono-Q ion exchanger in FPLC and exhibits antimicrobial activity against *Bacillus cereus* and *Escherichia coli* by inhibiting endogenous respiration as well as exogenous glucose oxidation. In both cases oxygen consumption has been measured in an oxygraph. Scyllin has produced 50% inhibition of endogenous respiration at a concentration of 110 micrograms/mL and 125 micrograms/mL in *Bacillus cereus* and *Escherichia coli*, respectively. It also reduced the exogenous glucose oxidation by 50% at a concentration of 12 micrograms/mL and 80 micrograms/mL, respectively in *Bacillus cereus* and *Escherichia coli*. The mechanism of bacterial growth inhibitory property of scyllin is suggested though the other studies such as inhibition of nucleic acid biosynthesis, cell wall biosynthesis *etc*. to evaluate its total mode of inhibitory action are not yet obtained[34].

2.8. Artemocyanin

Artemocyanin, the extracellular hemolymph biliprotein of *Artemia*, is demonstrated in the fairy shrimp *Streptocephalus*, the clam shrimp *Leptestheria* and the water flea *Daphnia*. Artemocyanins can be purified from hemolymph as intact polypeptides (MW 170-190 000), but are degraded upon homogenization of the whole animal by partial proteolysis to polypeptides with MW of 102 000 and 85 000. The aminoterminal sequence of the intact artemocyanin polypeptide was determined, but no clear-cut relationships with arthropod biliproteins or other protein families could be demonstrated[35].

2.9. Penaidin

Penaeidins are members of a family of antimicrobial peptides, originally isolated from the shrimp *Litopenaeus vannamei*, which exhibit both gram-positive antibacterial and antifungal activities[36]. Penaeidins appear to be a family of antimicrobial peptides ubiquitous among penaeid shrimps where they are major actors of the immune response. Penaeidins have been initially characterized from *Litopenaeus vannamei* by biochemical approach and molecular cloning. The three peptides (Litvan PEN2-2, -2-1 and -3-1; initially named Pen-1,-2,-3) were isolated in their active and mature form (5.48-6.62 kDa) from the hemocytes of animals collected from intensive shrimp farms in Ecuador[37]. The penaeidins are highly cationic molecules composed of a N-terminal proline-rich domain, followed by a C-terminal domain containing 6 cysteine residues organized in two doublets. This overall structure is quite unique among the antimicrobial peptide families and this originality has led to the identification of the new family of penaeidins. Very recently, the solution structure of Litvan PEN3-1 and Litvan PEN4-1 has been determined revealing the overall organization of the two domains and the arrangement of the disulfide bonds[38]. The N-terminal domain is unstructured, in contrast to the C-terminal domain that is shown to be a highly constrained and well-structured domain. The structure of the peptide suggests that the two domains of the penaeidin may have different and complementary properties, which may contribute to a multifunctional character of the peptides. Penaeidins display post-transcriptional modifications. Litvan PEN2-1 and -3-1 are C-terminally amidated. The role of this modification is not yet clearly understood. It would be a common feature of peptides constitutively present and stored in hemocytes. This modification is also observed in peptides forming alpha-helices where the amidation would enhance the helical structure and may consequently have a significant contribution to the electrostatic interaction between the peptide and the microorganism membrane. When penaeidins are not C-terminally amidated, their antifungal activity is not affected whereas the antibacterial activity is reduced two-fold[39]. In addition, Litvan PEN3-1 is blocked at the N-terminus by a pyroglutamic acid resulting from the cyclization of a glutamine residue. The penaeidins, initially characterized from the shrimp *Litopenaeus vannamei*, appear to be a family of antimicrobial peptides ubiquitous among penaeid shrimp. Penaeidin chitin-binding ability could participate both in antimicrobial activity and in wound healing and chitin assembly. The peptides may play a role in the protection of the animals during molting cycle when the animals are particularly exposed to potential infections. This dual function of penaeidins is likely determinant for the

survival of the animals.

2.10. Clotting protein

The clottable protein (CP) was isolated from white shrimp, *Penaeus vannamei* plasma as a 400-kDa protein that splits to two identical 200-kDa subunits when it is reduced with 2-ME. However, using DTT as reducing agent, four main bands were observed; two of them (179 and 125 kDa) had the same N-terminus sequence of the intact CP, indicating that most fragmentation occurs in the carboxy-terminus. The proteinase activity of reduced CP was detected using azoalbumin as substrate. Proteinase activity was only detected in the reduced, but not alkylated protein. Trypsin and papain, as well as soybean trypsin inhibitor and E64, were included for comparison. Proteolytic activity of reduced CP was inhibited by E64, but not by STI, indicating that such activity corresponds to a cysteine type proteinase[40].

2.11. Pernin

A protein, designated pernin, found in the New Zealand green-lipped mussel, comprises almost all of the protein in cell-free haemolymph. It occurs as large, aggregate structures of several hundred units resembling small virus-like particles. Pernin is a non-pigmented, glycosylated protein, composed of 497 amino acids, which has an estimated molecular mass of 60 kDa. It is exceptionally rich in histidine Ž13.7%. and aspartic acid Ž12.3%, amino acids both known to participate in the binding of divalent metal cations. In addition, pernin has serine protease inhibitor activity, likely due to a sequence of eight N-terminal amino acid residues, separated from the remainder of the protein *via* a histidine aspartate spacer. The pernin monomer comprises three regions of obvious sequence duplication. These make up approximately 95% of the pernin molecule and have sequences clearly homologous to the active-site domain of CuZn SODs Z superoxide dismutases. The presence of pernin in the haemolymph at high concentration, its derivation from a protein Z SOD with a high affinity for activated oxygen, its aggregation into multimeric units associated with Fe and the lack of any known oxygen-transport system in Perna Z or other Mytilidae all suggest that the main role of pernin may be as an oxygen storage and transport protein[41].

2.12. Scygonadin

Scygonadin is an anionic antimicrobial peptide recently identified from the seminal plasma of *Scylla serrata*. To gain more detailed information on its antimicrobial activity, scygonadin mature peptide was expressed in *Escherichia coli* in order to obtain a large quantity of biologically active product. An approximately 43 kDa fusion protein CKS-scygonadin was obtained in a highly stable and soluble form[42]. The soluble component of the fusion CKS-scygonadin was purified by immobilized metal affinity chromatography (IMAC). A single 11 kDa recombinant scygonadin was cleaved from CKS-scygonadin and purified from the cleavage mixture using an affinity chromatography column with a yield of 10.6 mg/L. Alternatively, a recombinant scygonadin was purified from pET28-scygonadin by one-step Ni^{2+} affinity chromatography and 65.9 mg/L pure recombinant scygonadin was obtained which was higher than that purified from pTrc-CKS/scygonadin in bacteria culture. The recombinant scygonadin was confirmed using SDS-PAGE analysis and MS-fingerprinting. Both recombinant products of scygonadin from different expressed plasmids showed the activity against both gram-positive and gram-negative bacteria, but no activity against yeast and fungi tested. The kinetic studies showed that the recombinant scygonadin was strong active against *Staphylococcus aureus* and the killing of *Staphylococcus aureus* appeared time and dose dependent. Considering the quantity of recombinant product and the applicability of purification, the pET28-scygonadin expression system is a better choice to produce large quantities of recombinant scygonadin for commercial use in future[43].

2.13. Glycoproteins

Glycoproteins are proteins that contain oligosaccharide chains (glycans) covalently attached to polypeptide side-chains. In proteins that have segments extending extracellularly, the extracellular segments are often glycosylated. Glycoproteins are often important integral membrane proteins, where they play a role in cell-cell interactions. Copepods are crustaceans that dominate marine zooplankton communities. They use dissolved and surface-bound chemical cues for mate recognition[44]. Studies detailing the chemical identity of cue molecules and their role in communication between the sexes have been conducted in few species. Most work has focused on oligosaccharide residues of surface-bound glycoproteins and their use by copepods as sex pheromones. Glycoproteins bound to harpacticoid copepod exoskeleton surfaces, particularly the anntenule, genital pore, and caudal ramus, are involved in the recognition of species, sex and stage of potential mates[45,46]. The functional importance of carbohydrate residues on surface-bound glycoproteins of harpacticoid copepods (*Tigriopus japonicus* and *Coullana* spp.), particularly N-acetylglucosamine, has been demonstrated by lectin and monosaccharide binding assays. Based on these, and studies with proteolytic enzymes, the receptors for binding oligosaccharide residues on glycoproteins are hypothesized to be lectin-like proteins. Small molecule cues may also serve as pheromones in copepods[47]. However, little is known about the chemical nature of these cues.

3. Discussion

The emergence of new infectious diseases and resistance to the antibiotics by the existing ones led to the new sources for drug discovery. Many organisms possess antimicrobial

properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with conventional antimicrobials obtained from microorganisms[48]. With meager amounts of material no single analytical technique is capable of complete characterization of peptide structure. As a consequence, structure elucidation is usually performed by using various techniques, of which mass and NMR spectrometry are two of the most powerful methods. Mass spectrometry has been a primary technique in peptide structure analysis for more than three decades[49] with the information available. Recently marine peptides have opened a new perspective for pharmaceutical developments. It was fractionated and its various fractions were found to be rich in ninhydrin positive spot indicating the possibility of containing peptides. It was further confirmed by the presence of doublets in the region of its NMR spectrum[50].

Clearly, to resolve the enigmas about hemolymph, more directed functional and proteomic studies need to be undertaken, especially with respect to the diversity of bioactivities of the natural proteins, as well as considering how expression, processing and bioactivities relate to different types of threats to homoeostatic integrity. This study indicates that the haemolymph of crab would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics. Future research on marine invertebrate immunology would greatly benefit from knowledge acquired concerning vertebrates, invertebrates such as insects and also plants.

Conflict of interest statement

We declare that we have no conflict of interest.

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