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REVIEW

The role of lysosomal cysteine proteases in crustacean immune response

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Abstract

Over the long course of evolution and under the selective pressure exerted by pathogens and parasites, animals have selectively fixed a number of defense mechanisms against the constant attack of intruders. The immune response represents a key component to optimize the biological fitness of individuals. Two decades ago, prevention and control of diseases in crustacean aquaculture systems were considered priorities in most shrimp-producing countries, but knowledge was scarce and various pathogens have severely affected aquaculture development around the world. Scientific contributions have improved our understanding of the crustacean immune response. Several studies confirm the central role played by proteases in the immune response of animals, and the cooperative interaction of these enzymes in a wide variety of organisms is well known. This review summarizes the current information regarding the role of cysteine proteases in the immune system of Crustacea and points to aspects that are needed to provide a better integration of our knowledge.

Key Words: cathepsin; cysteine proteases; immune response; lysosome; regulation

Introduction

Metazoa comprise approximately 1.3 million living species and the phylum Arthropoda accounts for almost 95 % of the total. Insecta, the most diverse class among this phylum, has a large evolutionary history; these species have colonized all environments and their immune systems are partly responsible for their biological success (Vilmos and Kuruz, 1998). In the sea, Crustacea is a large and diverse subphylum that comprises about 67,000 species and could be considered aquatic counterparts of insects, but with a lower number of reported species. These species survive in an environment filled with a vast and diverse number of pathogenic agents. Both groups, insects and crustaceans, share a close phylogenetic relationship, and consequently, similarities in their innate immune systems (Regier *et al*., 2010).

The American Academy of Allergy, Asthma and Immunology defines immune system as: "The network of cell types working together to defend and protect the body from invaders, such as viruses, infections and disease" (http://www.aaaai.org/conditions-and-treatments/conditionsdictionary/immune-system.aspx). This definition applies to all species of arthropods; however, knowledge concerning the immune system of insects and crustaceans is still scarce, in contrast to their large number of species. Moreover, the structure and function of the immune system differs among taxa and relates to their environmental conditions; thus, comparative studies are necessary to understand the way individual systems work and how they were shaped by natural selection.

In the last 20 years, hundreds of studies about the immune system response of Dipteran insects, as the fruit fly *Drosophila melanogaster*, and the malaria mosquito *Anopheles gambiae,* have been published (see Jules Hoffman publications http://www-ibmc.u-strasbg.fr/ridi/profil.php?equipe_id=10&lang=en) (Dimopoulos *et al*., 2000; Watson *et al*., 2005; Pham *et al*., 2007; Li *et al*., 2013). Studies about crustaceans immune system sharply increased in the last 10 years, hundreds of studies on the freshwater crayfish *Pacifastacus leniusculus* and some shrimp species of the genera *Penaeus*, *Litopenaeus*, *Marsupenaeus*, and *Fenneropenaeus* spp., are aimed to describe new proteins, specific-receptors and molecules participating as part of response mechanisms that regulate activation of the proPO system, melanization and hematopoiesis among others

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(Söderhäll and Cerenius, 1998; Supungul *et al*., 2002; Lin and Söderhäll, 2012; Jin *et al*., 2013).

Early studies of the crustacean immune system found proteases as part of the innate response. Serine proteases were mainly described as participating in a series of enzymatic cascade pathways, but information about cysteine proteases and their physiological roles is scarce. The discovery of the first lysosomal cysteine protease, cathepsin C, in animal's tissues, occurred in 1948 by Gutman and Fruton. No other physiological roles for cathepsins were described until cathepsins B and H were identified in 1970 (Barret *et al.,* 1998; Turk *et al*., 2001). These enzymes were considered non-selective, being responsible for protein degradation in cells and only participating in lysosomes. A series of new functions and new enzymes have been identified recently, and some cathepsins and other lysosomal proteases are now recognized as a part of the immune system in vertebrate and invertebrate species.

This review provides a comprehensive overview on the current state of knowledge related to crustacean lysosomal cysteine proteases, including those that have recently been described and whose roles include immune response. The most recent findings are discussed and new perspectives about further study of these enzymes are suggested.

Characteristics of lysosomal cysteine proteases

Proteases are involved in a plethora of physiological functions and pathologies. Regarding the specificity of the enzyme, they are involved in two main functions: those proteases catalyzing nonspecific hydrolysis of proteins, like digestive proteases, and those catalyzing highly selective, limited, and efficient cleavage of specific substrates that control cell physiology (Fuchs *et al.,* 2013). The most abundant classes of proteases in human are, in order of abundance, metallo-, serine, and cysteine proteases (186, 176, and 143 genes, hence proteases). Some may be grouped according to the site they act on and their function (Puente *et al.,* 2003).

The cysteine proteases are those enzymes that include a cysteine residue in the catalytic site to hydrolyze peptide bonds (Dunn, 1989). These proteases are distributed in all taxa and the Clan CA, based on papain, is by far the largest among cysteine proteases, which are single polypeptide chain proteins around 30 kDa. All those cysteine proteases, which are involved in immune reactions, belong to the Clan CA, commonly associated with the innate and adaptive immunity processes. Many of them are found in the lysosome as part of a group that, in concert with the proteasome, plays an important role in the cellular protein turnover pathway.

Among the lysosomal proteolytic enzymes, the aspartic proteases, in combination with the cysteine and serine proteases, are usually detected in vesicles of the endocytic pathway. Besides their role in protein degradation within lysosomes, these enzymes in vertebrates are tissue-specific. They participate in antigen processing and presentation within early endosomes, zymogen processing, degradation of matrix constituents in the extracellular space, cytokine regulation, and in the initiation of apoptotic processes within the cytosol (Brix, 2005; Colbert *et al*., 2009).

Lysosomal cysteine proteases (LCPs) have optimum activity in the slightly acidic and reducing milieu of lysosomes. As other proteases, these enzymes are synthesized as zymogens in order to yield activity at the right spot and time, preventing thus unwanted tissue damage (Turk *et al*., 2001). Some of the most studied members of the LCPs family include cathepsins. Various lysosomal cathepsins from vertebrates, such as L, S, C, F, B, X, H, K, V and W are cysteine proteases. Cathepsins D and E are aspartic proteases, and cathepsin G, granzymes, and thymus-specific serine proteases (TSSP) are serine proteases (Colbert *et al*., 2009).

Interestingly, as several of the above mentioned enzymes perform the same task of protein degradation, it appears that the proteolytic processing machinery may be functionally redundant, which is supported by the fact that no effect can be detected by the lack of any cathepsin. However, specific functions have been reported for cathepsins expressed in a tissue-specific manner. Some examples are bone remodeling by cathepsin K, processing of the major histocompatibility complex class II by cathepsin S, and a lesser role by cathepsin L. The lysosomal cathepsin C is a major processing device that activates serine granule proteases, granzymes A and B, cathepsin G, neutrophil elastase, and chymase (Turk *et al*., 2001); thus, some of them are required to activate others, and the loss of homeostasis in enzyme activity leads to pathologies.

As with other LCPs, cathepsins are also synthesized as inactive precursors that become activated after a limited proteolysis that removes the N-terminal propeptide. This propeptide is located on the active site (Turk *et al*., 2001), and activation happens under acidic pH, auto-catalytically in trans (one molecule activating another) (Rawlings and Salvesen, 2012). Cathepsin disturbances in mammals have been associated with several malignancies. These enzymes are also involved in apoptosis; pathologies may appear when cathepsin inhibitors, instead, are down-regulated (Colbert *et al*., 2009).

Since cathepsins B, L and C are the only lysosomal cysteine proteases identified and studied in crustaceans to date, they will be briefly described below (Table 1). Although caspases are predominantly cytosolic, these cysteine proteases were included in this review since they are involved in the response against infectious agents in vertebrates. Recent evidence has shown that they also play a key role in apoptosis in arthropods.

Cathepsin B

Cathepsin B (EC 3.4.22.1) was one of the first described members of the large C1 family of LCPs. The protein is synthesized as a pre-proenzyme and targeted to lysosomes, and it may exhibit both,

Table 1 Lysosomal cysteine proteases from Crustacea species

Enzyme	Species	Tissue/organ	Protein size (aas)	Suggested function	Reference
Cathepsin B	*Pandalus borealis*	MG exclusive	328	Hydrolysis of food protein	Aoki *et al.,* 2003
Cathepsin B	*Litopenaeus vannamei*	Hm, Gills, Amp, M, MG, Gut, GJ	331	Hydrolysis of food protein/Intracellular protein hydrolysis	Stephens *et al.,* 2012
Cathepsin B	*Fenneropenaeus chinensis*	Muscle, Gills, MG	331	May participate in anti-WSSV immune reactions	Li *et al*., 2013
Cathepsin C	*Penaeus monodon*	O, H, MG, M, P, B	449	Perhaps involved in the immune defense	Qiu *et al.,* 2008
Cathepsin C	*Eriocheir sinensis*	Hm	427	Involved in the innate immune system	Li *et al*., 2010
Cathepsin C	*F. chinensis*	Hm, MG, Gills, Gut	451	May play a role in the antiviral immune response	Wang *et al*., 2012
Cathepsin L	*Homarus americanus*	MG, GJ	323, 322, 321	No details	Laycock *et al.,* 1991
Cathepsin L	*Nephrops norvegicus*	ES, S	324	Cathepsin L may not work as a digestive enzyme	Le Boulay *et al.,* 1995
Cathepsin L	*L. vannamei*	MG	326, 322	Digestive enzyme	Le Boulay *et al.,* 1996
Cathepsin L	*Artemia franciscana*	Embryos and young larvae	217	Non lysosomal	Butler *et al*. 2001
Cathepsin L	*Metapenaeus ensis*	S, MG, H, ES, T, O, G	322	Hydrolysis of food protein intra and extracellularly	Hu and Leung, 2004, 2007
Cathepsin L	*F. chinensis*	S, Gills, Hm, Gut, MG	---	May participate in anti-WSSV immune reactions	Ren *et al*., 2010

ES (eye stalk), MG (midgut gland), G (gut), GJ(gastric juice), Gills (gills), M (muscle), Hm (hemocytes), S (stomach), H (heart), T (testis), O (ovary), P (plasma), B (brain), Amp (terminal ampule)

endopeptidase or dipeptidyl-carboxipeptidase activities (Koga *et al*., 1991; Hasain *et al.,* 1992).

The human cathepsin B proenzyme is a glycoprotein with 339 amino acids. All cathepsin Bs include an extra 20-residue sequence encoding the highly flexible “occluding loop”, which is characterized by two adjacent histidine residues (His352, His353) and includes amino acid residues Pro344 to Cys371. The occluding loop confers dipeptidyl-carboxipeptidase activity to the enzyme (Musil *et al.*, 1991).

Cathepsin B compartmentalization within the lysosomes suggests an extensive role for intracellular protein degradation; however, its presence in the nucleus, cytoplasm, and plasmatic membrane indicates alternative roles in physiological and pathological processes. Involvement of this enzyme in lysosomal-mediated apoptosis is supported by studies where cathepsin B is knocked out in mice. This enzyme is also found in tumor cells, and a role in invasion and metastasis has been proposed (Guicciardi *et al*., 2000; Colbert *et al*., 2009).

Cathepsin C

Cathepsin C (EC 3.4.14.1), better known as dipeptidyl peptidase I (DPP-I), removes dipeptides from N-terminus substrates and activates zymogens. As cathepsin B, it also belongs to the C1 family, and the mature enzyme is a tetramer with a tetrahedral arrangement already confirmed by crystallography (Molgaard *et al*., 2007). In mammals, it is involved in the immune response and inflammatory disorders as arthritis (Adkinson *et al*., 2002). There is evidence that indicates that cathepsins L and S are part of the activation mechanism of human cathepsin C, whose propeptide is removed to activate the mature enzyme (Dahl *et al*., 2001). Once active, this enzyme activates neutrophil-derived serine proteases and regulates development of acute experimental arthritis (Adkinson *et al*., 2002).

Cathepsin L

Cathepsin L (EC 3.4.22.15) is an endopeptidase with a preference for aromatic residues. It is synthesized as a single chain

zymogen that is activated by limited hydrolysis (Rawlings and Salvesen, 2012).

Cathepsin L possesses two potential glycosylation sites, however only Asn221 is derivatized. The catalytic site is formed by Cys138, His276, and Asn300. Besides been located within the lysosomes at mannose-6-phosphate receptors, it is also located in the nucleus, cytoplasm, and outside the cell.

Cathepsin L specifically hydrolyzes autophagosomal membrane markers in lysosomes (Takahashi *et al.,* 2009). Lack or inhibition of the enzyme in mammals leads to several biochemical and cellular alterations, including up-regulating of signaling pathways or turnover of defective collagen. The enzyme is also involved in exogenous protein digestion occurring before the presentation of specific antigen peptides by the major histocompatibility complex (MHC) to CD4+ T cells (Onishi *et al*., 2004).

Caspases

As one of the two major families of cysteine endoproteases, caspases are cysteinyl aspartate-specific proteases in (X-X-X-Asp) motifs. These enzymes recognize tetra-peptide sequences on their substrates and hydrolyze peptide bonds after aspartic acid residues. They belong to the Clan CD Family C14 and rely on the catalytic dyad of His and Cys, which is characteristic of this enzyme family (Rawlings and Salvesen, 2012; McIlwain *et al.*, 2013). Although they are predominantly cytosolic, these cysteine proteases were included in this review because they are involved in the response against infectious agents in vertebrates, and recent evidence points out they play also a key role in apoptosis in arthropods.

The study of caspases in the nematode *Caenorhabditis elegans* led to the discovery of apoptosis. Fourteen nematode and insect caspases are listed in the Handbook of Proteolytic Enzymes (Rawlings and Salvesen, 2012). Since caspases may differ in the length of activation peptides and mature enzymes, they have been classified as procaspases-1, -2, -4, -5, -8, -9, -10, and -14, possessing longer activation peptides, and procaspases-3, -6, -7 -11, and -13 possessing shorter activation peptides (McIlwain *et al.,* 2013).

Activation of procaspases with a short activation peptide is a two-step process: at first, a cleavage takes place by caspases or granzyme B, followed by an autoproteolytic step (Zhivotovsky *et al*., 1999). Apoptotic caspases can be divided into two classes: initiator and executioner caspases. Initiator caspases (caspase-2, -8, and -9) are the apical caspases in apoptosis signaling cascades, and their activation is normally required for executioner caspase (caspase-3, -6, and -7) activation (Stephen *et al.*, 2010).

Executioner caspases are paramount in apoptosis; however, this is not their only function, since they are involved in a plethora of cellular functions, including proinflamatory cytokine interleukin processing, counteracting necroptosis, and mediation of cell immunity (Rodrigue-Gervais and Saleh, 2013).

Participation of proteases in the immune system

Differences between vertebrate and invertebrate immune systems

Understanding the immune response of crustaceans has become a major issue in recent years because several pathogens have become catastrophic to shrimp farmers around the world. These include the White Spot Syndrome Virus (WSSV; Sanchez-Paz, 2010) and the recently described Early Mortality Syndrome provoked by a bacterium (Tran *et al.,* 2013). Efforts by researchers, institutes, and government bureaus have attempted to solve this problem.

Immune response, as all physiological functions, evolved gradually from a relatively simple immune system to more intricate and complex forms (Kaufman, 2010). Immunity in vertebrates involves two mechanisms: innate and adaptive immunity. For a long time, studies have demonstrated significant differences between vertebrate and invertebrate immune systems (Vilmos and Kuruz, 1998; Müller *et al*., 2008). One of the more important differences is the lack of an adaptive immune response in insects and crustaceans, which means that there is no ability in invertebrates to produce immune cells specially designed to attack a specific antigen in a long term response, also called “immunological memory”, whose main purpose is to protect the host from re-infections and protect the immunologically immature offspring (Welsh *et al*., 2004).

To date, only the innate immune response present in flies, beetles, shrimp, crayfish, and crabs has been studied (Faye, 1990; Rinkevich and Weissman, 1990; Iwanaga *et al*., 1998; Witteveldt *et al*., 2004). However, recent evidence indicates that these species have a resemblance of an adaptive immunity that is based on molecules other than antibodies, T-cell receptors, and MHC molecules, which was first discovered in snails (Adema *et al*., 1997; Zhang *et al.*, 2004).

An “alternative” adaptive immunity in Crustacea was suggested in 1998 by Flegel and Pasharawipas, that hypothesized an “acquired tolerance of shrimp to viral pathogens”. Subsequently, Venegas *et al.* (2000) suggested a “quasi-immune response” of the kuruma prawn (*Marsupenaeus japonicus*) to WSSV. Arala-Chaves and Sequeira (2000), after comparing vertebrate and invertebrate responses, concluded that an alternative adaptive immune response occurs in invertebrates, which is quite different to the adaptive system in vertebrates; invertebrates have a small number and less diverse set of receptors for immunostimulants than vertebrates. Flegel (2009) suggested a heritable, antiviral immunity in crustaceans and insects that explains, through a viral accommodation process, the mechanism of heritable resistance to pathogens in arthropods; this is also one on the main characteristics of the adaptive immune response of vertebrates.

Recent studies report the first Dscam (Down Syndrome cell adhesion molecule), a member of the immunoglobulin superfamily (IgSF) in *Litopenaeus vannamei* and *Penaeus monodon* shrimp (Chou *et al.,* 2009, 2011), and the *E. sinensis* crab (Jin et al. 2013). The Dscam immunoglobulins in invertebrates are quite different from their vertebrate counterparts,

since they lack the transmembrane domain and the cytoplasmic tail; however, the single gene encoding Dscam generates thousands of variants by alternative splicing; these molecules are found on the cell surface of hemocytes, and gene expression is induced by the presence of lipopolysaccharides, peptidoglycans, and glucans (Brites *et al.,* 2008; Jin *et al.,* 2013). Watthanasurorot (2013) emphasized that the role of Dscam in the crayfish *P. leniusculus* is similar to the generation of antibody diversity in vertebrates and that this specificity in immune recognition is part of the innate immune system of crustaceans.

Originally, the innate immune system of Arthropoda was considered less sophisticated; currently, there is scientific evidence that supports a more complex role because it includes the protection exerted by physical barriers (epithelium) and rapid, efficient, and systematic responses to distinguish and destroy foreign (non-self) materials. Innate immunity includes humoral and cellular components, that are triggered by signal transduction pathways and activated following an infectious challenge, mainly in hemocytes and plasma, as originally studied in *D. melanogaster* (for comprehensive reviews, see Borregaard *et al*., 2000; Liu, 2008; Vazquez *et al*., 2009).

The innate response in the crayfish *P. leniusculus* has been studied (Liu *et al*., 2009; Watthanasurorot, 2013) and includes responses, such as melanization, blood coagulation, production of antimicrobial peptides, phagocytosis of small pathogens, and encapsulation of invading parasites that are too large to be phagocytized by individual hemocytes (Liu, 2008). A complex array of factors is activated when molecules are released from microbial structures, such as bacterial cell wall or double-stranded RNA in viruses. In general, these factors are pattern-recognition proteins, such as ß-glucan-binding protein, lipopolysaccaride- and glucan-binding protein, masquerade-like protein/serine proteinase homologues, and lectins (Maftuch *et al*., 2013).

Among Invertebrate strategies to expand immune responsiveness is to produce a wide variety of antimicrobial peptides (AMPs; Ghosh *et al*., 2011). AMPs are small, cation amphipatic molecules synthesized in the fat body of insects (Hoffman and Reichhart, 1997). In crustaceans, the first described AMPs were found in crabs, *Carcinus maenas* (Schnapp *et al*., 1996) and *Callinectes sapidus* (Khoo *et al.,* 1999), and later in shrimp *L. vannamei* (Destoumieux *et al.,* 1997), these molecules specifically recognize chemical entities from microbial structures as part of an initial humoral response of the host against invasions of Gram-negative bacteria, fungi, yeast, viruses, and protozoa (Liu, 2008).

In crustacean hemocytes AMPs are released to the hemolymph as a response to an infection (Destoumieux *et al.,* 2000; Bulet *et al*., 2004). Two major groups of shrimp AMPs are known: penaeidins and crustins. Four classes and several isoforms of penaeidins have been identified in crustaceans, all are small peptides (5 - 7 kDa), with specific characteristics as the C-terminal cysteine-rich domain. Penaeidin diversity relies on the variation of gene sequences and gene expression and in their activity against different microbes (Ghosh *et al*., 2011).

Crustins are larger than penaeidins (7 - 14 kDa) that contain whey acidic protein (WAP) domains, including a four-disulfide-core motif of around 50 amino acids and eight highly-conserved cysteine residues (Smith *et al*., 2008). Several crustins have been classified into various types, according to their amino acid sequences and their WAP domains; new proteins are described in freshwater and marine crustaceans containing up to two WAP domains that confer the ability of inhibiting proteinases and exhibit antiviral and antimicrobial activities (Chen *et al*., 2008; Li and Xiang, 2013).

Proteases as part of the crustacean immune system

Some of the more important molecules involved in the innate response of the Arthropoda, mainly crustaceans, have been already mentioned. Now, this review will focus on the proteases used to regulate defense responses, including antimicrobial peptide synthesis, melanization, and coagulation, which are carried in the hemolymph and are rapidly mobilized to activate the immune system (Kawabata *et al*., 1996; Iwanaga *et al.,* 1998; Gorman and Paskewitz, 2001).

Over recent decades, a large amount of fundamental scientific information on the role of proteases in the immune system of invertebrates has been reported (Gorman and Paskewitz, 2001). Studies in insects, mainly those species whose complete genome has been sequenced, such as the malaria mosquito *Anopheles gambiae,* have identified a series of proteases as part of the responsive immune system, including serine proteases that participate in activating the prophenoloxidase (ProPO) cascade, trypsin-like proteases, serpins and their specific inhibitors, as part of the coagulation cascade (Dimopoulos *et al.*, 2000). The serine protease Sp22D from *A. gambiae* is one of the first enzymes linked to the immune response; its transcriptional up-regulation occurs within 1 h after infection in immune related cell types, and its multi-domain organization suggests that it interacts with pathogen surfaces (Gorman *et al*., 2000).

In crustaceans, the lack of a completely sequenced genome has limited the identification and characterization of immune-related genes; therefore, there is less information available for these species. Recently, Tassanakajon *et al.* (2013) and Jeswin *et al.* (2013) reported a list of molecules that may be involved in shrimp immunity, some of which will be described.

In crayfish, the process of binding-pattern recognition proteins to a microbial molecular target causes proPO activation to phenoloxidase (PO), which is the terminal enzyme of the system that leads to the synthesis of melanin (Sritunyalucksana and Söderhall, 2000; Cerenius *et al*., 2010). Production of melanin, melanization, as a response to invaders, is a ubiquitous innate immune response that starts in hemocytes with the conversion of inactive proPO to active PO by a serine protease, the prophenoloxidase activating enzyme (ppA) and depends on a proteolytic cascade similar to the one

activating the Toll pathway (Söderhäll, 2010). Shrimp genes encoding a pro-phenoloxidase, prophenoloxidase-activating factor, and a masquerade-like serine proteinase-like protein are involved in detecting and clearing bacteria, mediating hemocytes adhesion, and binding to bacteria cell walls (Tassanakajon *et al*., 2013).

Serine proteases function as important regulatory proteins in activating the proPO and clotting systems of the fleshy prawn *Fenneropenaeus chinensis,* giant tiger prawn *P. monodon*, and whiteleg shrimp *L. vannamei* (Supungul *et al*., 2002; Jimenez-Vega *et al*., 2005; Dong and Xiang, 2007; Xue *et al*., 2013). Trypsins and chymotrypsins participate in the immune response of marine and freshwater shrimp and crayfish by rapidly activating the immune response when pathogens are detected (Xue *et al.,* 2013). Newly discovered enzymes, such as a chymotrypsin-like enzyme, have been found in crustaceans; the enzyme was isolated from the midgut gland of the redclaw crayfish *Cherax quadricarinatus*. It has been suggested that it participates in the innate immune reactions of adult and juvenile crayfish since its transcripts were detected in all tissues, but mainly in midgut gland, intestine, hemocytes and muscle where this enzyme is over-expressed after a WSSV challenge (Fang *et al.,* 2013).

Apoptosis and Toll pathways

Two physiological mechanisms evolved to lead embryos development and deal with microbial infections in larval and adult stages. They are apoptosis and the Toll pathway, with participation of cysteine proteases.

Apoptosis is a complex mechanism of defense against viral infections by inhibiting viral replication. Although not exclusively, apoptosis is a process of cell destruction requiring cysteine proteases called caspases (Wang *et al*., 2008). Apoptosis in crustaceans is a highly regulated mechanism of cellular suicide that includes a series of consecutive steps, from sensing invading agents to mitochondrial changes in membrane permeability (Menze *et al*., 2010; Leu *et al.,* 2013).

Caspases act as initiators and executioners in the process of apoptosis (see enzyme description above). In the Crustacea, different caspases have been identified, all of them include the typical domain structure of mammalian caspases (Wang *et al.,* 2013). In the tiger prawn *P. monodon,* experimentally inoculated with the WSSV, there is an increase in gene expression and enzyme activity of hemocyte caspase-3, which is present until apoptotic cell death (Wongprasert *et al*., 2007). In the whiteleg shrimp *L. vannamei*, four enzymes were recently identified as Lv-caspases 2 - 5, expressed in a tissue-specific manner. These were up-regulated after a viral challenge, suggesting a protective role in the defense against viruses (Wang *et al.*, 2013). Various conserved molecules, acting as apoptotic regulators, have also been identified in crustacean proteomes, but further research should reveal their functions because they have not been confirmed.

The Toll pathway uses toll-like receptors (TLRs) to recognize Gram-positive bacteria, fungi, and viruses to initiate an innate defensive reaction. In a review by Valanne *et al.* (2011), nine Toll receptors were found in the fly genome. Invading pathogens activate the cellular response and systemic synthesis of antimicrobial peptides. Activation happens when the proteolytically cleaved ligand Spätzle attaches to the Toll receptor, which then leads to activation of the NF-k B (dorsal) factor. The vertebrate TLR signaling pathway and the invertebrate Toll signaling pathway share similarities and it is suggested they share similar function.

Recent information demonstrates a functional Toll signaling pathway in crustaceans. Identification of molecules, such as Toll-like receptors (TLRs), the cytokine-like molecule Spätzle, and the Pelle protein, among others in several shrimp species, are similar to proteases reported in *Drosophila melanogaster*, which suggests that a signal transduction system is part of the innate immune response of crustaceans (Li *et al*., 2013).

Crustacean lysosomal cysteine proteases

Studying transcriptomes in shrimp has contributed to the ESTs list of proteins whose genes may be involved in the immune functions when organisms are infected by a virus, bacteria, or both (Dong and Xiang, 2007; Tassanakajon *et al.,* 2013). Besides caspases, cathepsins are the more frequently reported cysteine proteases in the crustacean list of immune-related genes.

Probably the first reported cysteine protease was found in the gastric fluid of the American lobster *Homarus americanus* (Laycock *et al*., 1989). Later studies report cathepsins L, C, and B; these are the only lysosomal cysteine proteases that have been identified and studied in shrimp tissues and species (Table 1).

In the Crustacea, cathepsin C was first suggested to be involved in maturation of oocytes of the kuruma shrimp *Marsupenaeus japonicus* (Qiu *et al*., 2005). Qiu *et al.* (2008) characterized the cathepsin C cDNA in the giant tiger prawn *P. monodon* and proposed, for the first time, that it participates in the immune response of shrimp. Experimental evidence indicates that cathepsin C mRNA is over-expressed in midgut gland when stimulated by lipopolysaccharides (LPS). Increased levels of mRNA of this enzyme were detected in hemocytes of the crab *E. sinensis* after a *Vibrio anguillarum* challenge, peaking at 6 h after injection (Li *et al.,* 2010).

Other studies that analyzed the structure and phylogenetic relationships of shrimp cathepsin B are conclusive about its conserved regions, characteristic domains, and similarity to insect homologues; however, its function seems to vary among species. Aoki *et al.* (2003) reported the first cathepsin B exclusively expressed in the midgut gland of the ghost shrimp *Pandalus borealis* and suggest that the enzyme is a food protein hydrolase. Later, Stephens *et al*. (2012) found that cathepsin B mRNA is expressed and the enzyme activated in several shrimp tissues, and suggested that the enzyme participates in extracellular hydrolysis of food protein as they compared the enzyme with other digestive enzymes as trypsin and

chymotrypsin. Li *et al.* (2013) showed that cathepsin B participates in the antiviral immune response of the fleshy prawn as the amount of mRNA increased after a WSSV challenge.

Although cathepsin L has been proposed as part of the immune response of the fleshy prawn (Ren *et al.,* 2010), there is no information about the relationship between the enzyme digesting exogenous proteins and the production of antigenic peptides or any similar role, as observed in mammals, to date.

To better understand the role of crustacean enzymes in the innate immune system, biochemical studies are required. Acquiring data about the protein structure and their kinetic parameters of cysteine proteases will be useful, including their abilities to hydrolyze different substrates and to be inhibited by specific compounds.

Inhibition studies are essential, but there are no reports for these enzymes in crustaceans. Since specific inhibitors have been tested on cathepsins in human cell lines in an attempt to control their activity in cancer cells, inhibition assays may help to determine how cathepsins function in uninfected/infected shrimp.

As mentioned above, proteases do not act individually; instead, protease cascades may be activated through a mechanism of pattern recognition that controls coagulation, melanization, and activation of Toll and apoptosis pathways. Since experimental evidence shows a series of unidentified active proteases in zymogram gels of shrimp extracts, it is likely that not all molecules that form part of these complex mechanisms have been identified or their functions in crustaceans understood.

Differences between terrestrial and aquatic environments may explain some adaptive characteristics related to the structure and function of these enzymes, as observed by Rojo *et al.* (2013) who demonstrated that cathepsin D in the midgut gland of clawed lobsters is a cold-adapted enzyme having structural modifications.

Immunohistochemical studies should provide information about extracellular enzymes functions as food protein hydrolases in the midgut gland and/or their role as intracellular lysosomal proteases actively participating in the innate immune response.

Concluding Remarks

Some of the roles of LCPs, as part of the immune system, were reviewed for the most studied vertebrate models and compared with roles of crustacean cysteine proteases.

The absence of an adaptive immune system in crustaceans as that of mammals, which includes a MHC, T cells, and other components, may limit the roles of cysteine proteases, as they have been described; thus, we suggest that the enzymes are: (1) devoted only to essential functions, such as hydrolyzing food protein extracellularly and hydrolyzing lysosomal proteins intracellularly; and (2) involved in somewhat-related functions in the immune system, acting in a less specific manner than mammalian enzymes.

Studies of the crustacean immune system, including recent findings of molecules, such as the Dscam, toll pathway receptors, and cathepsins suggest the innate immune response is more complex than expected. As research continues, there is an important opportunity to find new cysteine proteases, probably including a large group of caspases, unreported cathepsins, and other lysosomal aspartic and serine proteases. All the team players, their identities, characteristics, and functions may be integrated into the physiological and pathological mechanisms that allow crustaceans to overcome pathogens and develop resistance-states to viruses and bacteria in the sea.

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