

The proPO and clotting system in crustaceans

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Received 1 March 2000; accepted 9 May 2000

Keywords: Prophenoloxidase activating system; Pattern recognition proteins; Peroxinectin; Clotting protein; Crustacean

1. Introduction

Aquaculture production has made impressive progress during the past two decades especially with regards to crustaceans, such as shrimp, prawn and lobster. Research in terms of immunity has received a high priority to control disease and to ensure long-term survival of shrimp culture. Invertebrates, including crustaceans, do not have acquired immunity, instead they have an innate immune system, which includes melanization by activation of the prophenoloxidase activating system (proPO system), a clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action and cell agglutination (Söderhäll, 1999). Research in the area of innate immunity in arthropods is rapidly progressing, whereas shrimp immunity research has been a subject of minor interest compared to similar research performed on other crustaceans and insects. The penaeid shrimp can be a good model to use mainly because they are short lived compared to many other crustaceans. In this paper, we review what is known so far about crustacean defence mechanisms, particularly of penaeid shrimp, with special emphasis on the proPO and the clotting system.

2. The prophenoloxidase activating system

It has been recognised that defence reactions in many invertebrates are often accompanied by melanization. In arthropods, melanin synthesis is involved in the process of sclerotization and wound healing of the cuticle as well as in defence reactions

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(nodule formation and/or encapsulations) against invading microorganisms entering the hemocoel (Söderhäll, 1982; Ratcliffe et al., 1985; Sugumaran, 1996). The enzyme involved in melanin formation is phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) and has been detected in the hemolymph (blood) or coelom of both protostomes and deutereostomes, as well as in the cuticle of arthropods (Söderhäll and Cerenius, 1998). PO is a bifunctional copper containing enzyme, which catalyses both the *o*-hydroxylation of monophenols and the oxidation of phenols to quinones (Sugumaran, 1996). Thus, this enzyme is able to convert tyrosine to DOPA, as well as DOPA to DOPA-quinone, followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment. PO is the terminal enzyme of the so-called proPO system, a non-self recognition system present in arthropods and other invertebrates (Söderhäll, 1982; Ashida, 1990; Söderhäll et al., 1996). The activation of this proPO cascade is exerted by extremely low quantities (pg/l) of microbial cell wall components (lipopolysaccharides (LPS), β -1,3-glucans or peptidoglycans (PG)) and results in the production of the melanin pigment, which can often be seen as dark spots in the cuticle of arthropods (Söderhäll, 1982; Sugumaran and Kanost, 1993). During the formation of melanin, toxic metabolites are formed which have fungistatic activity (Söderhäll and Ajaxon 1982; St. Leger et al., 1988; Rowley et al., 1990; Nappi and Vass, 1993). Several components or associated factors of the proPO system have been found to play several important roles in the defence reactions of the freshwater crayfish, *Pacifastacus leniusculus* (Söderhäll and Cerenius, 1998; Söderhäll et al., 1996).

Biochemical studies on shrimp proPO system has been carried out in *Penaeus californiensis* (Vargas-Albores et al., 1993a, 1996; Hernández-López et al., 1996; Gollas-Galván et al., 1999), *P. paulensis* (Perazzolo and Barracco, 1997), *P. stylirostris* (Le Moullac et al., 1997) and *P. monodon* (Sritunyalucksana et al., 1999b). In the penaeid shrimp, enzymes of the proPO system are localized in the semigranular and granular cells (Vargas-Albores et al., 1993a; Perazzolo and Barracco, 1997). This is in agreement with a recent study showing that *P. monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana et al., 1999a).

The characteristics of proPO in arthropods that have been purified and cloned so far are shown in Table 1. Recently, *P. monodon* proPO was cloned (Sritunyalucksana et al., 1999a). Sequence analysis with the BLAST algorithm shows that the *P. monodon* proPO deduced amino acid sequence has highest similarity to crayfish proPO (74%). Significant similarity is also shown to other insect proPOs. The shrimp proPO has a 3002 bp cDNA and contains an open reading frame of 2121 bp encoding a putative polypeptide with 688 amino acids and with a molecular mass of 78.7 kDa. No hydrophobic signal sequence was present in the putative N-terminus, as was the case with all arthropod proPO's cloned so far. Shrimp proPO has been purified from *P. californiensis* hemocytes (Gollas-Galván et al, 1999) and is a monomeric protein with a

Notes to Table 1:

^a Molecular masses calculated from deduced amino acid sequence.

^b Molecular masses estimated by SDS-PAGE.

^c Direct submission of sequence. The numbers in parentheses show the accession numbers.

Table 1
Arthropod prophenoloxidasases

Species	Type of proPO	Cloned	Molecular masses (kDa) ^a	Reference	Purified	Molecular masses (kDa) ^b	Reference
Crustaceans							
<i>Penaeus monodon</i>	proPO	Yes	78.7	Sritunyalucksana et al., 1999a	No		
<i>Penaeus californiensis</i>	proPO	No			Yes	114	Gollas-Galván et al., 1999
<i>Pacifastacus leniusculus</i>	proPO	Yes	80.7	Aspán et al., 1995	Yes	76	Aspán and Söderhäll, 1991
Insects							
<i>Anopheles gambiae</i>	proPO2	Yes	78.1	Jiang et al., 1997b	No		
	proPO3	Yes	78.7	Jiang et al., 1997b	No		
	proPO1	Yes	79.3	Lee et al., 1998	No		
	proPO4–6	Yes	78.1–79.2	Müller et al., 1999	No		
<i>Armigeres subalbatus</i>	proPO	Yes	78.1	Cho et al., 1998	No		
<i>Anopheles stephensi</i>	proPO	Yes	78.8	Cui, 1998 ^c (AF062034.1)	No		
<i>Blaberus discoidalis</i>	proPO	No			Yes	76	Durrant et al., 1993
<i>Bombyx mori</i>	proPO1	Yes	78.6	Kawabata et al., 1995	No		
	proPO2	Yes	80.0	Kawabata et al., 1995	Yes	80	Ashida, 1971
<i>Calliphora vicina</i>	proPO	No			Yes	87	Naqvi and Karlson, 1979
<i>Drosophila melanogaster</i>	proPO A1	Yes	78.9	Fujimoto et al., 1995	Yes	78	Fujimoto et al., 1993
	proPO A2	No			Yes	77	Fujimoto et al., 1993
<i>Galleria mellonella</i>	proPO	No			Yes	80,83	Kopáček et al., 1995
<i>Holotrichia diomphalia</i>	proPO	No			Yes	79	Kwon et al., 1997
<i>Hyalophora cecropia</i>	proPO	No			Yes	76	Andersson et al., 1989
<i>Hyphantria cunea</i>	proPO1	Yes	78.2	Park et al., 1997	No		
	proPO2	Yes	80.2	Park et al., 1997	No		
<i>Manduca sexta</i>	proPO1	Yes	78.0	Jiang et al., 1997a	Yes	71,77	Aso et al., 1985
	proPO2	Yes	80.0	Hall et al., 1995b	Yes	98,100	Hall et al., 1995b
<i>Musca domestica</i>	proPO	No			Yes	60	Hara et al., 1993
<i>Sarcophaga bullata</i>	proPO1	Yes	79.8	Chase et al., 1999 ^c (AF161260.1)	No		
	proPO2	Yes	79.0	Chase et al., 1999 ^c (AF161261.1)	No		
<i>Tenebrio molitor</i>	proPO1	Yes	79.1	Lee et al., 1999	No		

molecular mass of 114 kDa on SDS-PAGE. The active form with a molecular mass of 107 kDa was produced after hydrolysis with a commercial proteinase preparation. The molecular mass of purified proPO from *P. californiensis* (114 kDa) is quite different compared to the calculated molecular mass of cloned proPO from *P. monodon* (78.7 kDa) (Sritunyalucksana et al., 1999a), which either suggests that proPO has a post-translational modification process, i.e. glycosylation since glycosylation sites were found in the shrimp proPO cDNA sequence (Sritunyalucksana et al., 1999a) or alternatively, the proPO from *P. monodon* has a much lower mass than that of *P. californiensis* (Gollas-Galván et al., 1999), which however seems less likely.

The thiol ester-like motif (GCGEQNMI) present in the complement components; C3, C4 and α 2-macroglobulins (Belt et al., 1984; Sottrup-Jensen et al., 1984; de Bruijn and Fey, 1985) was also observed in crustacean proPO (GCGWPQHM) (Aspán et al., 1995; Sritunyalucksana et al., 1999a). Upon cleavage by their activating protease, the thiol ester in the vertebrate complement molecule becomes highly active and it can react with the hydroxyl or amino group on biological surfaces, which leads to immobilization of this molecule to a foreign surface. Whether this mechanism occurs with invertebrate proPOs has to be further clarified.

By comparison of amino acid sequences, arthropod proPOs can be classified into two major groups; insect and crustacean proPO, respectively, as shown in Fig. 1. As first demonstrated for crayfish proPO, arthropod proPOs are also highly similar to arthropod hemocyanins and the cloned shrimp proPO has 46% similarity to hemocyanin from another penaeid shrimp, *P. vannamei* (Sellos et al., 1997). The six histidine residues within the two copper binding sites of proPO and hemocyanin are highly conserved in all arthropod proPOs, including shrimp proPO (Sritunyalucksana et al., 1999a). These two copper binding sites have been shown to be functional only in crayfish (Aspán et al., 1995). Tyrosinase found in the ascidian, *Halocynthia roretzi*, resembles vertebrate tyrosinases rather than arthropod proPO (Sato et al., 1997), since this enzyme has a signal peptide and a transmembrane domain like vertebrate tyrosinases.

The conversion of inactive proPO to active PO is by a serine protease named the prophenoloxidase activating enzyme (ppA), which has been isolated and purified from several arthropods; from the cuticle of *Bombyx mori* (Ashida and Dohke, 1980; Satoh et al., 1999) and *Manduca sexta* (Jiang et al., 1998), from a crayfish hemocyte lysate (Aspán et al., 1990), from *Drosophila* pupae (Chosa et al., 1997) and from plasma of *Holotrichia diomphalia* (Lee et al., 1998a,b). Shrimp proPO has been shown to be activated by commercial trypsin proteinases in vitro (Perazzolo and Barracco, 1997; Sung et al., 1998), but no endogeneous enzyme has been reported so far. The common feature of arthropod ppA enzymes are that they are serine proteases and have clip-like domains (Jiang et al., 1998; Satoh et al., 1999). It was shown in crayfish by Aspán et al. (1990) that only ppA is sufficient for the activation of crayfish proPO, but in two insects; *Hyalophora cecropia* (Andersson et al., 1989) and *H. diomphalia* (Lee et al., 1998a,b) ppA and an additional unknown factor are required for the activation of the proPO system. However, the mechanism by which this proteinase converts proPO to active enzyme is still unclear.

Among the most interesting structures in the deduced protein sequences of ppA from cuticle of *M. sexta* (Jiang et al., 1998) and silkworm, *B. mori* (Satoh et al., 1999) were

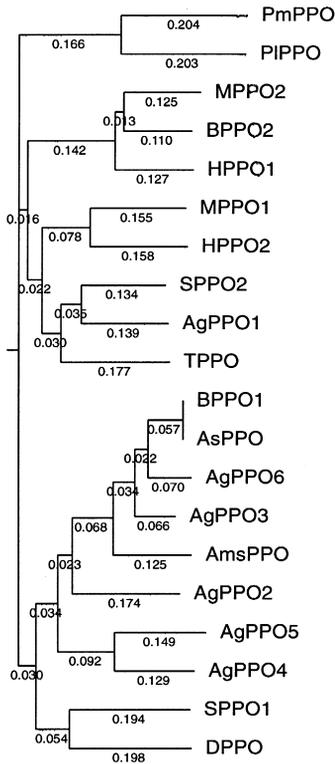


Fig. 1. A comparison of 20 arthropod proPOs based on Clustal W analysis (Mac Vector version 6.5.1) of their deduced amino acid sequences. Numbers in parentheses show the accession numbers. AgPPO1-6: *A. gambiae* proPO1-6 (L76038.1, AF004915, AF004916, AJ010193.1, AJ010194.1, AJ010195.1); AsPPO: *A. stephensi* proPO (AF062034.1); AmsPPO: *subalbatus* proPO; BPPO1, 2: *B. mori* proPO 1, 2 (D49370.1, D49371.1); DPPO: *melanogaster* proPO A1 (D45835.1); HPPO1, 2: *Hyp. cunea* proPO 1, 2 (U86875.1, AF02039.1); MPPO1, 2: *sexta* proPO 1, 2 (AF003253.1, L42556); PmPPO: *P. monodon* proPO (AF099741.1); PIPPO: *P. leniusculus* proPO (X83494.1); SPPO1, 2: *S. bullata* proPO1, 2 (AF161260.1, AF161261.1); TPPO: *T. molitor* proPO (AB020738.1).

that they were similar to *Drosophila easter*, which is the proteinase involved in determining dorsoventral polarity of the *Drosophila* embryo (Belvin and Andersson, 1996) and which contains a carboxyl terminal serine protease domain and an amino-terminal clip domain. The clip-like domain is also present in β -defensins, an antimicrobial peptide in mammals (Tang and Selsted, 1993). Taken together, it leads to the speculation that ppA might have an antimicrobial activity.

3. Recognition molecules

Invertebrates do not have immunoglobulins, the specific recognition molecules found in higher animals, although proteins containing immunoglobulin-like domains have been

identified in many species (Lanz-Mendoza and Faye, 1999). Instead they have the so-called pattern recognition proteins (PRPs) to recognize and respond to microbial intruders by the presence of signature molecules on the surface of the intruders (Janeway, 1989). Several pattern recognition molecules have been isolated and characterized for example, β -1,3-glucan binding proteins (BGBP) (Ochiai and Ashida, 1988; Söderhäll et al., 1988; Duvic and Söderhäll, 1990, 1993; Seki et al., 1994), LPS-binding proteins (Sun et al., 1990, Natori and Kubo, 1996; Lee et al., 1996; Dimopoulos et al., 1997) and Peptidoglycan-binding proteins (Yoshida et al., 1996; Kang et al., 1998; Ochiai and Ashida, 1999). So far, BGBPs have been cloned from two arthropods; the horseshoe crab; *Tachypleus tridentatus* (Seki et al., 1994) and the freshwater crayfish (Cerenius et al., 1994). BGBPs from two insects, *Blaberus craniifer* (Söderhäll et al., 1988) and *B. mori* (Ochiai and Ashida, 1988) and from *P. leniusculus* (Duvic and Söderhäll, 1990) were shown to enhance the activity of both the ppA and PO when β -1,3 glucans were present. Crayfish BGBP is a monomer of 100 kDa (Duvic and Söderhäll, 1990) and although it has some glucanase-like motifs, it has no function as a glucanase enzyme (Cerenius et al., 1994). A plausible hypothesis is that the BGBPs developed from a primitive glucanase and then evolved into proteins without glucanase activity, but instead bind glucans and after binding, operate as elicitors of defence responses. The BGBP- β -1,3-glucan complex (BGBP-L) could bind specifically to hemocytes and then induce spreading and partial degranulation of isolated crayfish granular hemocytes, whereas the BGBP itself had no such activity (Barracco et al., 1991). BGBP-L binds to the hemocyte surface (Duvic and Söderhäll, 1992) through its RGD motif (Arg-Gly-Asp) which may indicate that it may bind to an integrin-like protein (Ruoslahti, 1996). An integrin β -subunit was recently identified in crayfish hemocytes (Holmblad et al., 1997) which may be a candidate receptor for BGBP-L. The BGBP also functions as an opsonic protein in crayfish (Thörnqvist et al., 1994).

PO activity in *P. californiensis* (Vargas-Albores et al., 1996) and *P. paulensis* (Perazzolo and Barracco, 1997) was shown to increase after treatment with β -1,3-glucans and BGBP has been identified in plasma of the brown shrimp, *P. californiensis* by using a polyclonal antiserum against BGBP from the freshwater crayfish, *P. leniusculus* (Vargas-Albores et al., 1996). Shrimp BGBP has the same characteristics as that of crayfish since it is a 100-kDa monomeric protein and shows high similarities in both amino acid composition and N-terminal sequence to that of crayfish and it is involved in the activation of shrimp proPO system (Vargas-Albores et al., 1996; Yepiz-Plascencia et al., 1998).

Two major lipoproteins, one high density lipoprotein (HDL) and one very high density lipoprotein (VHDL), isolated from male crayfish, were found to be identical to BGBP and the clotting protein (CP), respectively (Hall et al., 1995a). Moreover, Lubzens et al. (1997) showed that crayfish BGBP (HDL) seems to be identical to the shrimp, *P. semisulcatus*, protein LP1, which is involved in lipid transport to the ovary. Recently, Yepiz-Plascencia et al. (1998) determined the biochemical characteristics of BGBP and HDL in shrimp hemolymph. The N-terminal sequence of purified *P. vannamei* HDL is identical to that of BGBP from the same species, and both are similar to BGBP from the brown shrimp, *P. californiensis* and crayfish as shown in Fig. 2.

<i>P. vannamei</i> HDL/BGBP	DAGQAS LAGNFNSLR
<i>P. californiensis</i> BGBP	DAGQAS FAGNFNSLR
<i>P. leniusculus</i> HDL/BGBP	DAGEAS LVTNFNSAK

Fig. 2. Alignment of N-terminal sequence of shrimp HDL/BGBP (Yepiz-Plascencia et al., 1998) compared with that of crayfish HDL/BGBP (Hall et al., 1995a). The corresponding residues in the shrimp BGBP are in boldface.

Thus, it appears as if the same protein can be involved in both reproduction and immunity.

Another group of PRPs are the LPS-binding proteins which have been found in several arthropod species (Sun et al., 1990; Natori and Kubo, 1996; Lee et al., 1996; Dimopoulos et al., 1997). At present, only four inducible gram negative bacterial binding proteins (GNBPs) have been discovered in insect hemolymph; a *Periplaneta americana* LPS binding protein (Natori and Kubo, 1996), hemolin, a bacterial surface binding protein belonging to the immunoglobulin superfamily (Sun et al., 1990), a 50-kDa GNBPs from the silkworm, *B. mori* (Lee et al., 1996), and GNBPs from *Anopheles gambiae* (Dimopoulos et al., 1997). These binding proteins from insects appear to be functionally similar by having affinity to the gram negative bacterial cell wall and are inducible during injury or infection. Recently, two molecules isolated and cloned from the coelomic fluid of the earthworm, *Eisenia foetida* (Beschin et al., 1998) and from the hemocytes of crayfish, *P. leniusculus* (Lee et al., 2000) showed affinity to both β -1,3-glucans and LPS and both molecules have been shown to be involved in the activation of the proPO system. In shrimp, a 175-kDa LPS-binding protein from the hemolymph of *P. californiensis* was isolated, but its function in immunity is still unclear (Vargas-Albores et al., 1993b).

Peptidoglycan-recognition proteins (PGRP) have been characterized and cloned from *Trichoplusia ni* (Kang et al., 1998) and *B. mori* (Ochiai and Ashida, 1999) and since PG can induce activation of the proPO system in shrimps, it may indicate that PGRP may be present in shrimp (Sritunyalucksana et al., 1999b).

4. Associated factors of the proPO system

Peroxinectin, a 76-kDa cell adhesion factor with peroxidase activity was first purified from hemocyte lysate supernatant (HLS) of crayfish, *P. leniusculus* (Johansson and Söderhäll, 1988). Crayfish peroxinectin is synthesized in the blood cells, stored in secretory granules of granular hemocytes in an inactive form, released in response to stimulus, and activated outside the cells to mediate hemocyte attachment and spreading. The biological activities of peroxinectin is generated concomitant with activation of the proPO system (Johansson and Söderhäll, 1988). Peroxinectin is a multifunctional protein containing five different biological activities shown in vitro: (a) a cell adhesion activity (Johansson and Söderhäll, 1988), (b) a degranulation activity (Johansson and Söderhäll, 1989), (c) an encapsulation-promoting activity (Kobayashi et al., 1990), (d) an opsonic activity (Thörnqvist et al., 1994), and (e) peroxidase activity (Johansson et al., 1995).

However, it is important to emphasize that the peroxidase activity is not a prerequisite for these biological activities. A factor found in shrimp HLS can also trigger partial degranulation and spreading of granular cells (Perazzolo and Barracco, 1997) and in that respect, this activity mimics that of peroxinectin. Recently, a peroxinectin was identified and cloned from the hemolymph of the shrimp, *P. monodon*, which shows high similarity to crayfish peroxinectin (69%) and vertebrate peroxidases (45–55%) (Sritunyalucksana et al., unpublished data). The cysteine residues forming six intra-chain disulfide bonds in myeloperoxidase (MPO) (Zeng and Fenna, 1992) are also conserved in the shrimp protein. A putative cell adhesive site, KGD (Lys–Gly–Asp) that have been shown in crayfish to be an important triplet for adhesion activity was also present in the shrimp protein.

So far, a few blood cell adhesion molecules in arthropods have been cloned as shown in Table 2 and for a recent review, see Johansson (1999). Holmblad et al. (1997) reported the presence of an integrin β -subunit on surfaces of the crayfish hemocytes. Integrins are common receptors and recognize motifs such as RGD (Arg–Gly–Asp), KGD (Lys–Gly–Asp) or LDV (Leu–Asp–Val) (Ruoslahti, 1996). Moreover, crayfish extracellular superoxide dismutase (EC-SOD) may function as a receptor for peroxinectin (Johansson et al., 1999). Peroxinectin, BGBP and EC-SOD all contain integrin-

Table 2
Arthropod blood cell adhesion molecules

Species (protein)	Cloned	Reference	Cell adhesion activity ^a	Reference
<i>P. leniusculus</i> (Peroxinectin)	Yes	Johansson et al., 1995	Yes	Johansson and Söderhäll, 1988
<i>P. monodon</i> (Peroxinectin)	Yes	Sritunyalucksana et al., unpublished data	Yes ^b	Sritunyalucksana et al., unpublished data
<i>P. paulensis</i>	No		Yes ^b	Perazzolo and Barracco, 1997
<i>Limulus polyphemus</i> (Limulus agglutination–aggregation factor)	Yes	Fujii et al., 1992	Yes	Fujii et al., 1992
<i>L. polyphemus</i> (Limunectin)	Yes	Liu et al., 1991	No	
<i>Carcinus maenus</i>	No		Yes	Thörnqvist et al., 1994
<i>Blaberus craniifer</i>	No		Yes	Rantamäki et al., 1991
<i>B. mori</i> (Hemocytin)	Yes	Kotani et al., 1995	Yes ^c	Kotani et al., 1995
<i>Pseudoplasia includens</i> (plasmatocyte spreading peptide)	Yes	Clark et al., 1998	Yes	Clark et al., 1997

^aCell adhesion activity detected from purified protein.

^bCell adhesion activity detected in hemolymph.

^cCell adhesion activity detected in recombinant protein expressed in baculovirus vector.

binding motifs and then two possible models are proposed for how an integrin could interact with these molecules involved in defence mechanisms in this animal (Thörnqvist and Söderhäll, 1997; Holmblad and Söderhäll, 1999). Either peroxinectin and BGBP could bind to integrin and to EC-SOD separately, or peroxinectin and BGBP first bind to EC-SOD, which then in turn binds to the integrin present in the hemocyte membrane. Peroxinectin has peroxidase activity and works as an opsonin (Thörnqvist et al., 1994; Johansson et al., 1995) and thus peroxinectin might produce hypohalic acid from H_2O_2 produced by SOD and as a consequence, these toxic substances would be produced in close proximity to the invading microorganisms.

5. The clotting system

Only two different coagulation mechanisms have been characterized in molecular detail in invertebrates so far; the hemocyte-derived clotting cascade in horseshoe crab, *Tachypleus tridentatus* (Kawabata et al., 1996) and the transglutaminase (TGase)-dependent clotting reaction in crayfish, *P. leniusculus* (Kopáček et al., 1993; Hall et al., 1999). The proteins participating in the horseshoe crab clotting system all reside in the hemocytes and, upon activation they are released from the cytoplasmic L-granules into the hemolymph through rapid exocytosis (Kawabata et al., 1996). The horseshoe crab clotting system is a proteolytic cascade and is activated by microbial cell wall components. Gram negative bacteria and fungi invading the horseshoe crab hemolymph activate factor C and factor G, respectively, which results in subsequent activation of proclotting enzyme and the resulting clotting enzyme catalyses the conversion of a soluble protein (coagulogen) into an insoluble aggregate (coagulin) (Iwanaga, 1993; Kawabata et al., 1996). In crustaceans, the clottable proteins were found in several species; the freshwater crayfish (Kopáček et al., 1993), *P. monodon* (Yeh et al., 1998), the sand crayfish, *Ibacus ciliatus* (Komatsu and Ando, 1998) and in the lobster, *Panulirus interruptus* (Doolittle and Fuller, 1972; Doolittle and Riley, 1990). They were shown to be homodimeric glycoproteins of about 380–400 kDa and appeared to have similar amino acid compositions and N-terminal sequences (Fig. 3). The crayfish CP, a dimeric protein consisting of 210 kDa subunits, is a VHDL (Hall et al., 1995a; Kopáček et al., 1993) and each of the 210-kDa subunits has both free lysine and glutamine, which are recognized and become covalently linked to each other by TGases. The capability of crayfish CP to polymerize and form clot in the presence of Ca^{2+} and TGases released from hemocyte was shown (Table 3) (Kopáček et al., 1993). CP was clearly shown to be a substrate for TGases in crayfish by using fluorescent TGase substrates (Kopáček et al.,

<i>P. monodon</i> CP	LQPGLEYQYR
<i>P. leniusculus</i> CP	LHSNLEYQYR
<i>I. ciliatus</i> VHDL	LQPGLEYQYR
<i>P. interruptus</i> fibrinogen	LQPKLEYQYK

Fig. 3. Alignment of N-terminal sequence of *P. monodon* CP (Yeh et al., 1999) compared with that of *P. leniusculus* CP (Kopáček et al., 1993), *I. ciliatus* VHDL (Komatsu and Ando, 1998) and *P. interruptus* fibrinogen (Doolittle and Riley, 1990). The corresponding residues in the shrimp CP are in boldface.

Table 3
The clotting ability of crayfish plasma and the CP (Kopáček et al., 1993)

Sample	Gel formation
Plasma + HLS + Ca ²⁺	+
CP + HLS + Ca ²⁺	+
CP-depleted plasma + HLS + Ca ²⁺	-
CP-depleted plasma + CP + HLS + Ca ²⁺	+

(+) Samples which formed a stable clot that resisted shaking.
(-) Samples which remained liquid and did not form a clot.

1993). The TGase-dependent clotting reaction in crayfish is induced when a TGase is released from hemocytes or tissues, becomes activated by the Ca²⁺ in plasma and starts to crosslink the CP molecules into large aggregates (Fig. 4). The polymerization of CP molecules were clearly shown by using electron microscopy after the incubation of purified CP molecules with crayfish hemocyte lysate containing endogeneous TGase activity (Hall et al., 1999). TGases (EC 2.3.2.13) are Ca²⁺-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins. A TGase has been characterized and cloned from *Limulus* hemo-

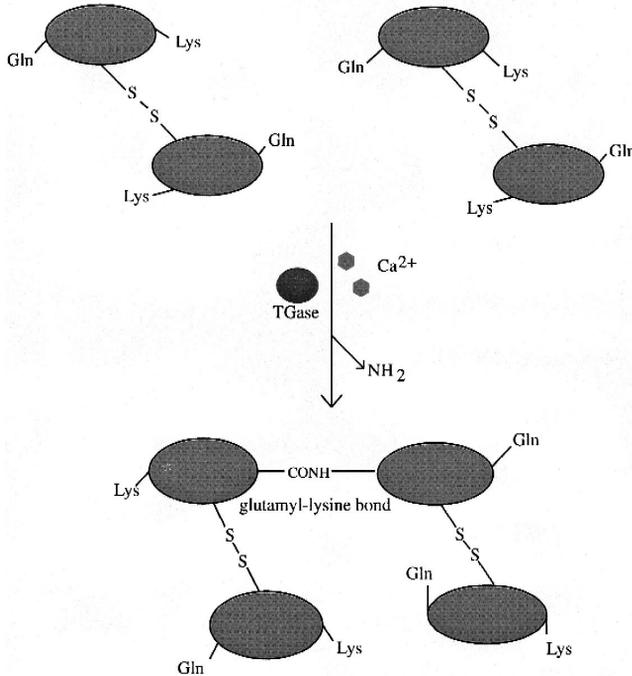


Fig. 4. The TGase-mediated clotting reaction in crayfish. CP, a dimeric protein consisting of two identical 210-kDa subunits held together by disulfide bond (●-s-s-●) are cross-linking by hemocyte TGase (●) in the presence of Ca²⁺ (filled hexagon).

cytes, but it does not appear to use coagulogen as a substrate (Tokunaga et al., 1993a,b) and its role during clotting is unclear. In the spiny lobster, *Panulirus japonicus*, TGase was localized in the hemocytes, especially in hyaline and semigranular cell, and was shown to be involved in the clotting process (Aono and Mori, 1996).

CP was first cloned and characterized from *P. leniusculus* (Hall et al., 1999) followed by CP from *P. monodon* (Yeh et al., 1999). Crayfish CP has been isolated from crayfish hepatopancreas cDNA library and its cDNA consists of 6396 nts, which correspond to a 7-kb transcript from hepatopancreas in Northern blots. It encodes a polypeptide with 1721 amino acids with a molecular mass of 193 kDa and 15-amino acids signal peptide. Shrimp CP cDNA has a total length of 6124 nts and it encodes a polypeptide with 1670 amino acids, including a 14-amino acid signal peptide (Yeh et al., 1999). Sequence analysis shows that shrimp CP deduced amino acid sequence has 36% identity and about 57% similarity to that of crayfish. Northern blot analysis revealed the 6.2-kb CP transcript is expressed in most of the shrimp tissues but not in the mature hemocytes (Yeh et al., 1999). Besides having similar functions, the CP does not share any characteristics with fibrinogen or coagulogen, the protein forming clots in vertebrate animals and horseshoe crab, respectively (Hall et al., 1999). Instead, sequence analysis reveals a significant similarity of crayfish CP, as well as shrimp CP, to insect vitellogenins. Since the crayfish CP is present in both sexes of crayfish with identical functions as a CP, it should not be considered as a true vitellogenin, which is a female-specific protein. However, female crayfish have a true vitellogenin, which has been isolated and partially characterized (Hall, Wang and Söderhäll, unpublished data). Vitellogenins are proteins found in various egg-laying animals and they appear not to be involved in any clotting reactions (Sappington and Raikhel, 1997). Both CP and vitellogenins have a cysteine-containing stretch with sequence similarity to the D domain of von Willebrand factor (vWF) (Sadler, 1991). vWF is a large multimeric protein involved in the blood coagulation process in mammals, and its D domains are important for the multimer formation of human vWF (Voorberg et al., 1990; Mayadas and Wagner, 1992). It appears therefore as if the CP is very similar in crustaceans, for example, in crayfish and shrimp. However, the clotting reaction has only been fully characterized in crayfish (Hall et al., 1999), the mechanism in other crustaceans have to be elucidated in more detail for comparative studies of the clotting reaction in crustaceans.

6. Conclusion

Penaeid shrimp is an interesting animal model to use for the study of defence mechanisms in crustaceans mainly because of its economical value, its short life-time, its ability to survive in microbe-rich marine environments without gross signs of disease and the high amount of samples that can easily be collected from shrimp farms. Research to characterize the active molecule(s) involved and to explain how the defence machinery is turned on/off is a primary need before going to study the stimulation of the system. The significant similarities of the primary structures of proPO, peroxinectin and CP from shrimp and crayfish (Sritunyalucksana et al., 1999a; Sritunyalucksana et

al., unpublished data; Yeh et al., 1999) demonstrate that the immune genes and immune defence are likely to be very similar and hence much knowledge can be learned from studies on crustaceans in general.

Acknowledgements

This work was supported by the Swedish Natural Science Research Council.

References

- Andersson, K., Sun, S.-C., Boman, H.G., Steiner, H., 1989. Purification of *Cecropia* phenoloxidase and four proteins involved in its activation. *Insect Biochem.* 19, 629–638.
- Aono, H., Mori, K., 1996. Interaction between hemocytes and plasma is necessary for hemolymph coagulation in the spiny lobster *Panulirus japonicus*. *Comp. Biochem. Physiol.* 113, 301–305.
- Ashida, M., 1971. Purification and characterization of prophenoloxidase from hemolymph of the silkworm *Bombyx mori*. *Arch. Biochem. Biophys.* 144, 749–762.
- Ashida, M., 1990. The prophenoloxidase cascade in insect immunity. *Res. Immunol.* 141, 908–910.
- Ashida, M., Dohke, K., 1980. Activation of pro-phenol oxidase by the activation enzyme of the silkworm *Bombyx mori*. *Insect Biochem.* 10, 37–47.
- Aso, Y., Kramer, K.J., Hopkins, T.L., Lookhart, G.L., 1985. Characterization of haemolymph protyrosinase and a cuticular activator from *Manduca sexta* (L.). *Insect Biochem.* 15, 9–17.
- Aspán, A., Söderhäll, K., 1991. Purification of prophenoloxidase from crayfish blood cells, and its activation by an endogenous serine proteinase. *Insect Biochem.* 21, 363–373.
- Aspán, A., Sturtevant, J., Smith, V.J., Söderhäll, K., 1990. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. *Insect Biochem.* 20, 709–718.
- Aspán, A., Huang, T.S., Cerenius, L., Söderhäll, K., 1995. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. *Proc. Natl. Acad. Sci. U.S.A.* 92, 939–943.
- Barracco, M.A., Duvic, B., Söderhäll, K., 1991. The β -1,3-glucanbinding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a β 1, 3glucan, induces spreading and degranulation of crayfish granular cells. *Cell Tissue Res.* 266, 491–497.
- Belt, K., Carroll, M., Porter, R., 1984. The structural basis of the multiple forms of human component C4. *Cell* 36, 907–914.
- Belvin, M., Andersson, K.V., 1996. A conserved signaling pathway: the *Drosophila* Toll–Dorsal pathway. *Annu. Rev. Cell Dev. Biol.* 12, 393–416.
- Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L., Gomez, J., De Baetselier, P., Timmermans, M., 1998. Identification and cloning of a glucan-and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. *J. Biol. Chem.* 273, 24948–24954.
- Cerenius, L., Liang, Z., Duvic, B., Keyser, P., Hellman, U., Palva, E.T., Iwanaga, S., Söderhäll, K., 1994. A β -D-glucan binding glucan in crustacean blood. Structure and biological activity of a fungal recognition protein. *J. Biol. Chem.* 269, 29462–29467.
- Cho, W.L., Liu, H.S., Lee, C.H., Kuo, C.C., Chang, T.Y., Liu, C.T., Chen, C.C., 1998. Molecular cloning, characterization and tissue expression of prophenoloxidase cDNA from the mosquito *Amigeres subalbatus* inoculated with *Dirofilaria immitis* microfilaria. *Insect Mol. Biol.* 7, 31–40.
- Chosa, N., Fukumitsu, T., Fujimoto, K., Ohnishi, E., 1997. Activation of pro-phenol-oxidase A1 by an activating enzyme in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 27, 61–68.
- Clark, K.D., Pech, L.L., Strand, M.R., 1997. Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect *Pseudoplasia includens*. *J. Biol. Chem.* 272, 23440–23447.

- Clark, K.D., Witherell, A., Strand, M.R., 1998. Plasmatocyte spreading peptide is encoded by an mRNA differentially expressed in tissues of the moth *Pseudoplasia includens*. *Biochem. Biophys. Res. Commun.* 250, 479–485.
- de Bruijn, M.H.L., Fey, G.H., 1985. Human complement component C3: cDNA coding sequence and derived primary structure. *Proc. Natl. Acad. Sci. U.S.A.* 82, 708–712.
- Dimopoulos, G., Richman, A., Müller, H.-M., Kafatos, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl. Acad. Sci. U.S.A.* 94, 11508–11513.
- Doolittle, R.F., Fuller, G.M., 1972. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies on lobster fibrinogen and fibrin. *Biochim. Biophys. Acta* 263 (3), 805–809.
- Doolittle, R.F., Riley, M., 1990. The amino-terminal sequence of lobster fibrinogen reveals common ancestry with vitellogenins. *Biochem. Biophys. Res. Commun.* 167, 16–19.
- Durrant, H.J., Ratcliffe, N.A., Hipkin, C.R., Aspán, A., Söderhäll, K., 1993. Purification of the pro-phenol oxidase enzyme from haemocytes of the cockroach *Blaberus discoidalis*. *Biochem. J.* 289, 87–91.
- Duvic, B., Söderhäll, K., 1990. Purification and characterization of a β -1,3-glucan binding protein from plasma of crayfish *Pacifastacus leniusculus*. *J. Biol. Chem.* 265, 9327–9332.
- Duvic, B., Söderhäll, K., 1992. Purification and characterization of a β -1,3-glucan-binding protein membrane receptor from blood cells of the crayfish *Pacifastacus leniusculus*. *Eur. J. Biochem.* 207, 223–228.
- Duvic, B., Söderhäll, K., 1993. β -1,3-glucan binding proteins from the plasma of the freshwater crayfish *Astacus astacus* and *Procambarus clarkii*. *J. Crustacean Biol.* 13, 403–408.
- Fujii, N., Minetti, C.A.S.A., Nakhasi, H.L., Chen, S.-W., Barbehenn, E., Nunes, P.H., Nguyen, N.Y., 1992. Isolation, cDNA cloning, and characterization of an 18-kDa hemagglutinin and amebocyte aggregation factor from *Limulus polyphemus*. *J. Biol. Chem.* 267, 22452–22459.
- Fujimoto, K., Masuda, K.I., Asada, N., Ohnishi, E., 1993. Purification and characterization of prophenoloxidase from pupae of *Drosophila melanogaster*. *J. Biochem.* 113, 285–291, (Tokyo).
- Fujimoto, K., Okino, N., Kawabata, S., Iwanaga, S., Ohnishi, E., 1995. Nucleotide sequence of the prophenoloxidase A1 of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7769–7773.
- Gollas-Galván, T., Hernández-López, J., Vargas-Albores, F., 1999. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. *Comp. Biochem. Physiol.* 122B, 77–82.
- Hall, M., van Heusden, M.C., Söderhäll, K., 1995a. Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. *Biochem. Biophys. Res. Commun.* 216, 939–946.
- Hall, M., Scott, T., Sugumaran, M., Söderhäll, K., Law, J.H., 1995b. Proenzyme of *Manduca sexta* phenoloxidase: purification, activation, substrate specificity of the active enzyme, and molecular cloning. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7764–7768.
- Hall, M., Wang, R., Antwerpen, R.K., Sottrup-Jensen, L., Söderhäll, K., 1999. The crayfish plasma clotting: a vitellogenin-related protein responsible for clot formation in crustacean blood. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1965–1970.
- Hara, T., Miyoshi, T., Tsukamoto, T., 1993. Comparative studies of larval and pupal phenoloxidase of the housefly, *Musca domestica*. *L. Comp. Biochem. Physiol.* 106B, 287–292.
- Hernández-López, J., Gollas-Galván, T., Vargas-Albores, F., 1996. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes) haemolymph. *Comp. Biochem. Physiol.* 104B, 407–413.
- Holmblad, T., Söderhäll, K., 1999. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture* 172, 111–123.
- Holmblad, T., Thörnqvist, P.-O., Söderhäll, K., Johansson, M.W., 1997. Identification and cloning of an integrin β subunit from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. *J. Exp. Zool.* 277, 255–261.
- Iwanaga, S., 1993. The limulus clotting reaction. *Curr. Opin. Immunol.* 5 (1), 74–82.
- Janeway, C.A. Jr., 1989. Approaching the asymptote? Evolution and revolution in Immunology. *Cold Spring Harbor Symp. Quant. Biol.* 54, 1–3.
- Jiang, H., Wang, Y., Kanost, M.R., 1998. Pro-phenol oxidase activating proteinase from an insect *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12220–12225.

- Jiang, H., Wang, Y., Ma, C., Kanost, M.R., 1997a. Subunit composition of pro-phenoloxidase from *Manduca sexta*: molecular cloning of subunit proPO-P1. *Insect Biochem. Mol. Biol.* 27, 835–850.
- Jiang, H., Wang, Y., Korochkina, S.E., Benes, H., Kanost, M.R., 1997b. Molecular cloning of cDNAs for two pro-phenol oxidase subunits from the malaria vector, *Anopheles gambiae*. *Insect Biochem. Mol. Biol.* 27, 693–699.
- Johansson, M.W., 1999. Cell adhesion molecules in invertebrate immunity. *Dev. Comp. Immunol.* 23, 303–315.
- Johansson, M.W., Söderhäll, K., 1988. Isolation and purification of a cell adhesion factor from crayfish blood cells. *J. Cell Biol.* 106, 1795–1803.
- Johansson, M.W., Söderhäll, K., 1989. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. *Insect Biochem.* 19, 183–190.
- Johansson, M.W., Lind, M., Holmblad, T., Thörnqvist, P.-O., Söderhäll, K., 1995. Peroxinectin, a novel cell adhesion protein from crayfish blood. *Biochem. Biophys. Res. Commun.* 216, 1079–1087.
- Johansson, M.W., Holmblad, T., Thörnqvist, P.-O., Cammarata, M., Parrinello, N., Söderhäll, K., 1999. A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish. *J. Cell Sci.* 112, 917–925.
- Kang, D., Liu, G., Lundstrom, A., Geluis, E., Steiner, H., 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10078–10082.
- Kawabata, S.-I., Muta, T., Iwanaga, S., 1996. The clotting cascade and defense molecules found in the hemolymph of the horseshoe crab. In: Söderhäll, K., Iwanaga, S., Vasta, G.R. (Eds.), *New Directions in Invertebrate Immunology*. SOS Publications, Fair Haven, pp. 255–283.
- Kawabata, T., Yasushara, Y., Ochiai, M., Matsura, S., Ashida, M., 1995. Molecular cloning of insect pro-phenoloxidase: a copper containing protein homologous to arthropod hemocyanin. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7774–7778.
- Kobayashi, M., Johansson, M.W., Söderhäll, K., 1990. The 76 kDa cell-adhesion factor from crayfish haemocytes promote encapsulation in vitro. *Cell Tissue Res.* 260, 113–118.
- Komatsu, M., Ando, S., 1998. A very-high-density lipoprotein with clotting ability from hemolymph of sand crayfish, *Ibacus ciliatus*. *Biosci. Biotechnol. Biochem.* 62 (3), 459–463.
- Kopáček, P., Hall, M., Söderhäll, K., 1993. Characterization of a clotting proteins isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. *Eur. J. Biochem.* 213 (1), 591–597.
- Kopáček, P., Weise, C., Götz, P., 1995. The prophenoloxidase from the wax moth *Galleria mellonella*: purification and characterization of the proenzyme. *Insect Biochem. Mol. Biol.* 25, 1081–1091.
- Kotani, E., Yamakawa, M., Iwamoto, S.-i., Tashiro, M., Mori, H., Sumida, M., Matsubara, F., Taniai, K., Kadono-Okuda, K., Kato, Y., Mori, H., 1995. Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. *Biochim. Biophys. Acta* 1260, 245–258.
- Kwon, T.H., Lee, S.Y., Lee, J.H., Choi, J.S., Kawabata, S.I., Iwanaga, S., Lee, B.L., 1997. Purification and characterization of prophenoloxidase from the hemolymph of coleopteran insect, *Holotrichia diomphalia* larvae. *Mol. Cells* 7, 90–97.
- Lanz-Mendoza, H.L., Faye, I., 1999. Physiological aspects of the immunoglobulin superfamily in invertebrates. *Dev. Comp. Immunol.* 23, 359–374.
- Lee, H.S., Cho, M.Y., Lee, K.M., Kwon, T.H., Homma, K.-i., Natori, S., Lee, B.L., 1999. The pro-phenoloxidase of coleopteran insect, *Tenebrio molitor*, larvae was activated during cell clump/cell adhesion of insect cellular defense reactions. *FEBS Lett.* 444, 255–259.
- Lee, S.Y., Wang, R., Söderhäll, K., 2000. A lipopolysaccharide- and β -1,3-protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization and cDNA cloning. *J. Biol. Chem.*, (in press).
- Lee, S.Y., Kwon, T.H., Hyun, J.H., Choi, J.S., Kawabata, S.-I., Iwanaga, S., Lee, B.L., 1998a. In vitro activation of pro-phenol-oxidase by two kinds of pro-phenol-oxidase-activating factors isolated from hemolymph of coleopteran, *Holotrichia diomphalia* larvae. *Eur. J. Biochem.* 254, 50–57.
- Lee, S.Y., Cho, M.Y., Hyun, J.H., Lee, K.M., Homma, K.-I., Natori, S., Kawabata, S.-I., Iwanaga, S., Lee, B.L., 1998b. Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3- β -glucan in coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem.* 257, 615–621.

- Lee, W.J., Lee, D.J., Kravchenko, V.V., Ulevitch, R.J., Brey, P.T., 1996. Purification and molecular cloning of an inducible Gram negative bacteria-binding protein from the silkworm *Bombyx mori*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7888–7893.
- Lee, W.J., Ahmed, A., Torre, A., Kobayashi, A., Ashida, M., Brey, P.T., 1998. Molecular cloning and chromosomal localization of a prophenoloxidase cDNA from the malaria vector *Anopheles gambiae*. *Insect Mol. Biol.* 7, 41–50.
- Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P., Aquacop, 1997. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. *Fish Shellfish Immunol.* 7, 227–234.
- Liu, T., Lin, Y., Cislo, T., Minetti, C.A.S.A., Baba, J.M.K., Liu, T.-Y., 1991. Limunectin. *J. Biol. Chem.* 266, 14813–14821.
- Lubzens, E., Ravid, T., Khayat, M., Daube, N., Tietz, A., 1997. Isolation and characterization of the high-density lipoproteins from the hemolymph and ovary of the penaeid shrimp *Penaeus semisulcatus* (de Haan): apoproteins and lipids. *J. Exp. Zool.* 278, 339–348.
- Mayadas, T.N., Wagner, D.D., 1992. Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3531–3535.
- Müller, H.M., Dimopoulos, G., Blass, C., Kafatos, F.C., 1999. A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes. *J. Biol. Chem.* 274, 11727–11735.
- Nappi, A., Vass, E., 1993. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigm. Cell Res.* 6, 117–126.
- Naqvi, S.N.H., Karlson, P., 1979. Purification of the prophenoloxidase in the haemolymph of *Calliphora vicina* (R&D). *Arch. Int. Physiol. Biochim.* 87, 687–695.
- Natori, S., Kubo, T., 1996. Role of lectins in development and morphogenesis in insects. In: Söderhäll, K., Iwanaga, S., Vasta, G.R. (Eds.), *New Directions in Invertebrate Immunology*. SOS Publications, Fair Haven, pp. 175–187.
- Ochiai, M., Ashida, M., 1988. Purification of a β -1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of silkworm, *Bombyx mori*. *J. Biol. Chem.* 263, 12056–12062.
- Ochiai, M., Ashida, M., 1999. A pattern recognition protein for peptidoglycan: cloning the cDNA and the gene of the silkworm, *Bombyx mori*. *J. Biol. Chem.* 274, 11854–11858.
- Park, D.S., Shin, S.W., Kim, M.G., Park, S.S., Lee, W.J., Brey, P.T., Park, H.Y., 1997. Isolation and characterization of the cDNA encoding the prophenoloxidase of fall webworm, *Hyphantria cunea*. *Insect Biochem. Mol. Biol.* 27, 983–992.
- Perazzolo, L.M., Barracco, M.A., 1997. The prophenoloxidase activating system of the shrimp, *Penaeus paulensis* and associated factors. *Dev. Comp. Immunol.* 21, 385–395.
- Rantamäki, J., Durrant, H., Liang, Z., Ratcliffe, N.A., Duvic, B., Söderhäll, K., 1991. Isolation of a 90 kDa protein from haemocytes of *Blaberus craniifer* which has similar properties to the 76 kDa protein from crayfish haemocytes. *J. Insect Physiol.* 37, 627–634.
- Ratcliffe, N.A., Rowley, A.F., Fitzgerald, S.W., Rhodes, C.P., 1985. Invertebrate immunity: basic concepts and recent advances. *Int. Rev. Cytol.* 97, 183–350.
- Rowley, A.F., Brookman, J.L., Ratcliffe, N.A., 1990. Possible involvement of the prophenoloxidase system of the locust, *Locusta migratoria*, in antimicrobial activity. *J. Invertebr. Pathol.* 56, 31–38.
- Ruoslahti, E., 1996. RGD and other recognition sequences for integrins. *Annu. Rev. Dev. Cell Biol.* 12, 697–715.
- Sadler, J.E., 1991. von Willebrand factor. *J. Biol. Chem.* 266 (34), 22777–22780.
- Sappington, T.W., Raikhel, A.S., 1997. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol.* 28 (5–6), 277–300.
- Sato, S., Masuya, H., Numakunai, T., Satoh, N., Ikeo, K., Gojobori, T., Tamura, K., Ide, H., Takeuchi, T., Yamamoto, H., 1997. Ascidian tyrosinase gene: its unique structure and expression in the developing brain. *Dev. Dyn.* 208, 363–374.
- Satoh, D., Horii, A., Ochiai, M., Ashida, M., 1999. Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*: purification, characterization and cDNA cloning. *J. Biol. Chem.* 274, 7441–7453.
- Seki, N., Muta, T., Oda, T., Iwaki, D., Kuma, K., Miata, T., Iwanaga, S., 1994. Horseshoe crab (1,3)

- β -D-glucan-sensitive coagulation factor G. A serine protease zymogen heterodimer with similarities to β -glucan-binding proteins. *J. Biol. Chem.* 269, 1370–1374.
- Sellos, D., Lemoine, S., Van Wormhoudt, A., 1997. Molecular cloning of hemocyanin cDNA from *Penaeus vannamei* (crustacea, Decapoda): structure, evolution and physiological aspects. *FEBS Lett.* 407, 153–158.
- Söderhäll, K., 1982. Phenoloxidase activating system and melanization — a recognition mechanism of arthropods? A review. *Dev. Comp. Immunol.* 6, 601–611.
- Söderhäll, K., 1999. Editorial. Invertebrate immunity. *Dev. Comp. Immunol.* 23, 263–266.
- Söderhäll, K., Ajaxon, R., 1982. Effect of quinone and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci*, a parasite of crayfish. *J. Invertbr. Pathol.* 39, 105–109.
- Söderhäll, K., Cerenius, L., 1998. Role of the prophenoloxidase activating system in invertebrate immunity. *Curr. Opin. Immunol.* 10, 23–28.
- Söderhäll, K., Cerenius, L., Johansson, M.W., 1996. The prophenoloxidase activating system in invertebrates. In: Söderhäll, K., Iwanaga, S., Vasta, G.R. (Eds.), *New Directions in Invertebrate Immunology*. SOS Publications, Fair Haven, pp. 229–253.
- Söderhäll, K., Rögner, W., Söderhäll, I., Newton, R.P., Ratcliffe, N.A., 1988. The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a β -1,3-glucan. *Insect Biochem.* 323–330.
- Sottrup-Jensen, L., Stepanik, T.M., Kristensen, T., Wierzbicki, D.M., Jones, C.M., Lønblad, P.B., Magnusson, S., Petersen, T.E., 1984. Primary structure of the human alpha 2-macroglobulin: V. The complete structure. *J. Biol. Chem.* 259, 8318–8327.
- Sritunyalucksana, K., Cerenius, L., Söderhäll, K., 1999a. Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. *Dev. Comp. Immunol.* 23, 179–186.
- Sritunyalucksana, K., Sithisarn, P., Withayachumnarnkul, B., Flegel, T.W., 1999b. A preliminary study on activation of agglutinin and antibacterial activity in hemolymph of the black tiger prawn, *Penaeus monodon*. *Fish Shellfish Immunol.* 9, 21–30.
- St. Leger, R.J., Cooper, R.M., Charnely, A.K., 1988. The effect of melanization of *Manduca sexta* cuticle on growth and infection by *Metarhizium anisopliae*. *J. Invertbr. Pathol.* 52, 459–470.
- Sugumaran, M., 1996. Roles of the insect cuticle in host defense reactions. In: Söderhäll, K., Iwanaga, S., Vasta, G.R. (Eds.), *New Directions in Invertebrate Immunology*. SOS Publications, Fair Haven, pp. 355–374.
- Sugumaran, M., Kanost, M., 1993. Regulation of insect hemolymph phenoloxidases. In: Beckage, N.E., Thompson, S.M., Federici, B.A. (Eds.), *Parasites and Pathogens of Insects*. Academic Press, San Diego, pp. 317–342.
- Sun, S.C., Lindström, I., Boman, H.G., Faye, I., Schmidt, D., 1990. Hemolin — an insect immune protein belonging to the immunoglobulin superfamily. *Science* 250, 1729–1732.
- Sung, H.-H., Chang, H.-J., Her, C.-H., Chang, J.-C., Song, Y.-L., 1998. Phenoloxidase activity of hemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. *J. Invertbr. Pathol.* 71, 26–33.
- Tang, Y.Q., Selsted, M.E., 1993. Characterization of the disulfide motif in BNBD-12, an antimicrobial β -defensin peptide from bovine neutrophils. *J. Biol. Chem.* 268, 6649–6653.
- Thörnqvist, P.-O., Söderhäll, K., 1997. Crustacean Immune Reaction, a short review. In: Flegel, T.W., MacRae, I.H. (Eds.), *Diseases in Asian Aquaculture III*. Fish Health Section, Asian Fishery Society, Manila, pp. 203–218.
- Thörnqvist, P.O., Johansson, M.W., Söderhäll, K., 1994. Opsonic activity of cell adhesion proteins and β -1,3-glucan-binding proteins from two crustaceans. *Dev. Comp. Immunol.* 18, 3–12.
- Tokunaga, F., Yamada, M., Miyata, T., Ding, Y.L., Hiranaga-Kawabata, M., Muta, T., Iwanaga, S., Ichinose, A., Davie, E.W., 1993a. Limulus hemocyte transglutaminase. Its purification and characterization, and identification of the intracellular substrates. *J. Biol. Chem.* 268, 252–261.
- Tokunaga, F., Muta, T., Iwanaga, S., Ichinose, A., Davie, E.W., Kuma, K., Miyata, T., 1993b. Limulus hemocyte transglutaminase. cDNA cloning, amino acid sequence, and tissue localization. *J. Biol. Chem.* 268, 262–268.
- Vargas-Albores, F., Guzmán, M.-A., Ochoa, J.L.A., 1993a. An anticoagulant solution for hemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). *Comp. Biochem. Physiol.* 106A, 299–303.

- Vargas-Albores, F., Guzmán, M.-A., Ochoa, J.-L., 1993b. A lipopolysaccharide-binding agglutinin isolated from brown shrimp (*Penaeus californiensis* Holmes) haemolymph. *Comp. Biochem. Physiol.* 104B, 407–413.
- Vargas-Albores, F., Jiménez-Vega, F., Söderhäll, K., 1996. A plasma protein isolated from brown shrimp (*Penaeus californiensis*) which enhances the activation of prophenoloxidase system by β -1,3-glucan. *Dev. Comp. Immunol.* 20, 299–306.
- Voorberg, J., Fontijn, R., van Mourik, J.A., Pannekoek, H., 1990. Domains involved in multimer assembly of von Willebrand factor (vWF): multimerization is independent of dimerization. *EMBO J.* 9, 797–803.
- Yeh, M.S., Chen, Y.L., Tsai, I.H., 1998. The hemolymph clottable proteins of tiger shrimp, *Penaeus monodon*, and related species. *Comp. Biochem. Physiol.* 121B, 169–176.
- Yeh, M.S., Huang, C.J., Leu, J.H., Lee, Y.C., Tsai, I.H., 1999. Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (*Penaeus monodon*). *Eur. J. Biochem.* 266, 624–633.
- Yepiz-Plascencia, G.M., Vargas-Albores, F., Jimenez-Vega, F., Ruiz-Verdugo, L.M., Romo-Figueroa, G., 1998. Shrimp plasma HDL and β -glucan binding protein (BGBP): comparison of biochemical characteristics. *Comp. Biochem. Physiol.* 121B, 309–314.
- Yoshida, H., Kinoshita, K., Ashida, M., 1996. Purification of a peptidoglycan recognition protein from the hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.* 271, 13854–13860.
- Zeng, J., Fenna, R.E., 1992. X-ray crystal structure of canine myeloperoxidase at 3 Å resolution. *J. Mol. Biol.* 226 (1), 185–207.