The Prophenoloxidase Activating System and Its Role in Invertebrate Defence[a]

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INTRODUCTION

Invertebrates have an open circulatory system and must therefore have rapid and immediate noninducible defence and coagulation mechanisms to entrap parasites and prevent blood loss after wounding. As in most animals, these processes are mainly carried out by the blood cells or, as they are called in arthropods, hemocytes. The hemocytes of arthropods and other invertebrates have been shown to be important in defence, since they are responsible for phagocytosis of small foreign particles such as bacteria or fungal spores and form capsules around parasites that are too large to be internalized by individual hemocytes.

Because the hemocytes obviously can react to and remove a foreign particle that has succeeded in gaining entry into the body cavity of an arthropod, it appears as if these animals can differentiate non-self from self, and thus a system that can carry out this process ought to be present. Agglutinins or lectins may be one candidate for such a system.[1-5] Another likely candidate is the so-called prophenoloxidase (proPO) activating system,[6] and recently evidence has accumulated mainly from work done on crustaceans that this may be the case. The prime reason why this system early was proposed to function in recognizing foreign particles was the finding that the enzyme phenoloxidase in crayfish blood was turned into its active form by fungal cell wall β-1,3-glucans.[7,8] This was later also confirmed to be the case in several insect species[9-11] and other invertebrates.[12-14] Other microbial products such as lipopolysaccharides and peptidoglycans from bacterial cell walls are also active as elicitors of the proPO system.[15,16] Thus, regardless of which events follow after the proPO system is activated, it is clear that it can react to foreign microbial polysaccharides and as such can be defined as a recognition system.

Recent research has also provided clear evidence that, upon activation of the proPO system, factors are produced that will aid in the elimination of foreign particles such as parasites within the body cavity. This brief overview will report some of these studies, which have mainly been carried out on arthropods, and where

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appropriate make comparisons with the vertebrate immune system as well as with other invertebrates.

THE PROPHENOLOXIDASE SYSTEM: LOCALIZATION AND ACTIVATION

The activity of the terminal component of the proPO system, phenoloxidase, has been detected in several invertebrate groups, such as in crustaceans, insects, millipedes, mollusks, bivalves, brachiopods, echinoderms, and ascidians,[12–14,17,18] but the biochemical mechanism of proPO system activation has been studied in greatest detail in the silkworm *Bombyx mori* and the freshwater crayfish *Pacifastacus leniusculus.* Prophenoloxidase has been isolated in a homogeneous form from the insects *B. mori,*[19] *Manduca sexta,*[20] *Hyalophora cecropia*[21]; *Blaberus discoidalis*[22]; and the freshwater crayfish *P. leniusculus*[23] (TABLE 1). The molecular mass is 76 kDa for the cockroach and crayfish proPO and 80 kDa for the silkworm proenzyme. A prophenoloxidase-activating enzyme (ppA), a serine proteinase with a mass of 36 kDa, was purified from crayfish blood cells,[24] and this proteinase could cleave the proPO into two peptides with masses of 60 and 62 kDa, which both exhibited phenoloxidase activity.[23] In *B. mori,* Ashida and colleagues have shown that a cuticular proteinase,[25] which may not be the endogenous proPO-activating enzyme, could cleave and thus activate proPO from silkworm blood into an active PO by removing a 5-kDa peptide from the zymogen.[26] Nevertheless, it has to be emphasized that the complete mechanism of proPO-system activation is by no means clear, and, for example in freshwater crayfish, yet another hemocyte serine proteinase with a mass of 38 kDa also seems to be able to convert proPO into its active form.[23] In addition, at least two other

TABLE 1. Components of the Prophenoloxidase Activating System Purified from Arthropods

Protein and Species	Molecular Mass (kDa)	Reference
Prophenoloxidase		
Bombyx mori	80	Ashida, 1971[19]
Manduca sexta	71 & 77	Aso *et al.*, 1985[20]
Hyalophora cecropia	76	Andersson *et al.*, 1989[21]
Pacifastacus leniusculus	76	Aspán & Söderhäll, 1991[23]
Blaberus discoidalis	76	Durrant *et al.*, 1993[22]
Prophenoloxidase Activating Enzyme		
Bombyx mori (cuticle)	*ca.* 35	Dohke, 1973[25]
Pacifastacus leniusculus	36	Aspán *et al.*, 1990[24]
Prophenoloxidase Activating Enzyme Inhibitor		
Pacifastacus leniusculus	155	Aspán *et al.*, 1990[24]
Locusta migratoria	3.76 & 3.8	Boigegrain *et al.*, 1992[51]

serine proteinases with masses of 50 and 67 kDa are present in the crayfish blood cells,[23] but their function, if any, in the proPO system is as yet unknown.

Both proPO and ppA are present in the secretory granules of the semigranular and granular blood cells of crayfish[27]; thus, the compartmentalization of the system in these vesicles may be one important way to control and regulate the release of the proPO system at least in crustaceans. Other means by which the activation of the proPO system may be regulated are proteinase inhibitors present in crayfish plasma such as an α-macroglobulin[28] and a high-molecular-mass inhibitor of trypsin-like proteinases.[29] The trypsin inhibitor, which has a mass of 155 kDa, is by far the most efficient endogenous inhibitor of ppA.[30]

Another way to control production and activity of proPO factors is proteolytic degradation, and in crayfish this occurs with the 76-kDa cell-adhesion protein,[31] which is rapidly degraded in a hemolymph sample in which the proPO system is activated into a 30-kDa peptide with lower biological activity.[32] (See TABLE 2 for list of defence proteins purified from crayfish blood.)

BIOLOGICAL FUNCTION OF FACTORS OF THE proPO SYSTEM

Upon activation of the proPO system by microbial elicitors, the terminal component of the system, phenoloxidase, can oxidize phenols into quinones, and then these will autocatalyze into melanin. Melanin and its precursors have been shown to inhibit growth of fungi and bacteria.[33–35]

More importantly, proteins that are associated with the proPO system have been shown to be directly involved in the communication between different blood cell types and also in the removal of foreign particles within the hemocele of crayfish.

TABLE 2. Defence Proteins Purified from Crayfish Blood

Protein	Molecular Mass (kDa)	Reference
Prophenoloxidase	76	Aspán & Söderhäll, 1991[23]
Prophenoloxidase activating enzyme (ppA)	36	Aspán *et al.*, 1990[24]
Cell adhesion, degranulating, and opsonic protein	76	Johansson & Söderhäll, 1988,[31] 1989[48] Kobayashi *et al.*, 1990[32]
β-1,3-Glucan binding protein (βGBP)	100	Duvic & Söderhäll, 1990[39]
α-Macroglobulin	2 × 190	Hergenhahn *et al.*, 1988[28]
ppA inhibitor	155	Hergenhahn *et al.*, 1987[29]
Subtilisin inhibitor	23	Häll & Söderhäll, 1982[52]
Receptor for βGBP and cell adhesion protein	340[a]	Duvic & Söderhäll, 1992[42] Cammarata *et al.*, unpublished
Clotting protein	2 × 210	Kopacek *et al.*, 1993[53]
Hemagglutinin	420[b]	Kopacek *et al.*, 1993[54]

[a]Multimer of 90-kDa subunits.
[b]Multimer of 65–85-kDa subunits.

Thus, it has been demonstrated that proteins associated with the proPO system have, for example, encapsulation-promoting activities and can act as opsonins.

ELICITORS OF PROPO SYSTEM ACTIVATION

The proPO system is induced to its active form by different microbial polysaccharides, such as β-1,3-glucans[6] and peptidoglycans or lipopolysaccharides.[16,36] The activation exerted by β-1,3-glucans is very specific; most other glycans that do not contain β-1,3-D-glucopyranosyl residues fail to elicit any activation of the proPO system.[8] Consequently, the proPO system can specifically recognize different types of microorganisms by the presence of different polysaccharides on their surface; for example, the cell walls of almost all fungi contain β-1,3-glucans. Thus, it was not surprising to see that proteins that can bind to β-1,3-glucans and induce activation of the proPO system are present in the plasma of both insects and crustaceans.

THE β-1,3-GLUCAN-BINDING PROTEIN

A protein that specifically binds β-1,3-glucans has been isolated and characterized from a number of different arthropods such as the insects *B. mori,*[37] and *B. craniifer*[38] and several crustaceans[39,40] (Thörnqvist *et al.,* unpublished) (TABLE 3). This protein, besides inducing activation of the proPO system *in vitro,* also functions as a degranulation factor for crayfish granular cells.[41] Similarly to the 76-kDa factor, this protein can function as a phagocytosis-stimulating opsonin for crustacean hyaline cells (Thörnqvist *et al.,* unpublished).

The most detailed information regarding both the biochemical characteristics and the biological activities of any β-1,3-glucan-binding protein (βGBP) has been obtained from the crayfish *P. leniusculus.*[39,41,42] Recently, the cDNA cloning of the *P. leniusculus* protein was achieved and a sequence established. This protein is synthesized in the hepatopancreas from which it appears to be secreted into the plasma.

Sequence analysis against data banks have not revealed any strong homology to any known protein. Interestingly, however, the sequence of the crayfish βGBP

TABLE 3. β-1,3-Glucan Binding Proteins Purified from Arthropods

Species	Molecular Mass (kDa)	Reference
Insects		
Blaberus craniifer	90	Söderhäll *et al.,* 1988[38]
Bombyx mori	62	Ochiai & Ashida, 1988[37]
Crustaceans		
Pacifastacus leniusculus	100	Duvic & Söderhäll, 1990[39]
Procambarus clarkii	100	Duvic & Söderhäll, 1992[42]
Astacus astacus	95, 105	Duvic & Söderhäll, 1992[42]
Carcinus maenas	110	Thörnqvist *et al.,* unpublished

contains an RGD (arginine-glycine-aspartic acid) sequence. Such sequences are known to be involved in ligand binding to receptors of the integrin family in vertebrates.[43] We have earlier demonstrated that a pentapeptide containing the RGD sequence triggers degranulation and cell-substratum attachment of crayfish granular hemocytes.[44] Whether the degranulation provoked by the binding of β-1,3-glucan-treated βGBP to the hemocytes and the effects obtained by the RGD peptide involve the same receptor has not been established.

The βGBPs from different crustaceans are very similar. For example, an antiserum raised against the *P. leniusculus* βGBP reacts with βGBP from other crayfish and crab species[40] (Thörnqvist *et al.*, unpublished). In addition, crab βGBP exerts the same biological activities on crayfish hemocytes as it does on crab cells and vice versa (Thörnqvist *et al.*, unpublished). Furthermore, the NH_2-terminal amino acid sequence of βGBP from *P. leniusculus* and the crab *Carcinus maenas* are similar (Thörnqvist *et al.*, unpublished). In TABLE 3 β-1,3-glucan binding proteins isolated so far from different arthropods are shown.

THE 76-kDa PROTEIN

This crayfish protein (for reviews, see Johansson and Söderhäll[45,46]), which is synthesized by the blood cells, stored in a biologically inactive form in their secretory granules,[47] and is released from them during degranulation, gains its biological activity concomitant with proPO system activation[31] (TABLE 4). How this protein is converted to its active form is still unknown, although some recent data clearly indicate that a limited proteolysis by a serine proteinase appears to be involved (Johansson & Söderhäll, unpublished). If this turns out to be the case, then only a very small peptide may be cleaved off from the 76-kDa protein to generate the active factor. In its active form this protein functions as a cell-adhesion factor for crayfish semigranular and granular blood cells[31] and as a strong degranulation factor for the same cells.[48] Kobayashi *et al.*[32] could demonstrate that the 76-kDa protein also is an encapsulating promoting factor, since particles coated with the purified protein were more avidly encapsulated by the semigranular cells. The 76-kDa protein also functions as a phagocytosis-stimulating opsonin for crab hyaline cells (Thörnqvist *et al.*, unpublished), and polyclonal antiserum to the 76-kDa protein recognizes two bands

TABLE 4. Cell Communication Proteins in Crayfish Blood

76-kDa Protein	
Biosynthesis:	Hemocytes
Localization of inactive protein:	Hemocyte granules
Activation:	Concomitant with proPO-system activation
β-1,3-Glucan Binding Protein	
Biosynthesis:	Hepatopancreas
Localization of protein:	Plasma
Generation of hemocyte receptor binding activity:	After reacting with β-1,3-glucans

of 81 and 85 kDa in the crab *C. maenas*[46] (Thörnqvist *et al.*, unpublished). From the insect *B. craniifer,* a similar cell-adhesion and degranulating protein with a mass of 90 kDa has been isolated, and it reacts with the anti-crayfish 76-kDa protein antiserum.[49] Furthermore, the crayfish 76-kDa protein cross-reacts with the 75-kDa mammalian cell-adhesion protein vitronectin[46] (Thörnqvist *et al.*, unpublished), which is involved in the coagulation and complement systems.[50]

A MEMBRANE RECEPTOR FOR THE βGBP AND THE 76-kDa PROTEIN

In hemocyte membranes we were able to identify a receptor protein for the βGBP.[42] Interestingly, the βGBP did only bind the receptor if it had previously been reacted with a β-1,3-glucan. The receptor protein could be isolated, purified, and partially characterized. It was shown to be composed of subunits of 90 kDa, and its native molecular mass is around 340 kDa[42] (Cammarata *et al.*, unpublished). Recently, we have also been able to demonstrate that the 76-kDa protein binds to the same receptor with a higher apparent affinity and that βGBP that has been reacted with β-1,3-glucans competes with the 76-kDa protein for this binding (Cammarata *et al.*, unpublished). The membrane receptor is presently being cloned and sequenced to possibly reveal its structural similarity with receptor proteins from other animals.

CELLULAR COMMUNICATION

In crustaceans, two proteins, which are associated with proPO system activation in some as yet unknown way, have been demonstrated to be directly involved in the communication between blood cells (TABLE 4). More structural data, however, need to be obtained about these two molecules, their receptors, and their tentative analogues in other arthropods and invertebrates before we can reveal the fine details of ligand–receptor interaction during these defence processes. Such work is presently under way in our laboratory. We are currently also investigating the intracellular signaling pathways triggered by binding of these ligands to the cells.

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