Immune properties of invertebrate phenoloxidases

Lage Cerenius, Kenneth Söderhäll
Department of Organismal Biology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden

A R T I C L E   I N F O
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- Complement system
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- Tyrosinase
- Pattern recognition
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A B S T R A C T
Melanin production from different types of phenoloxidases (POs) confers immunity from a variety of pathogens ranging from viruses and microorganisms to parasites. The arthropod proPO expresses a variety of activities including cytokine, opsonin and microbiocidal activities independent of and even without melanin production. Proteolytic processing of proPO and its activating enzyme gives rise to several peptide fragments with a variety of separate activities in a process reminiscent of vertebrate complement system activation although proPO bears no sequence similarity to vertebrate complement factors. Pathogens influence proPO activation and thereby what types of immune effects that will be produced. An increasing number of specialised pathogens – from parasites to viruses – have been identified who can synthesise compounds specifically aimed at the proPO-system. In invertebrates outside the arthropods phylogenetically unrelated POs are participating in melanization reactions obviously aimed at intruders and/or aberrant tissues.

Infections and tissue damage are commonly seen accompanied by melanization in many invertebrates. A flurry of recent findings has highlighted the importance of melanization reactions and associated activities as key contributors to immunity in invertebrates, in particular arthropods such as insects and crustaceans. By the use of carefully designed infection experiments, mutant analyses and RNA interference some earlier doubts of the crucial role for phenoloxidase (PO) in providing resistance to microbial organisms, parasites and viral infections have been fully overcome. Although the resistance towards pathogens in many cases is linked to melanin production or the toxic reaction intermediates of the melanogenesis some intriguing evidence obtained in several insects and crustaceans strongly suggest that some prophenoloxidase (proPO) gene products confer host immunity even without a visible melanization. In line with this, recent detailed data demonstrate that crustacean proPO upon limited proteolysis gives rise to peptide fragments exhibiting cytokine, opsonic as well as microbial agglutinating and killing activities lending credit to the view that the arthropod proPO-system fulfils many of the duties that the complement system carries out in vertebrates and that even the proPO-protein itself exhibits some striking functional similarities to the vertebrate complement proteins in spite of lacking structural similarity to such proteins. In this review we will emphasise the immunological contribution of the melanization reaction and associated activities by the proPO activating system. We will begin with some general characteristics of invertebrate POs followed by a discussion of the activation cascade of arthropod proPOs that begins with pattern recognition and/or tissue damage that initiates a complex proteolytic cascade ending with processing the proPO. Finally, we will consider the different biological activities that arise by the processing of the proPO protein and compare them to the vertebrate complement.

1. Invertebrate phenoloxidases
Melanin is produced from low molecular weight aromatic compounds such as tyrosine by a combination of enzymatic and non-enzymatic reactions. The enzymatic conversion (hydroxylation) of monophenols and the subsequent oxidation of the ortho-phenols produced are both carried out by POs. For more specialised reviews of the chemical reaction mechanisms involved in melanin formation the reader is referred to e.g. Nappi and Christensen (2005) and a more general discussion of various type 3 copper proteins and their phylogeny (to which phenoloxidases belong) can be found in Aguilera et al. (2013). In vertebrates this enzyme, a tyrosinase, is a membrane-bound enzyme acting in a confined membrane-enclosed compartment, the melanosome (D’Alba and Shawkey, 2019). Melanosome-bound tyrosinases have been characterised in some invertebrates such as cuttlefish (Palumbo et al., 2000) or Ciona (Racioppi et al., 2019). However, in several invertebrates, such as insects, crustaceans and other arthropods,
corresponding PO-activities are instead carried out by soluble proteins lacking an ER-localisation pro-peptide (Cerenius and Söderhäll, 2004). Arthropod POs are well-studied from an immunological viewpoint whereas the immunological role of melanization in other invertebrates is less investigated. However, the PO-activity of tunicate morula cells in rejection reactions towards foreign tissue explants (Franchi et al., 2019) or melanization reactions in corals (Bailey et al., 2019; Palmer et al., 2011) molluscs (Coaglio et al., 2018 and reviewed in Luna-Acosta et al., 2017) or in annelids (reviewed in Prochazkova et al., 2020) towards pathogens suggest a role for the melanization reaction beyond the arthropods. A recent transcriptome analysis after grafting operations in the mantle of the pearl mussel Hystioptis cumingi is one additional example that clearly supports a role for mollusc tyrosinases in immune reactions (Shen et al., 2020).

Obviously due to these differences in cellular localisation, regulation and activation of PO activities between arthropods and many non-arthropod invertebrates, control of melanization in the latter must at least partly occur along other paths than the well-investigated arthropod PO-activation system.

1.1. Laccases

To comply with the present use in the immunological literature and to distinguish them from the vertebrate membrane-bound tyrosinases the term PO will be used for the cytosolic invertebrate enzymes with tyrosinase (EC 1.14.18.1) activities. It should be noticed that technically catechol oxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2) are pheno-

1.2. Hemocyanin

Type 3 copper proteins to which both tyrosinases and hemocyanins (as well as tyrosinase-related proteins and catechol oxidases) belong possess a conserved site with 2 copper atoms, each coordinated by three histidine residues. The copper atoms serve as O2 carriers or are transporting electrons in the reactions of the phenolic substrate for hemocyanin and POs, respectively. Structural data and modelling suggest that access into the copper is more restricted in hemocyanin since some crucial amino acids are hindering the relatively bulky phenolics to reach them although it is well established that hemocyanins from many different sources can be converted into enzymatically active POs in vitro by a variety of different treatments and hereby produce antimicrobial activities (for reviews see Decker and Jaenicke, 2004; Coates and Nairn, 2014). It seems likely that these in vitro treatments serve to shift the position of an active site block (Li et al., 2009) residue such as the phenylalanine 371 in the crustacean Panulirus japonicus hemocyanin (Masuda et al., 2020) that is denying access to the copper to more bulkier substrates. This Phe residue is not present in crustacean POs; its absence and the presence of conserved glutamic acid and aspartagine acid residues and a conserved water molecule around the Cu have been suggested to contribute to the enzyme activity of arthropodian PO (Masuda et al., 2020; Hu et al., 2016). The immunological relevance of hemocyanin-to-PO conversions remains to firmly establish in many cases. In a few cases endogenous substances with capacity to induce PO-activity in the corresponding hemocyanin (Nagai et al., 2001) have been identified although their physiological significance remains to clarify. However, the possible immunological role of hemocyanin goes beyond its PO-activity, proteolytic processing of the protein may produce antimicrobial substances such as the crayfish-derived astacidin 1 (Lee et al., 2003) and several shrimp hemocyanin-derived peptides (Destoumieux-Garzon et al., 2001 and reviewed in Tassanakajon et al., 2018).

2. The number and distribution of invertebrate PO genes vary

The number of proPO-genes in different arthropods varies greatly from one to more than 10 in some insects such as mosquitoes. A few arthropods are even lacking them, possibly hemocyanins or laccases may instead fulfill their immunological role. By increasing the numbers some species may obtain adaptability letting different proPO genes be under separate regulation and tissue localisation. A clear example of such a partial division of function and localisation has been unravelled in Drosophila melanogaster. In this species two PO-genes, PPO1 and PPO2, are expressed in crystal cells whereas PPO3 expression is localised to the lamellocytes (Dudzic et al., 2015). The latter gene was suggested to have arisen recently in the melanogaster group by a gene duplication of PPO2 providing means more or less exclusively aimed at larger parasites, in particular parasitoid wasps. An additional division of labour between the PPO gene products is suggested by the fact that the PPO2 protein is stored in large protein inclusions in the cell and released by cell rupture whereas the PPO1 protein may be released continuously into the hemolymph by an undetermined mechanism (Dudzic et al., 2019). Of note, the amounts of different POs may vary even within what appear to be a homogenous hemocyte type by morphological criteria. In the mosquito A. gambiae for example there are different subpopulations of granulocytes, some of which contain high amounts of PPO6 protein whereas other possess much lower concentrations (Severo et al., 2018). The study by Severo et al. (2018) also suggests that some granulocytes can transfer PPO6 (their in vitro study with isolated granulocyte sub-populations demonstrated transfer of PPO6 transcripts between these sub-populations) to other granulocytes via micro-vesicles (exosomes) thereby enhancing the melanization potential of some hemocyte populations and indicating a route via which different hemocytes may cooperate during immune reactions.

In another insect Nipponaphis monseni a soldier specific PO-gene has undergone an accelerated evolution compared to the other PO-genes of this species, presumably an adaptation to its apparent specialised role in coagulation (see section below on immunological effects) of the body fluid of self-sacrificing soldier nymphs (Katsukake et al., 2019). Indirectly some functional diversion is suggested among the 9 PPO genes in Anopheles gambiae mosquitoes since silencing of 3 of them, PPO2,3 or 9 of them increased Plasmodium berghei survival in the insect host whereas, in contrast, knock-downs of three other genes, PPO4,5 or 6 did not affect parasite survival (Kwon and Smith, 2019).
Although their role in immunity as indicated above is less clear it is noteworthy that in some mussels the number of PO (tyrosinase) genes has undergone an enormous expansion with some species harbouring more than 20 PO-genes (Aguliera et al., 2014).

3. Pattern recognition in the activation of the proPO-activation cascade

3.1. β-1,3 glucan-binding proteins

Interest into the proPO system grew considerably once it was demonstrated that β-1,3 glucans, a polysaccharide characteristic of fungal cell walls and a few other organisms had the capacity to specifically and in very low concentrations initiate its activation in crustaceans (Unestam and Söderhäll, 1977). It soon become evident that β-1,3 glucans triggered proPO-activation in insects as well and several proteins capable of binding β-1,3 glucans and thereafter mediate proPO-activation were isolated and characterised biochemically in insects, first by Söderhäll et al. (1988) and shortly thereafter by Ochiai and Ashida (1988) and later also in crustaceans (Duvic and Söderhäll, 1990) to be followed by the cloning and structural characterisation of a glucan-binding protein (BGBP) from freshwater crayfish (Cerenius et al., 1994). BGBP, with dual functions as lipid carrier and pattern recognition protein, can agglutinate fungal cells (Hall et al., 1995; Goncalves et al., 2012) and will after binding to the β-1,3-glucans both initiate proPO-activation and through binding to a hemocyte receptor act as an opsonin and as a promotor of hemocyte degranulation (Duvic and Söderhäll, 1992). BGBP seems to be unique for crustaceans but extensive studies have revealed the wide distribution of a family of proteins with capacity to bind both β-1,3 glucans and bacterial cell wall products such as LPS followed by activation of the proPO-cascade are present in crustaceans (Lee et al., 2000; Sritunyalucksana et al., 2002) as well as in insects (Ochiai and Ashida, 2000; Ma and Kanost, 2000). These proteins, variously often called lipopolysaccharide and glucan-binding proteins (LGBP) or β-glucan recognition proteins (GRPs) or gram-negative bacteria binding-proteins (GNBPs) belong to members of a small family of ca 50–60 kD proteins with some similar structure to glucanases (Lee et al., 1996), although the glucanase-like domain apparently is not involved in the binding of the glucans (Takashashi et al., 2009). LGBPs have also been found in other invertebrates e.g. annelids (Beschin et al., 1998) and molluscs (reviewed by Wang et al., 2018). LGBPs are likely to trigger many other immune reactions such as the expression of genes for antimicrobial peptides as shown in fruit flies (Kim et al., 2000), Manduca sexta GRP3 was shown by use of the recombinant protein to agglutinate and retard growth of Bacillus cereus (Rao et al., 2014). Purified shrimp LGBP, although capable of binding bacteria and activate the proPO-system, does not inhibit the growth of the bacterial pathogen Vibrio parahaemolyticus (Phupet et al., 2018). GRPs from different insect species differ with respect to which conformation variants of β-1,3 glucans they bind to (Adachi et al., 2019) which, is suggesting the possibility that they may have evolved to recognise different pathogens. There is often a small family of different LGBP/GRP genes in many species but the functional significance of this diversity within a single species remains to a large extent to investigate. There are also at least a few examples of peptidoglycan-binding proteins (PGRPs see below) that are capable of binding β-1,3 glucans and activate the proPO-system (Lee et al., 2004; Zhao et al., 2018).

3.2. Peptidoglycan binding proteins

Peptidoglycans may activate the proPO-cascade through peptido-glycan recognition proteins, PGRPs, a family of proteins widely distributed in the animal kingdom. They are structurally related to T7 lysozyme albeit some of them lack its amidase activity. Several PGRP family members participate in recognising peptidoglycan of both the diaminopimelic acid (DAP)-type and the Lys-type to initiate different immune reactions such as proPO-activation or synthesis of antimicrobial peptides. Activation of the proPO-system by a PGRP-mediated reaction was first demonstrated in silkworm (Yoshida et al., 1996) but is apparently common in insects, e.g. in D. melanogaster Takehana et al. (2002); Tenebrio molitor (Park et al., 2007); Ostrinia furnacalis (Sun et al., 2014); Helicoverpa armigera Li et al., (2015); Antheraea pernyi Zhao et al. (2018). Most insect species investigated possess several PGRP-genes that differ both structurally and functionally. In several insect species one PGRP protein, PGRP-SA has been identified in a complex with GNBP1 to mediate the proteolytic activation by Lys-type peptidoglycan of the proPO-system and the Toll-ligand Spätzle, as first demonstrated biochemically in the beetle T. molitor (Kim et al., 2008). In Drosophila PGRP-LE will mediate proPO-activation by DAP-containing peptidoglycan (Takehana et al., 2002). At least some crustacean groups however lack PGRP but a complex consisting of an LGBP and two SPHs appear to fulfil a corresponding role of mediating peptidoglycan-triggered proPO activation (Liu et al., 2011). Also, a QM-protein has been demonstrated using a recombinant such protein to bind peptidoglycans and promote phagocytosis and proPO-activation in the shrimp Penaeus monodon (Udompetchcharaporn et al., 2014). It is uncertain however whether the QM-protein is localised to the hemolymph in vivo and therefore in position to function as an extracellular pattern recognition protein. An interesting example of PRR activity among crustaceans is another large SPH, the masquerade-like protein (Huang et al., 2000) that upon binding bacterial cell walls is proteolytically cleaved into several fragments expressing different activities such as binding to hemocytes and bacterial cells hereby promoting removal of bacteria from circulation and one fragment functioning as a cell adhesion factor (Lee and Söderhäll 2001). Also, C-type lectins mediate proPO-activation e.g. the Manduca sexta immunolectins (Yu et al., 1999), the shrimp Fc and the crustacean mannos-binding lectin (MLB)-like protein (Wu et al., 2012). The latter is a collectin-like protein mediating LPS-induced activation of the proPO-system, i.e. a protein which both functionally and structurally has some resemblance to mannos-binding lectin associated serine protease (MASP), the initiator of the lectin pathway of vertebrate complement. An interesting observation is that the presence of high amount of a mannan binding lectin in the granular hemocytes of the crayfish P. leniusculus may play a role in regulating the proPO-system since a hemocyte lysate supernatant (HLS) prepared at 100 mM Ca²⁺ could become activated when the concentration of LPS was increased up to 3 mg/ml, this may indicate that PI-MLB acts as a scavenger for LPS to prevent spreading of LPS in the hemolymph to avoid further activation of the proPO-system (Wu et al., 2013).

3.3. Thiolester-proteins

In mosquitoites thiolester-proteins (TEPs) have been shown to influence the melanization reaction. It has been suggested, primarily by a genetical approach, that in A. gambiae TEP1 (Levashina et al., 2001) binds to foreign surfaces and interacts with a clip serine proteasine, SCPLI1, in a chain that via two downstream clip proteases homologues, CLIPA8 followed by CLIPA28, terminates in proPO cleavage by an uncharacterised protease (El Moussawi et al., 2019). The presence of SCPLI1 appears to stimulate the recruitment of the TEP to the microbial surface whereas an SPH, CLIPA2 negatively regulates TEP1 binding. In the hemolymph TEP1 circulates in a complex together with two leucine-repeat proteins (Povelones et al., 2009). Different TEP1 alleles however confers different degrees of melanization and resistance towards Plasmodium berghei and melanization requires proteolytic processing of TEP1 (Blandin et al., 2009; Volokhinsky et al., 2017). Several components in the mosquito pathway to proPO activation remain to identify, although recently one such the CLIP-domain protein C9 was characterised and found to act downstream of the SPHs (Sousa et al., 2020). Also, in the fruit fly several TEP proteins have been shown to influence the degree of melanization when the fly is experimentally infected with the bacterium Photobacterium (Shokal and Eleftherianos, 2020).
4. Release and activation of proPO in the extracellular milieu

Arthropod POs in general are synthesised as inactive proenzymes that become active by an elaborate chain of events brought about by pattern recognition as described above or by tissue damage, e.g. by danger signalling. In several insects and crustaceans a chain of associated proteinases and regulating proteins has been identified that bring about the initiation of the proPO cascade. Several of these factors are shared with the pathway that bring about the proteolytic activation of the Toll ligand Spätzle (Kan et al., 2008; Dudzic et al., 2019) and hereby trigger gene expression of many genes for antimicrobial peptides. Thus, there is a considerable amount of cross-talk between the pathways resulting in the melanization reaction and in activation of Toll.

4.1. Release of proPO from hemocytes

Most POs whose site of synthesis has been determined are produced in specific sub-sets of hemocytes that depending on species may be called granulocytes, oenocytoids or crystal cells. The enzyme is in most cases released in its pro-form either by cell rupture after activation of the c-Jun N-terminal kinase (JNK) pathway as seen in Drosophila oenocytoids (Bidda et al., 2007; Dudzic et al., 2015) or possibly by other still not characterised mechanisms of continuous release. One recent study in the insect Acyrthosiphon pisum suggests that the JNK-signalling may also influence proPO gene expression as RNAi of this pathway decreases expression of both proPO-genes in this species (Ma et al., 2020). This suggestion requires elaboration since the effects of JNK knockdown on PO activities on the other hand was minimal.

Data from a crustacean implicates a mechanism reminiscent of inflammasome-activation involving a caspase-1-like enzyme in the release of PO from granular hemocytes into the extracellular milieu (Jearaphunt et al., 2014). This caspase-1 enzyme can also modulate the activities of the released extracellular PO; cleavage by the caspase of proPO produces PO fragments without melanising ability but with capacity to agglutinate bacteria (Jearaphunt et al., 2014). Extracellular release of proPO may be brought about by cues such as microbial products or tissue damage (danger signalling). The enzyme gains its activity after a limited highly regulated proteolysis of the proPO (for details see e.g. review by Lu et al., 2014, Cerenius et al., 2008), often the result of a chain of events involving several proteinases, pattern recognition proteins and regulatory proteins. In crustaceans is the prophenoloxidase activating enzyme (ppA) and other components of the proPO-activating system (but not proPO!) are localised into granules that are released upon pattern recognition events in the plasma (Sriacharoen et al., 2005; Jearaphunt et al., 2014) (Fig. 1). The use of an extracellular PO in many invertebrates is likely to have contributed to relatively elaborate mechanisms for controlling both the proPO-activation and the PO-activity once it becomes activated in these animals.

4.2. Proteolytic activation of proPO

ProPO is activated by limited proteolysis, in most cases so far investigated by clip-containing serine proteinases. In a few insect species the chain of events from pattern recognition to proPO cleavage has been worked out (for a review of proteolytic activation of proPO in insects see Lu et al., 2014). They consist of upstream pattern recognition proteins (several previous section), a chain of proteinases activating each other terminating with one or several prophenoloxidase activating enzymes (PPAES or ppAs). In several instances the most upstream proteinase in the activation cascade is a modular proteinase containing some structural motifs resembling vertebrate complement initiating serine proteinase MASP or the complement component C1q as seen in the Tenebrio molitor modular serine protease (MSP) Park et al. (2007); D. melanogaster ModSP (Buchon et al., 2009); and M. sexta HP14 (Ji et al., 2004). These modular proteinases interact with pattern recognition proteins bound to e.g. a microbial cell wall component and as a result the proteinase becomes auto-activated as was first shown in T. molitor with MSP, Lys-PG, PGRP-SA and GNBP1 (Kan et al., 2008; Roth et al., 2009) and later in M. sexta with p1,3-glucans, jGRP2 and HP-14 (Takahashi et al., 2015). In addition, serine proteinase homologues (lacking the serine residue required for proteolytic activity) regulate activation of the system, e.g. being necessary in some systems to achieve melanization activity by the proteolytically processed PO. Both the serine proteases and the SPH contain one or several clip domains in the pro-region (reviewed in Kanost and Jiang, 2015). Unfolding of these cascades have made it clear that there is a large overlap between the extracellular part of the Toll signalling giving rise to AMP gene expression and the proPO cascade in that both pathways share several components and in many cases are activated simultaneously (Kan et al., 2008). An alternative way to achieve proPO-activation is through tissue damage, it is commonly observed in many invertebrates that injuries quickly lead to melanization of the damaged area. The signal(s) leading to this activation are mostly unknown but in D. melanogaster the protease Hayan (independently of the PGRP-SA/GNBP → ModSP → Grass pathway that mediates activation of PPO1 and pro-Spätzle via pattern recognition of peptidoglycan) is mediating activation of both PPO1 and PPO2 at the site of injury (Dudzic et al., 2019; Nam et al., 2012). It was suggested that H2O2 or other reactive oxygen species at the site of injury constituted a signal that affected other cells in the body (Nam et al., 2012). Reactive oxygen species may also regulate the hematopoietic formation of more proPO-producing granular hemocytes as in crustaceans (Junkunlo et al., 2016) and PO3-containing lamellocytes in fruit flies (Owuwu-Ansah and Banerjee, 2009). Besides Hayan a phylogenetically closely related proteinase, Persephe is

![Fig. 1.](https://example.com/fig1.png) Left shows a mixture of semigranular (SG, thin arrows) and granular cells (G, thick arrows) under the light microscope and stained with May-Grünwald Giemsa staining. Notice that granular cells are stained more avidly compared to semigranular cells. Photo Irene Soderhall. Right shows a transmission electron micrograph of a granular cell which contains most of the components of the proPO-system. The black stained vesicles contain the proPO-system components except proPO which is in the cytosol of the granular cell. Photo Oleg Chaga.
activated in particular by microbial proteases to mediate activation of the Toll-ligand as well as of melanization in *D. melanogaster* (Issa et al., 2018; Dudzic et al., 2019). As demonstrated in freshwater crayfish different bacteria may influence the outcome of the extracellular processing of proPO; an *Aeromonas hydrophila* infection led to a higher proportion of ppA-cleaved PO and a low amount of caspase-1-cleaved PO and thus higher PO-activity compared to an *E. coli* infection that produced proportionately more caspase cleaved PO (Jearaphunt et al., 2014). This result suggests that infections may trigger different proteolytic events of proPO-system components that may adjust what type of immune effectors that will appear. Results obtained with recombinant *D. melanogaster* PPO3 protein may point to similar conclusions; depending on how the recombinant protein was activated different amounts of melanization intermediates appeared (Wu et al., 2018). Together data so far appear to suggest that there are multiple ways to activate the proPO-system resulting in a variety of immune effectors produced.

5. Inhibitors and regulators of proPO-activation or activity

5.1. Protease inhibitors

Given the toxicity and reactivity of several components produced during the melanization reaction (Nappi and Christensen, 2005) strict control of the activation as well as the activity of the PO enzyme itself is of paramount importance. In several arthropods regulatory protease inhibitors to the different proteolytic steps in the proPO-system activation have been identified and characterised. Many of these are serpins, a superfamily whose members inhibit serine proteases by a specific mechanism that involves cleavage of the target protease at a specific bond, the scissile bond. Thus, some pathogens have evolved specific serpins with a specific capacity to prevent host melanization. One example is the baculovirus encoded Hesp018 serpin (Ardisson-Araujo et al., 2015) another the parasitoid venom LbSPINV (Colinet et al., 2009), see also Table 1. A few examples of serpins and their target proteases that are inhibiting the proPO-cascade (and sometimes the Toll pathway as well) in some detail are: *D. melanogaster* SPN27A, an inhibitor of both melanization and extracellular Toll signalling (De Gregorio et al., 2002; Ligoxygakis et al., 2002) and it specifically inhibits Sp7 (also called MP2) (An et al., 2013) and SPN28D inhibits melanization through an unidentified protease target (Scherfer et al., 2008). In *T. molitor* SPN40 inhibits modular serine protease (MSP), SPN55 inhibits spätzle-activating enzyme (SAE), SPN48 inhibits spätzle-processing enzyme (SPE) (Jiang et al., 2009) and as a result both melanization and Toll pathway is blocked by either

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Host</th>
<th>Pathogen factor(s)</th>
<th>Short description of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td><em>Galleria mellonella</em></td>
<td>a,1-3-gucan</td>
<td>The glucan is binding proPO-activating complex (Staszek et al., 2020).</td>
</tr>
<tr>
<td>Baculovirus</td>
<td><em>Helicoverpa armigera</em></td>
<td>unknown</td>
<td>Virus by increasing serpin-5,-9 expression prevents proPO-system activation by the clip domain proteases CSP46 (Yuan et al., 2017).</td>
</tr>
<tr>
<td>Baculovirus</td>
<td><em>Triaspisia ni</em></td>
<td>Hesp018</td>
<td>Virally encoded serpin inhibiting host melanisation (Ardisson-Araujo et al., 2015).</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td><em>Anaphaedes stephensi</em></td>
<td>mili1</td>
<td>An RNA that initially during infection is highly expressed and via binding to Argonaute 1 silences Spätzle and thus Toll activation, but will by subsequently lowering its expression avoid to trigger expression of the proPO-activator CLIP99 in the haemocoeel during later phases of infection (Cai et al., 2019).</td>
</tr>
<tr>
<td><em>Cotesia rubecula</em></td>
<td><em>Pieria rapae</em></td>
<td>Vn50 (SPH homologue)</td>
<td>A Lbenzoquinone derivate that inhibits PO-activity (Feng et al., 2015).</td>
</tr>
<tr>
<td><em>Isaria javanica</em></td>
<td><em>Aedes aegypti</em></td>
<td>Uncharacterized</td>
<td>Inhibitor of host proPO-activation. In a <em>M. sexta</em> model Vn50 down-regulates proPO-activation by PAP-1, SPH-1,2 (Asgari et al., 2003; Zhang et al., 2004).</td>
</tr>
<tr>
<td><em>Leptopilina boulardi</em></td>
<td><em>Drosophila yakuba</em></td>
<td>LhSPNY</td>
<td>Strain specific subversion of the melanisation response via reduced PPO3 and PPO expression and compromised Toll pathway (Ramirez et al., 2018).</td>
</tr>
<tr>
<td><em>Metarhizium robertsi</em></td>
<td><em>Galleria mellonella</em></td>
<td>MmP4</td>
<td>Parasitoid venom serpin that inhibits proPO-activation (Colinet et al., 2009).</td>
</tr>
<tr>
<td><em>Metarhizium robertsi</em></td>
<td><em>Drosophila melanogaster</em></td>
<td></td>
<td>Metalloproteaseinhibiting PO-activity via proPO-cleavage at other sites than positions used by proPO-activating serine proteases (Huang et al., 2020).</td>
</tr>
<tr>
<td><em>Micropis demolitor</em></td>
<td><em>Manduca sexta</em></td>
<td>Egf1.0, Egf1.5</td>
<td>Virally encoded inhibitors of proPO-activating proteins 1.2 (Beck &amp; Strand, 2007; Lu et al., 2008, 2019).</td>
</tr>
<tr>
<td><em>Nasonia vitripennis</em></td>
<td><em>Musca domestica</em></td>
<td>NvSPPI</td>
<td>Small pacifastin-type protease inhibitor that prevents host proPO-activation (Qian et al., 2017).</td>
</tr>
<tr>
<td><em>Nasonia vitripennis</em></td>
<td><em>Musca domestica</em></td>
<td>NvKSPI-1,2</td>
<td>Two Kazal type inhibitors preventing proPO-activation (Qian et al., 2015).</td>
</tr>
<tr>
<td><em>Nasonia vitripennis</em></td>
<td><em>Boettcherica pergireza</em></td>
<td>nasonin-3</td>
<td>Defensin-like peptide that inhibits PO-activity (Yian et al., 2019).</td>
</tr>
<tr>
<td><em>Nosema bombycis</em></td>
<td><em>Bombby mori</em></td>
<td>Serpin 6 (NbSPN6)</td>
<td>NBSNP6 will target the prophenoloxidase activating enzyme (Bao et al., 2019).</td>
</tr>
<tr>
<td><em>Photobahdus luminosca</em></td>
<td><em>Manduca sexta</em></td>
<td>(E)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene</td>
<td>Secreted hydroxystilbene compound with capacity to inhibit activated PO (Eleftherianos et al., 2007).</td>
</tr>
<tr>
<td><em>Photobahdus temperata</em></td>
<td><em>Galleria mellonella</em></td>
<td>Pthilic acid</td>
<td>Inhibits PO activity and nodule formation (Ullah et al., 2014).</td>
</tr>
<tr>
<td><em>Pseudonomas arragina</em></td>
<td>*Elastase B (IasB)</td>
<td></td>
<td>Infections by als30 mutants are causing much higher PO activities than infections by the wild type (Ma et al., 2019).</td>
</tr>
<tr>
<td><em>Sclerodermus guani</em></td>
<td><em>Tenebrion molitor</em></td>
<td>SgusaSPh</td>
<td>A serine protease homologue interfering with host proPO-activation (Wu et al., 2020).</td>
</tr>
<tr>
<td><em>Sclerodermus guani</em></td>
<td><em>Ostrinia furnacalis</em></td>
<td>Venom superoxide</td>
<td>One parasitoid superoxide, SgusSOD3, suppresses host melanisation as demonstrated with dismutase recombinant protein (Lu et al., 2019).</td>
</tr>
<tr>
<td><em>Venturia canecens</em></td>
<td><em>Lepidoptera</em></td>
<td>Serine protease</td>
<td>Endoparasitoids inhibits melanisation in calyx fluid (Beck et al., 2000).</td>
</tr>
<tr>
<td>White spot syndrome virus</td>
<td><em>Penasus monodon</em></td>
<td>WSSV164 protein</td>
<td>The viral protein suppresses PmPO2 and PmPO2 activities (Sangsuriya et al., 2018).</td>
</tr>
<tr>
<td>White spot syndrome virus</td>
<td><em>Penasus monodon</em></td>
<td>WSSV28 protein</td>
<td>VP 28 Interacts with a host clip serine protease, PceSP, leading to a compromised melanization response (Guo et al., 2017).</td>
</tr>
<tr>
<td>White spot syndrome virus</td>
<td><em>Penasus monodon</em></td>
<td>WSSV453 protein</td>
<td>Inhibits proPO-activation by preventing PAPAE2 to become enzymatically active and thereby activate both PmPO1 and PmPO2. WSSV453 does not interfere with already activated PAPAE2 (Suthangkul et al., 2015, 2017).</td>
</tr>
<tr>
<td><em>Wigglesworthia</em></td>
<td><em>Drosophila melanogaster</em>, <em>Glosina moritana</em>, <em>perhaps bacterial odorsants</em></td>
<td>unknown,</td>
<td>Obp expression indirectly regulate melanisation via lounge expression and hematopoiesis of crystal cells, a major proPO source (Besoit et al., 2017). Products of the gene cluster are secreted and will inhibit PO activity (Nunez-Valdez et al., 2019, Crawford et al., 2012).</td>
</tr>
</tbody>
</table>
inhibitor. In *M. sexta* serpins 1 J, 3, 4, 6 are all reported to inhibit different proPO-cascade proteases (reviewed in Kanost et al., 2004). *M. sexta* serpin 12 is an inhibitor of HP14 and thereby both proPO and Toll activation (Wang et al., 2020a) and serpin 7 an inhibitor of the PAP-3 in the same species (Suvanchaichinda et al., 2013). A large number of other serpins are known to inhibit melanization in many insect and crustacean species, e.g. serpin 32 in *B. mori* (Wang et al., 2019) and many other (reviewed in Meekins et al., 2017; Tassanakajon et al., 2018). Other types of proteinase inhibitors besides conventional serpins also regulate melanization, an interesting example is the crustacean high molecular weight inhibitor pacifastin, founding member of a currently characterised group of proPO activating enzymes (Liang et al., 1997). Pacifastins, characterised by multiple domains possessing six cysteine residues in a conserved pattern (Breugelmans et al., 2009) are present widely in arthropods and some other phyla as well although less in known about their possible function in immunity except for crustaceans. One functionally and structurally characterised insect pacifastin is *Nosema vitripennis* small pacifastin protease inhibitor, NvSSP1 (Qian et al., 2017). NvSSP1 is present in the venom of this parasitic wasp and becomes injected into the insect hosts whereupon it inhibits proPO-activation and thus aid parasite establishment (Table 1).

### 5.2. Melanization inhibition proteins

Both in insects (Zhao et al., 2005) and in crustaceans (Söderhäll et al., 2009) melanization inhibition proteins (MIPs) without any apparent homology to serpins or other proteinases have been characterised. These proteins negatively regulate the melanization reactions by reactions so far uncharacterised. Both proteins contain an Asp-rich domain that by mutational analysis in the crayfish *P. leniusculus* was found to be necessary for its inhibitory activity (Söderhäll et al., 2009). A recombinant shrimp MIP was able to inhibit PO-activity in vitro using crude hemolymph as source of proPO and LPS (Noothuan et al., 2017). Possibly MIPs or other so far uncharacterised factors may prevent the formation of reactive quinones without restricting factors with other immunologic activities to arise from the proPO protein.

### 5.3. Amyloid proteins

Amyloid proteins are well known to spatially regulate the deposition of melanin in vertebrate melanosomes (reviewed in Bissig et al., 2016) but have so far received a relatively restricted attention in invertebrate melanogenesis. However, a protein apparently homologous to p102 is known to participate in the melanization reaction of the morula cells in the ascidian *Botryllus schlosseri* (Franchi et al., 2018). A finding by Di Prisco et al. (2016) is suggestive of a role for an amyloid protein in arthropod melanization as well. Deformed wing virus is reducing the capacity of melanotic encapsulation in honey bee, after the virus was transferred there by its symbiont the bee parasitic mite *Varroa destructor*. The virus facilitates mite survival by suppressing the bee immunity measured as melanization and encapsulation capacities. The authors observed that Amel1:102, a transcript related to similar proteins found associated with melanin in both insects (Falabella et al., 2012; Di Lelio et al., 2014) and ascidians (Franchi et al., 2019), was downregulated in the presence of the virus. Lower levels of Amel1:102 was accompanied by a reduced capacity to encapsulate and melanize foreign objects (Di Prisco et al., 2016).

### 6. Immunologic effects of proPO gene products

An increasing amount of data is demonstrating the importance of the proPO-system in host defence against a variety of pathogens. Consequently, a large number of host mechanisms to avoid the effects of this system has evolved during numerous host-pathogens encounters. Table 1 contains more than 25 compounds produced by different invertebrate pathogens to counter the effects of the melanization cascade. Its diverse content, ranging from viruses, bacteria, fungi, parasites with respect to pathogens producing them and the variety of molecules employed by the pathogens ranging from low molecular weight compounds to microRNAs and proteins is a good illustration of the immunologic potential of the proPO-system.

#### 6.1. Effects against bacteria and parasites

An active PO will produce toxic intermediates and short-lived radicals (Nappi and Christensen, 2005). The melanin itself, although chemically inert, is shielding the pathogen or the animals’ own damaged tissues and creates a physical and perhaps a chemical barrier to the rest of the host tissues. Early knock-down experiments directed towards proPO genes by the use of RNAi showed that in crayfish proPO expression contributed significantly to the resistance of the animals against *Aeromonas hydrophila* (Liu et al., 2007) and similar experiments in shrimp demonstrated increased resistance towards *Vibrio harveyi* (Ampanyup et al., 2009). In an insect, *Manduca sexta*, a pathogenic bacterium, *Photorhabdus luminescens*, was shown to specifically target the proPO-system by secreted hydroxythibilenes in order to establish an infection (Eleftherianos et al., 2007) and another *Photorhabdus* strain was demonstrated to make phthalic acid, a compound with capacity to inhibit PO-activity (Ullah et al., 2014). As shown in Table 1 several other pathogenic bacteria have evolved means to interfere with the host proPO-system; *Xenorhabdus nematophila* and the above-mentioned *P. luminescens* are producing rhabduscin and related products capable of inhibiting PO (Nunez-Valdez et al., 2019; Crawford et al., 2012). A *Pseudomonas aeruginosa* strain pathogenic on silk worm is synthesising an elastase whose experimental deletion is causing much higher PO-activities and reduced virulence in the insect (Ma et al., 2019).

In the mosquito *Anopheles gambiae* there are 9 PPO genes making it more challenging to ascertain the role of POs in immunity. Silencing PPO2, PPO3 or PPO9 individually each increased the survival of the *Plasmodium berghei* oocysts (Kwon and Smith, 2019). The PPOs seem not to effect infection rates but more long term survival as no effects on parasite numbers were seen 2 days post infection but was evident 8 days post infection. Silencing of PPO5,6 or 8 was without effect. However, targeting some of the PPO genes had side effects on the expression of some of the PPO genes making it difficult to interpret the contribution of each PPO gene in resistance in each single case. More recently, analyses of mutants in *Drosophila melanogaster* have significantly broadened our understanding on how POs contribute to resistance towards both bacteria and parasites. In the fly three genes, PPO1,2,3, each has specific but often overlapping roles in the immunity. A double mutant defective for both PPO1 and 2 is more susceptible towards a *Staphylococcus aureus* infection than each single PPO null mutant (Dudzic et al., 2019). A triple PPO1-3 mutant was more susceptible to infections by the nematode *Steinernema carpocapsae* harbouring its symbiotic bacterium *Xenorhabdus nematophila* than any double or single PPO mutant (Cooper et al., 2019). However, when using axenic nematodes a compensatory activity by remaining PPOs were often seen in single and double PPO mutants.

#### 6.2. Antifungal effects

Melanization seems to be a general and effective weapon against pathogenic fungi in arthropods such as insects (for a treatise of the ecological implications the reader is referred to Gonzalez-Santoyo and Cordoba-Aguilar, 2012). Also, pathogenic oomycotan parasites, a group of organisms with a fungal-like life style may be effectively controlled by the melanization reaction as demonstrated in a comparison by parasite-resistant and susceptible freshwater crayfish species (*Söderhäll and Axjon, 1982; Cerenius et al., 2003*). In the mosquito *A. gambiae* will knock-down of TEP1 and CLIP8 genes, key regulators of the melanization response in this insect, greatly facilitate the establishment of a
Beauveria bassiana infection, an important fungal pathogen on insects and a potential biocontrol agent (Yassine et al., 2012). In a related mosquito species this pathogen was recently demonstrated to affect the Toll response and the melanization reaction via silencing RNA that was released via exosomes (microvesicles) from the pathogen to be taken up by host cells (Cui et al., 2019). This specialised pathogen controls timing of the silencing RNA in order not to stimulate synthesis of proPO activating enzyme, CLIPBP9, in the hemocoeel of the insect. In the mosquito A. aegypti an infection with B. bassiana results in increased expression of the PPO3 gene (Wang et al., 2015). Wang et al. (2017) followed up this finding by injecting recombinant A. aegypti PPO3 protein into the hemocoeel of the mosquito, a treatment that was found to increase the survival of fungus-infected animals. In D. melanogaster fungi can trigger a melanization reaction that appears effective against several types of fungi (Matkevich et al., 2010) and the double PO1/PO2 mutant mentioned above was found to be susceptible towards four different tested fungi (Binggeli et al., 2014). Several specialised pathogenic fungi have developed means to specifically interfere with the proPO-system and are thereby preventing the host to launch a fully effective immune response. Several examples of hormone derived compounds used by parasitic fungi and other pathogens to interfere with proPO-activation or PO-activity are listed in Table 1. One example of such an interference is demonstrated by the specialised entomopathogenic fungus, Metharizium robertsi that secretes a family of apparently dedicated metalloproteinases with capacity to proteolytically degrade insect proPO in a way that does not result in activation of the proPO (Huang et al., 2020). When a number of Isaria species, differing in pathogenicity towards A. aegypti mosquitoes, were tested with respect to PO activities and PO gene expression the most pathogenic fungi were able to reduce PO-activities without apparent effects on gene expression (Ramirez et al., 2018). The mechanism for this fungal inhibition of the mosquito defence is not known but some other fungal pathogens such as B. bassiana have been shown both to synthesise effective PO-inhibitors such as the bibenzoquinone oosporein (Feng et al., 2015) and to produce a microRNA with capacity to prevent Toll activation and modulate proPO-activation (Cui et al., 2019) whereas Aspergillus niger is making cell wall components that are binding the proPO-activating complex (Staczeok et al., 2020).

6.3. Antiviral effects

Although the precise mechanism(s) remains to establish the melanization reaction has strong effects against some viruses e.g. against several DNA and RNA viruses in the tobacco budworm (Ourth and Renis, 1993; Shelley and Popham, 2006), against arboviruses in mosquitoes (Rodriguez-Andres et al., 2012) and baculovirus in cotton bollworm (Yuan et al., 2008; Wang et al., 2020b). One possible mediator of this antiviral effect is the melanization intermediate 5,6-hydroxyindole as suggested by experiments performed in vitro with baculovirus and lambda phages (Zhao et al., 2011). A growing list of examples of viruses interfering with the proPO-system has emerged during the last years (Table 1) lending credit to the view that the system has antiviral capacity. An early example of this is the bracovirus-encoded Egf1.0 protein, an inhibitor of PAP1 and 2 in Manduca sexta (Lu et al., 2008). Several viruses prevent PO activation by interfering with host PO-activating proteinases (Table 1). In crustaceans the devastating white spot syndrome virus (WSSV) is suppressing PO-activity in shrimp (Suthangkul et al., 2015) and crayfish (Jiravanichpaisal et al., 2006). Two viral proteins were found to interact with the proPO-system, WSSV-G53 to the Pro-PO activating enzyme PmPAPAE2 thereby preventing PO-activation (Suthangkul et al., 2017) and WSSV164 which is reducing PO-activity by binding to both PO1 and PO2 (Sangsuriya et al., 2018). In crayfish a clip domain containing serine proteinase was found to bind to WSSV through VP28 and it was shown that this proteinase was necessary for WSSV internalisation to the hematopoietic cells (Guo et al., 2017). The WSSV is also targeting gene expression of another protease in the proPO-cascade, PmPAPAE3, via up-regulating a host microRNA, miR-315 (Jaree et al., 2018). It seems very likely that once investigated many further examples of viruses interfering with the host proPO-system will be detected. In silk worm (Bombyx mori) nucleopolyhedrovirus infectivity is increased by knock-downs of host serpin-2, an inhibitor of the proteolytic activation of proPO in this insect (Toufeeq et al., 2019). In control silk worms serpin-2 gene expression is enhanced by the nucleopolyhedrovirus leading to a suppressed melanization in this insect (Toufeeq et al., 2019). Another virus that actively prevents melanization is boilworm, Helicoverpa armigera, nucleopolyhedrovirus that was found to reduce host PPO2 expression by more than 18-fold (Xing et al., 2017) strongly suggesting that this gene is of crucial importance in anti-viral defence.

6.4. Effects in the gut and outside the body

The importance of the PO-activity stretches beyond the boundaries of the body itself; the melanization reaction helps to disinfect faeces and thereby the plant matter upon which the animal feeds as exemplified in PO residing in gut in several insect species (Zhao et al., 2012). The moulting fluids and the exuviae may contain PO and other proPO-system components to protect the insect from outside attack when its outer tissues are as most vulnerable during and shortly after ecysis (Zhang et al., 2014, 2017). Another example is detoxification of harmful plant phenolics by PO in the gut (Wu et al., 2015). Their importance was demonstrated by the fact that insects with PO deletion mutants were killed by plant phenolics in the feed (Wu et al., 2015). An interesting use of PO was recently demonstrated in the social aphid Nipponaphis mornzeni. This insect forms large galls on its plant host containing large numbers of the aphid. If the gall is broken specialised soldiers from the aphid colony seal the injured gall by releasing large volumes of body fluid that quickly coagulate and seal the injury. The insect has undergone large adaptations to achieve this remarkable process, its soldiers body cavity possesses large globular cells highly enriched in PO, the PO substrate tyrosine and a unique repeat containing protein (RCP) obviously serving as a scaffold in the clotting/melanization reaction (Kutsukake et al., 2020). It deserves consideration as hemolymph in other insects, e.g. fruit flies, after coagulation or clotting the clot becomes melanized although other proteins than the RCP such as fondue is involved in this case (Lindgren et al., 2008; Theopold et al., 2004).
corresponding to the proPO N-terminal part in *P. leniusculus* as produced by ppA cleavage, exhibits a strong agglutinating activity towards both Gram positive and negative bacteria and experiments with *E. coli* demonstrated that this peptide possesses a bactericidal activity and is causing cell wall damage (Jearaphunt et al., 2014). It is not known whether this peptide as vertebrate complement has a membrane perturbing effect as well. More recent data show that the proPO N-terminal has cytokine activities being active in inducing hemocyte differentiation as measured in vitro is far too slow for likely being of physiological importance, but the possibility of a proteinase activity inherent with the PO deserves further studies.

It was observed in several of the investigations described earlier (Dudzic et al., 2019; Kwon and Smith, 2019) that although the proPO-gene was required to achieve an immunological effect (as suggested by data obtained from null mutants of proPO-genes), its melanin formation is not necessarily required for the immune effect. In such cases other effects by parts of the PO protein or MIP activities towards the active enzyme may provide an explanation. Regardless how these effects beyond melanization were produced one should not overlook the fact that although the melanization reaction itself confers an immune effect other factors or, indeed, parts of the proPO molecule and other molecules of the proPO-system can give rise to important immune factors.

8. Conclusions

Melanin production from different types of phenoloxidases confer immunity from a variety of pathogens ranging from viruses and microorganisms to parasites.

The arthropod (insect and crustacean) phenoloxidase expresses a variety of activities including such influencing hemocyte proliferation and differentiation, opsonins and microbiocidal activities independent of and even without melanin production. Proteolytic processing of phenoloxidase and its activating enzyme gives rise to several peptide fragments with a variety of separate activities in a process highly reminiscent of vertebrate complement system activation in spite of being structurally unrelated to complement.

Pathogens influence phenoloxidase activation and thereby what types of immune effects that will be produced. An increasing number of specialised pathogens – from parasites to viruses – have been identified who can synthesise compounds specifically aimed at the phenoloxidase system.

In other invertebrates outside the arthropods phylogenetically unrelated phenoloxidases are participating in melanization reactions obviously aimed at intruders and/or aberrant tissues.  

Fig. 2. The phenoloxidase system as deciphered in crustaceans. In blue different biological activities generated by the system and in red factors that inhibit activation or the activity of phenoloxidase. Pattern recognition proteins (PRRs) will initiate a serine proteinase cascade that generates among other factors phenoloxidase (PO), peroxinectin, cytokine-like factors and proPO fragments with strong agglutinating capacities. AMP, antimicrobial peptide; MIP, melanization inhibiting protein; ppA, phenoloxidase activating enzyme; PAMP, pathogen-associated molecular patterns.


Soderhimer, K., Röger, W., Soderhimer, I., Newton, R., Ratcliffe, N.A., 1988. The properties and purification of a BmNPV inhibitor of proteolytic protein which enhances the activation of haemocyte prophenoloxidase by a β-1,3 glucan. Insect Biochem. 18, 323–330.