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Is activated hemocyanin instead of phenoloxidase involved in immune response in woodlice?

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ABSTRACT

In the Common woodlouse *Porcellio scaber* (Crustacea: Isopoda: Oniscidea), experimental immune challenge did not induce the expression of pro-phenoloxidase that, in most other invertebrates studied thus far, can be activated into phenoloxidase via an activation cascade upon immune challenge. Instead, *Porcellio* hemocyanin proved to exhibit catecholoxidase activity upon activation. However, none of the activating factors known from other invertebrates other than SDS-treatment resulted in activation of hemocyanin into a functional phenoloxidase *in vitro*. The distinct characteristics of isopod hemocyanin are reflected by the quaternary structure of the hemocyanin dodecamers that differs from that of other crustacean hemocyanins in that the two hexamers share a common 3-fold rotation axis and have an angular offset of 60° against each other. Accordingly, the sequence of *Porcellio* hemocyanin can be distinguished clearly from other crustacean hemocyanins and in a phylogenetic analysis forms a cluster with other isopod and amphipod hemocyanins. We propose a peracarid-type hemocyanin that may have evolved in response to its required multiple functions in respiration and immune response, while phenoloxidase *sensu strictu* is lacking.

1. Introduction

The immune response of arthropods comprises both cellular encapsulation and phagocytosis of pathogens by hemocytes and melanisation through the action of phenoloxidases [1–3]. Phenoloxidases are type-3-copper proteins that catalyse the o-hydroxylation of monophenols and the subsequent oxidation of the resulting o-diphenols to o-quinones [4]. The o-quinones produced by phenoloxidase polymerise non-enzymatically and form the brown pigment melanin by which pathogens are encapsulated [5,6]. Crystal structures of phenoloxidases, namely catecholoxidase and tyrosinase, are known, and based on these structural studies mechanistic models for substrate turnover have been proposed [7–9]. Phenoloxidases are expressed as pro-phenoloxidases, and their activity is tightly controlled by the phenoloxidase cascade, serving as a sensitive detection system for pathogens [10,11].

Phenoloxidases are generally found in insects and crustaceans, but there exist two groups of arthropods, namely chelicerates and isopods, which seem to lack phenoloxidase. In chelicerates, no phenoloxidase has been reported yet, and even though hemocytes from a tarantula were specifically examined for the presence of immune-related gene transcripts, no phenoloxidase was found [12–14]. While phenoloxidase is apparently lacking in two marine isopods, *Glyptonotus antarcticus* (Valvifera: Chaetiliidae) and *Bathynomus giganteus* (Cymothoida: Cirolanidae) [15,16], artificial pathogens are melanised – albeit weakly – in the terrestrial isopod *Porcellio scaber* [17].

The hemolymph of crustaceans and chelicerates contains hemocyanin as oxygen carrier, which is closely related to phenoloxidase [12,13,18]. In several different taxa, hemocyanin has been demonstrated to exert phenoloxidase activity upon activation [19,20]. In the chelicerate *Tachypleus tridentatus*, which lacks a phenoloxidase, it has been shown that hemocyanin can be activated by either clotting enzyme or antimicrobial peptides and thus seems to be the "functional phenoloxidase" of these animals [21,22]. Several activation mechanisms have been reported *in vitro* [19]. *In vivo* it seems that activation of hemocyanin is achieved by a conformational change: clotting enzyme, although being a protease, does not proteolyse hemocyanin, but activates it by forming a complex [21]. The activating conformational change in hemocyanin can be mimicked *in vitro* by SDS, which has been used as a standard activator for phenoloxidase *in vitro* for decades [23–26].

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Given that isopods and chelicerates follow the general body plan of arthropods, it seems unlikely that they can live without a phenoloxidase, which is necessary for very basic functions as in the primary immune response, wound-healing, cuticle sclerotisation or pigmentation.

With the present study, we aim at (1) elucidating whether phenoloxidase activity exists in the hemolymph of terrestrial isopods and at (2) identifying whether phenoloxidase or hemocyanin is responsible for phenoloxidase activity in these animals.

2. Materials and methods

2.1. Animals

For comparison of the Common woodlouse *P. scaber* with well-understood model organisms, we chose the American lobster and the House cricket. Woodlice (*P. scaber*, Crustacea: Isopoda: Oniscidea: Porcellionidae) were collected on the university campus in Kiel. They were kept in the laboratory at 20 °C (10 h L:14 h D) and were fed with a mix of leaf litter taken from the collecting sites. American lobsters (*Homarus americanus*, Crustacea: Decapoda: Homaridae) were obtained from a local seafood dealer and were kept in a seawater aquarium for a maximum of 2 days prior to experimental stimulation of the immune system. House crickets (*Acheta domesticus*, Insecta: Ensifera: Gryllidae) were obtained from a local pet shop.

2.2. Stimulation of the immune system

Several different immune stimuli have been used in studies of arthropod immune response. The aim of immune stimulation in the present study was to trigger melanisation of an (artificial) macro-pathogen through phenoloxidation. While there is no correlation between any particular experimental immune challenge and activation of hemocyanin into a functional phenoloxidase (if any), colourless nylon filaments have successfully been used to prompt melanisation in previous studies [17]. Thus, this approach was chosen herein, too.

American lobster (*H. americanus*) were infected with an artificial pathogen (colourless nylon filaments [17]) that was inserted dorsally between the 2nd and 3rd pleon segment. After 6 h, the artificial pathogen was removed and hemolymph was collected from the pleopodal base of the first abdominal segment using a syringe. The hemolymph was immediately dissolved in anticoagulant buffer (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES, pH 7.3) and centrifuged at 2000 × *g* for 20 min at 4 °C to remove hemocytes [35]. The supernatant was discarded and the cell pellet was homogenised in Trizol (Invitrogen) for RNA extraction (see below).

To stimulate the immune response of *P. scaber* and *A. domesticus*, artificial pathogens (colourless nylon filaments [17]) were implanted, dorsally between the 4th and 5th pereion segments and the 2nd and 3rd abdominal segments, respectively. Six hours after implantation of the nylon filaments, hemolymph was obtained by puncturing the intersegmental cuticle and collecting ca. 3 μL of the issuing hemolymph per animal with a glass capillary. The hemolymph was immediately dissolved in anticoagulant solution, and hemocytes were separated from the cell-free hemolymph plasma by centrifugation at 10,000 × *g* for 20 min at 4 °C.

2.3. Enzymatic assays

Catecholoxidase activity of *P. scaber* and *A. domesticus* hemolymph was measured by absorption spectroscopy at 20 °C. In all experiments, the diphenol dopamine was used as substrate and the formation of the reddish pigment dopachrome, which non-enzymatically forms from the reaction product dopaquinon, was followed at 475 nm [36]. In the standard assay, hemolymph was diluted at a ratio of 1:23 with a 4 mM dopamine solution in 40 mM HEPES (pH 6.5). The absorbance change at 475 nm was monitored immediately afterwards for 1 min. Often pro-phenoloxidase, but also hemocyanin, needs activation before catecholoxidase activity can be observed [19,37]. *In vitro* activation of catecholoxidase activity in hemolymph was tested by four methods:

2.3.1. Activation by SDS

Activation by the detergent SDS, which is used as a standard activator compound for phenoloxidases, was achieved by adding 2.1 mM SDS to the HEPES buffer of the standard assay [26,38,39]. This resulted in a final concentration of 2.0 mM SDS in the reaction mixture [17].

2.3.2. Activation by protease

In crustaceans, pro-phenoloxidase is activated in the phenoloxidase cascade by serine proteases [37]. This mode of activation was mimicked by addition of 1 mg chymotrypsin (≥40 U/mg, Sigma–Aldrich) to 40 mM HEPES buffer (pH 6.5). Hemolymph was diluted 1:23 with this solution and incubated for 5 min at room temperature. Then the reaction was initiated by adding dopamine to a final concentration of 4 mM.

2.3.3. Activation by pH-shift

According to previous findings, induction of catecholoxidase activity by a conformational change caused by a pH change of the buffer was assayed [25]. To this end, pH of the HEPES buffer of the standard assay was varied in the range from 6.5 to 8.5 in steps of 0.5 pH-units. The average pH of *Porcellio* hemolymph *in vivo* is 7.5 [40].

2.3.4. Activation by hemocyte components

Crustacean hemocyanin can be activated into a functional phenoloxidase by hemocyte components [35]. Thus, we obtained hemocyte components dissolved in 10 mM Tris–HCl by following [35]. In brief, 40 μL isopod hemolymph (pooled from 15 individuals) were mixed with 10 μL of cooled (4 °C) anti-coagulation buffer (see above) and centrifuged at 300 × *g* (4 °C) for 25 min. After 3-times washing with anti-coagulation buffer and subsequent centrifugation, the pellet was sonicated in a 5 mM $CaCl_2$/$MgCl_2$ solution in 10 mM Tris–HCl buffer, pH 7.8. After centrifugation at 12,000 × *g* for 45 min, supernatant and pellet were used separately to activate PO activity in untreated isopod hemolymph. To this end, hemolymph was diluted 1:16 with buffer (10 mM Tris–HCl, pH 7.8) containing 4 mM dopamine. This mixture was diluted further at a ratio of 1:1.15 with either water (control for hemolymph catecholoxidase activity), supernatant or pellet of the hemocyte extraction. Immediately after mixing, the absorption change at 475 nm was monitored for 1 min. As a further control, pellets of the hemocyte extraction were assayed in the absence of hemolymph.

2.4. Protein purification

Porcellio hemolymph from 15 animals (see above) was pooled, diluted in 500 μL stabilisation buffer (100 mM Tris–HCl, 20 mM $CaCl_2$, 20 mM $MgCl_2$, pH 7.5), and hemocytes and cellular debris were removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. Then 300 μL of the diluted hemolymph were applied to a Superose 6 10/300 GL SEC column (GE Healthcare, Piscataway, NJ, USA) connected to a Biologic Duoflow FPLC system (Bio-Rad, Munich, Germany). Proteins were eluted with stabilisation buffer at 20 °C at a flow rate of 0.5 mL/min. After eluting from the column, protein samples were concentrated in centrifugal filters (Biomax 30K; Millipore, Eschborn, Germany) when necessary.