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Characterization of hemolymph phenoloxidase activity in two *Biomphalaria* snail species and impact of *Schistosoma mansoni* infection

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Abstract

Background: *Biomphalaria* snails are the intermediate host of the blood fluke *Schistosoma mansoni*, which infect more than 67 million people in tropical areas. Phenoloxidase enzymes (POs), including tyrosinases, catecholases, and laccases, are known to play a role in the immune defenses of arthropods, but the PO activity present in *Biomphalaria* spp. hemolymph has not been characterized. This study was designed to characterize substrate specificity and reaction optima of PO activity in *Biomphalaria* spp. hemolymph as a starting point to understand the role of this important invertebrate enzyme activity in snail biology and snail-schistosome interactions.

Methods: We used spectrophotometric assays with 3 specific substrates (L-tyrosine for tyrosinase, L-DOPA for catecholase, and PPD for laccase) and diethylthiocarbamate (DETC) as specific PO inhibitor to characterize PO activity in the hemolymph of uninfected snails from two *Biomphalaria* species, and to determine the impact of the parasite *Schistosoma mansoni* on the PO activity of its *B. glabrata* vector.

Results: We identified laccase activity in hemolymph from uninfected *B. glabrata* and *B. alexandrina*. For both species, the activity was optimal at 45 °C and pH 8.5, and located in the plasma. The K_m and V_{max} of PO enzymes are 1.45 mM and 0.024 OD.min⁻¹ for *B. glabrata*, and 1.19 mM and 0.025 OD.min⁻¹ for *B. alexandrina*. When the snail vector is parasitized by *S. mansoni*, we observed a sharp reduction in laccase activity seven weeks after snail infection.

Conclusions: We employed a highly specific spectrophotometric assay using PPD substrate which allows accurate measurement of laccase activity in *Biomphalaria* spp. hemolymph. We also demonstrated a strong impact of the parasite *S. mansoni* on laccase activity in the snail host.

Keywords: *Biomphalaria*, Phenoloxidase activity, Laccase, Hemolymph, Spectrophotometric assay, *Schistosoma mansoni*

Background

Aquatic snails of the *Biomphalaria* genus are the intermediate hosts of the blood fluke *Schistosoma mansoni* [1, 2], trematodes that infect 67 million people in Africa and South America [3–5]. When parasite eggs are expelled with human faeces in water, miracidia larvae hatch and actively search for its snail vector. Larvae penetrate the snail head-foot, differentiate into primary sporocysts and then asexually proliferate to generate secondary sporocysts. After approximately a month of infection,

secondary sporocysts release the first cercariae, the human infective larval stage of the parasite, through the body of the snail, into the water. The *Biomphalaria* immune response is mounted both by cellular effectors (*i.e.* via the hemocytes, [6–10]) and humoral factors (for example FREPs [11, 12], SOD1 [13], Biomphalasin β -PFT [14], and lectins [15]). Many snail humoral factors have been carefully characterized in the *Biomphalaria* genus to identify the resistance mechanisms to schistosome infection. However no clear characterization of the phenoloxidase (PO) activity of the *Biomphalaria* snail hemolymph was attempted. This is surprising because PO activity is considered to be an important component of

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the humoral response [16] and an immunocompetence parameter in many arthropods [17–19]. The central aim of this work is to characterize the specific PO activity present in *Biomphalaria* hemolymph: this is an essential prerequisite for studies aiming to understand the role of PO activity in snail biology.

PO enzymes play a key role in wound healing [16], tissue pigmentation [20, 21], and reproductive process [22–25]. They are also involved in innate immune defense against intruding pathogens, being the last component of a reaction cascade called the “proPO activating system” [16, 26]. This cascade is triggered when pathogen molecules are detected and stimulate the activation of proPO enzymes into PO enzymes through the action of serine proteases. The active POs then convert phenolic or amine compounds in dopachrome and then melanin, which have cytotoxic activities damaging pathogen cells [16].

PO enzymes are copper-containing enzymes [27] and fall into three groups defined by their substrate specificity: (i) tyrosinases catalyze hydroxylation of monophenols and oxidation of *o*-diphenols, (ii) catecholases oxidize *o*-diphenols and (iii) laccases oxidize *o*-diphenols, *p*-diphenol and *p*-diamines [28]. Invertebrates possess the 3 different PO activities in various tissues [29–31], unlike vertebrates where only the tyrosinase activity is present [32]. Invertebrate PO activity can be easily measured and characterized in vitro using specific substrates. Assays conducted in the presence of exogenous serine proteases (like trypsin enzymes) measure all the PO activity present in an individual (total PO activity), while assays conducted in the absence of exogenous serine proteases measure the PO activity that can be activated during infection (intrinsic PO activity). Among all the substrates used to measure PO activity, L-DOPA (*o*-diphenol) is the most frequently used because it is non-specific and therefore does not require any prior knowledge about the enzyme involved in PO activity [28]. However, this substrate has multiple disadvantages because (i) its non-specific nature may lead to low oxidation efficiency and inaccurate measurement [28], (ii) it can be metabolized by other enzymes such as peroxidases [33, 34] and (iii) is highly susceptible to auto-oxidation [35].

Several studies have examined PO activity in pulmonate snails, but importantly all of these studies used L-DOPA, rather than specific substrates, and have therefore provided ambiguous results. For example, one study on *Lymnaea stagnalis* [36] suggests that peroxidase activity rather than PO activity is responsible for L-DOPA oxidation but suffers from methodological limitations, because PO and peroxidase activity cannot be distinguished. In this study the failure to detect PO inhibition may be due to (i) the inefficacy of the unique PO inhibitor used (phenylthiourea) and (ii) an unusually short time to dopachrome formation measurement for invertebrate hemolymph (5 min). In

another study on *Lymnaea*, Seppälä and Leicht [37] quantified PO-like activity but did not use a specific inhibitor to verify that the activity observed was due to oxidation of L-DOPA by PO enzymes. A study on *B. glabrata* snails [38] quantified PO activity in hemocytes six hours after adding L-DOPA, but without an L-DOPA auto-oxidation control, making it difficult to conclude true PO activity rather than substrate auto-oxidation. As a consequence, specific PO activity (tyrosinase, catecholase or laccase) remains poorly understood in snail hemolymph, and studies using specific substrates are required. Moreover, while infection with *S. mansoni* intra-molluscan stages has a negative impact on the tyrosinase activity in the *B. glabrata* albumen gland [39], nothing is known about the effects of the parasite on the PO activity in the snail hemolymph, a tissue in intimate contact with *S. mansoni* larvae.

To fill this knowledge gap, we characterized PO activity in the hemolymph of uninfected *Biomphalaria* spp. (*B. glabrata* and *B. alexandrina*) using three different specific substrates (L-tyrosine, L-DOPA and *p*-phenylenediamine (PPD)) and employed a specific and accurate assay to measure this activity. We then determined general characteristics of PO activity, including the temperature and pH optima, the exact location of action, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}). Finally, we measured the impact of *S. mansoni* infection on PO activity of the *B. glabrata*'s hemolymph over the pre-patent period (during 4 weeks after the exposure to the parasite, when primary sporocysts grow and produce several generations of secondary sporocysts in the snail tissues) and 5 weeks over the patent period (when secondary sporocysts produce cercariae released from the snail).

Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Biomedical Research Institute (permit number: 1419-MA-0).

Maintenance of snails *Biomphalaria glabrata* and *alexandrina*

We characterized phenoloxidase (PO) activity in the hemolymph of two different *Biomphalaria* species, both intermediate hosts of *Schistosoma mansoni*. We used 385 inbred albino *B. glabrata* (line Bg26 derived from 13-16-R1 line [40]) and 185 outbred pigmented *Biomphalaria alexandrina* (from Theodor Bilharz Research Institute, Egypt) in the experiments presented.

Uninfected snails were reared in 10-gallon aquaria containing aerated freshwater at 26–28 °C on a 12 L-12D

photocycle and fed ad libitum on green leaf lettuce. All snails used in this study had a shell diameter between 10–14 mm.

Hemolymph collection on *Biomphalaria* spp. snails

We collected hemolymph immediately before assaying PO activity. An advantage of *Biomphalaria* snails is their large size and the flat shape of their shell allowing an easy access to the heart and the collection of ~100 μ L of hemolymph. For each individual snail, we disinfected the shell with 70 % ethanol, and we collected total hemolymph by heart puncture using a 1 mL syringe and a 22 gauge needle. The hemolymph collected was immediately placed in a 1.5 mL microtube on ice.

Characterization of phenoloxidase activity in the hemolymph of *Biomphalaria* spp.

Three specific substrates for PO enzymes were tested: (i) L-tyrosine (monophenol; Sigma) metabolized by tyrosinase only, (ii) L-DOPA (*o*-diphenol; Sigma) metabolized by catecholase, tyrosinase and laccase enzymes, and (iii) *p*-phenylenediamine (PPD; *p*-diamine; Sigma) only metabolized by laccase enzyme [41, 42].

We detected and characterized PO activity in the hemolymph of *Biomphalaria* spp. by measuring the optical density (OD) of the colour reaction product (*i.e.* dopachrome) formed by the oxidation of each substrate. The OD was measured using a spectrophotometer at $\lambda = 465$ nm when using PPD (according to maximum absorption of the product of PPD oxidation by the PO enzyme, Additional file 1: Figure S1) and at $\lambda = 490$ nm when using L-tyrosine and L-DOPA [43].

In each sample test well of a 96-well optical plate (Corning), we added 10 μ L of hemolymph to 40 μ L of cacodylate buffer (10 mM sodium cacodylate (Sigma) and 10 mM calcium chloride (Sigma) in distilled water, pH = 8.4). Each sample test was coupled to a control test where 10 μ L of the same hemolymph sample was added to 40 μ L of 10 mM diethylthiocarbamate (DETC (Sigma) in cacodylate buffer, pH = 8.4). DETC is known to be a specific inhibitor of PO enzymes [38, 39, 44]. A substrate auto-oxidation control was also performed, where the hemolymph sample was replaced by 10 μ L of distilled water. The values obtained for this control were automatically subtracted from the test and control wells values for each experiment.

In order to measure the total PO activity in the hemolymph, we added an exogenous protease (40 μ L of trypsin prepared at 1 mg.mL⁻¹ in distilled water) mimicking the action of the serine protease of the PO cascade to each well (test and control). Following the addition of trypsin, the assay was incubated 45 min at 37 °C (the optimal temperature for trypsin protease). Fresh substrates (L-tyrosine, L-DOPA and PPD) were prepared at 10 mM in cacodylate buffer ten minutes before usage

and 120 μ L of substrate were added to the wells followed immediately by a plate reading. We tested each sample with the 3 substrates. Dopachrome formation was spectrophotometrically monitored every 15 min for 6 h at 37 °C and $\lambda = 465$ nm and $\lambda = 490$ nm, using a SpectraMax M1 (Molecular Devices). Comparisons between substrates were done using absorbance values obtained 4 h after adding the substrates at 10 mM, before reaching the plateau phase of the PO activity.

Influence of the temperature and pH on the laccase activity in the hemolymph of *Biomphalaria* spp.

To assess the effect of temperature on laccase activity in the hemolymph of *Biomphalaria* spp., we conducted laccase assays as described above (using 50 mM PPD, substrate in large excess). We determined dopachrome formation every 15 min for 6 h using a temperature range from 30 to 60 °C at $\lambda = 465$ nm.

We also examined the effect of pH on laccase activity. We used the same assays to those described but using cacodylate buffers with a pH ranging from 4.5 to 12.5 and spectrophotometric monitoring (every 15 min for 6 h) at 45 °C (optimum temperature) and $\lambda = 465$ nm.

Determination of the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the laccase-like enzyme from the hemolymph

To determine the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the laccase-like enzyme in the *Biomphalaria* hemolymph, we conducted spectrophotometric assays as described above using a final PPD concentration range from 0.891 mM to 14.28 mM. We monitored the enzymatic kinetics for both snail species during every 15 min for 6 h at 45 °C (optimal temperature) and $\lambda = 465$ nm. K_m and V_{max} were obtained from the Hanes-Woolf equation, a linear transformation of the Michaelis-Menten equation [45] using the formula:

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}}$$

where [S] is the substrate concentration in mM, V and V_{max} are the velocity and the maximum velocity in OD.min⁻¹ respectively, and K_m the Michaelis constant in mM.

Location of the laccase activity in the hemolymph of *Biomphalaria* spp.

Hemolymph collected from the snail comprises two fractions: the humoral fraction (plasma) and the cellular fraction containing the immune cells (hemocytes). Laccase activity was determined and characterized in the complete hemolymph, as well as in plasma and hemocytes. We centrifuged the samples at 300 $\times g$ (at 4 °C for 5 min) to

separate hemolymph into the two fractions. To be sure that the hemocyte fraction did not contain plasma, the cells were washed 2 times ($300 \times g$ at 4°C for 5 min) in PBS (pH 8.5) then resuspended in the same volume of PBS. We checked for the presence of cells in the hemocyte fraction as well as the integrity of the cells (mean viability of the hemocytes \pm sd: $87 \pm 8\%$) using an automatic cell counter (BioRad) ($10\ \mu\text{L}$ of the cellular fraction combined with $30\ \mu\text{L}$ of trypan blue 0.4 % (Sigma)). We used the same approach to check for the absence of hemocytes in the plasma fraction.

We conducted spectrophotometric assays both with trypsin (total laccase activity) and without trypsin (intrinsic laccase activity), using whole hemolymph and the two fractions (plasma and hemocyte). Note that our assay for laccase activity uses 37°C rather than optimal 45°C because trypsin activity is optimal at 37°C and because the spectrophotometer works efficiently at this temperature, and 37°C allows comparisons with previous PO activity studies.

Impact of *S. mansoni* on *B. glabrata* laccase activity in the hemolymph, across the infection

To assess the impact of the *S. mansoni* parasite to its intermediate host *B. glabrata*, we infected 100 snails with *S. mansoni* parasites from SmLE population. A control experiment was performed at the same time, using 100 uninfected snails. The SmLE schistosome population was originally isolated from a patient in 1965 in Belo Horizonte (Minas Gerais, Brazil), and has since been maintained in the laboratory [46], using NMRI line *B. glabrata* as intermediate host and syrian golden hamster (*Mesocricetus auratus*) as definitive hosts. Miracidia were hatched from eggs recovered from 45-day-infected hamster livers. The livers were homogenized and the eggs were filtered, washed with normal saline ($154\ \text{mM}$ calcium chloride (Sigma), pH 7.5), transferred to a beaker containing freshwater, and exposed to artificial light to induce hatching. Snails were individually exposed to 10 miracidia (to maximize the number of infected snails) then maintained in trays for 9 weeks. We covered trays with a black plexiglass lid after 3 weeks to reduce cercarial shedding. Four weeks post-exposure and then once a week, we exposed infected snails to artificial light to induce cercarial shedding.

We sampled 10 infected and 10 control (uninfected) snails per week for hemolymph collection and assessment of infection status. We collected hemolymph from both infected and uninfected snails as described above (see Hemolymph collection on *Biomphalaria* spp. snails section). The total and intrinsic laccase activity (with and without trypsin protease, respectively) was then spectrophotometrically monitored as described. Time-lapse series were built using absorbance values obtained

2 h after adding the PPD substrate, before reaching the plateau phase of the laccase activity.

During the pre-patent period (*i.e.* the first four weeks before the first cercarial shedding), we assessed the infection status of each snail after hemolymph collection by fixing each snail in Railey-Henry solution [47] and then dissecting them to observe the presence of primary sporocysts in the head-foot region, and secondary sporocysts in the hepatopancreas.

Statistical analyses

All statistical analyses and graphs were performed using R software (version 3.0.1). When data distribution did not follow a normal distribution (Shapiro test, $p < 0.05$), results were compared with a Kruskal-Wallis followed by Dunn's multiple comparison test or simple pairwise comparison (Wilcoxon-Mann-Whitney test). When data were normally distributed, results were compared with an ANOVA followed by Tukey's multiple comparison test or simple pairwise comparison *t*-test.

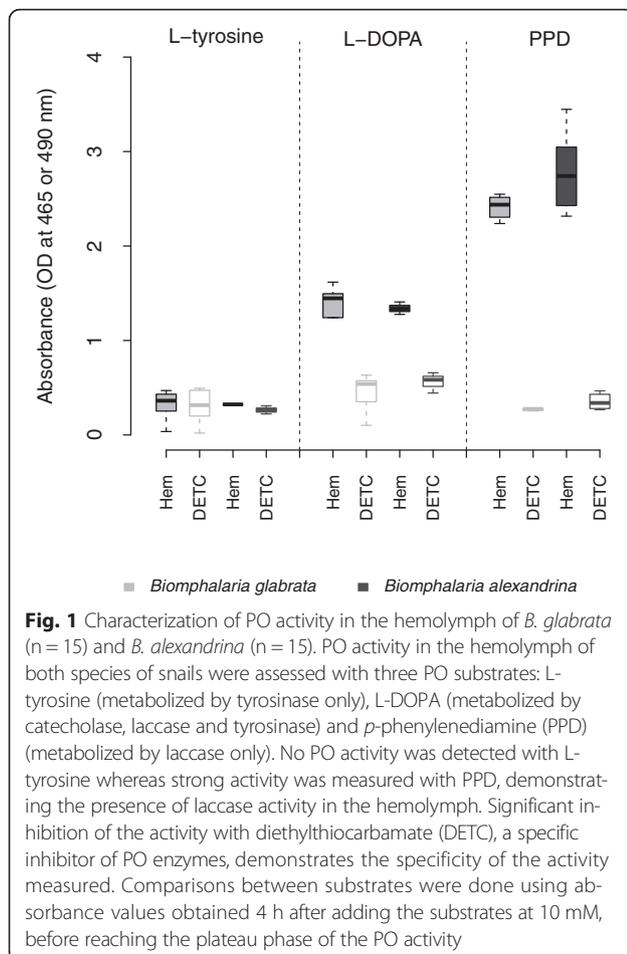
Results

Laccase activity characterized in the hemolymph of *Biomphalaria* spp.

We found the strongest phenoloxidase (PO) activity (*i.e.* dopachrome synthesis) in the hemolymph of both *B. glabrata* and *B. alexandrina* 4 h after adding the *p*-phenylenediamine (PPD) substrate, which is the specific substrate of laccase enzymes (Fig. 1). Test wells (containing hemolymph) and the inhibition control wells (containing hemolymph + diethylthiocarbamate (DETC) the specific competitive inhibitor of PO enzymes) showed a strong difference for both *B. glabrata* (*t*-test, $p < 1.10^{-10}$) and *B. alexandrina* (*t*-test, $p < 0.0001$), demonstrating that no other enzymes (such as peroxidases) were involved in this reaction.

We detected PO activity after 4 h using the L-DOPA substrate, which can be metabolized by all three types of enzyme, on the same hemolymph samples. Test wells and inhibition control well showed a strong difference for both *B. glabrata* and *B. alexandrina* (*t*-test, $p < 0.0001$ and $p = 0.004$ respectively; Fig. 1). We detected no PO activity when using L-tyrosine as substrate, showing that tyrosinase enzyme is not present in hemolymph of *Biomphalaria* spp. (Fig. 1).

For the two snail species, PO activity measured using PPD substrate is significantly higher than the activity assessed with L-DOPA (for *B. glabrata*, *t*-test, $p < 0.0001$ and for *B. alexandrina*, *t*-test, $p < 0.0001$). This demonstrates that a laccase-like enzyme is responsible for the PO activity measured in *Biomphalaria* spp. hemolymph.



General characteristics of the hemolymph laccase activity: temperature and pH optimum, Michaelis constant (K_m) and maximum velocity (V_{max}) of *Biomphalaria* spp. enzyme

We determined optimal parameters for the laccase-like enzyme from the hemolymph over a temperature range from 30 °C to 60 °C and a pH range from 4.5 to 12.5 (adjusted pH of the cacodylate buffer). The maximum activity of the hemolymph laccase-like enzyme is obtained at 45 °C for both snail species (Fig. 2a). Moreover, laccase activity was high from pH 6.5–8.5, but was the greatest at a pH of 8.5 (Fig. 2b), the physiological pH of *Biomphalaria* spp. hemolymph [38].

These parameters (temperature of 45 °C and pH of 8.5) were used to determine the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the laccase-like enzyme for both snail species, for PPD concentrations ranging from 0.891 mM to 14.28 mM. Using the Hanes-Woolf equation, a K_m of 1.45 mM and a V_{max} of 0.024 OD.min⁻¹ were calculated for the laccase-like enzyme of *B. glabrata* hemolymph, and a K_m of 1.19 mM and a V_{max} of 0.025 OD.min⁻¹ were determined for the enzyme of the *B. alexandrina* hemolymph. These enzyme parameters showed no difference between the two snail

species (χ^2 test, $p = 0.99$ and $p = 0.87$ for V_{max} and K_m respectively).

Laccase activity is located in the plasma of *Biomphalaria* spp. and enhanced by trypsin

We measured laccase activity in the hemolymph, plasma and hemocytes from individual snails of the two species. Four hours after adding the PPD substrate, laccase activity was the same in the hemolymph and the plasma fraction for both *B. glabrata* and *B. alexandrina*. This was consistent with or without trypsin (Fig. 3). We detected laccase activity in the hemocytes for both snail species (*B. glabrata*, *t*-test, $p = 0.0077$; *B. alexandrina*, *t*-test, $p = 0.0001$; Fig. 3) but this was very low compared to the hemolymph or plasma (~10 fold less; Kruskal-Wallis test, $p = 1.3 \cdot 10^{-9}$). These results demonstrated that the vast majority of laccase activity is located in the humoral component of the snail hemolymph; the laccase-like enzyme is circulating in the plasma.

Influence of parasite infection on laccase activity in the hemolymph of *B. glabrata*

To investigate the impact of parasitism on *B. glabrata* phenoloxidase activity we infected *B. glabrata* with *S. mansoni* (SmLE) parasites and monitored PO activity over 9 weeks of infection (Fig. 4).

Overall, we observed a strong negative impact of *S. mansoni* infection on both total laccase (*i.e.* with trypsin, Kruskal-Wallis test, $p = 1.03 \cdot 10^{-10}$) and intrinsic laccase activity (*i.e.* without trypsin, Kruskal-Wallis test, $p = 4.321 \cdot 10^{-7}$). However, the impact of infection on hemolymph laccase activity is not immediate. During the first six weeks we observed minimal differences between the uninfected control and infected snails with trypsin (Welsh *t*-test, $p = 0.048$) and no differences without trypsin. However, we observed very strong differences from week 7 (with trypsin: Welsh *t*-test, $p < 0.0001$ for week 7 to 9 and without trypsin: Welsh *t*-test, $p = 0.0233$ for week 7, $p = 0.0020$ for week 8 and $p < 0.0001$ for week 9). The differences in laccase activity between infected and uninfected snails results from a strong reduction in laccase activity over time after week 6 in the infected snails both with and without trypsin (Kruskal-Wallis test followed by a Dunn's post-hoc test, with trypsin: $p = 1.187 \cdot 10^{-10}$; without trypsin: $p = 5.280 \cdot 10^{-11}$). In comparison, we observed no change in laccase activity over time in the uninfected control snails with trypsin, and a slight change without trypsin (Kruskal-Wallis test followed by a Dunn's post-hoc test, $p = 0.01$).

We observed a global increase in the PO activity after trypsin treatment in both infected (Kruskal-Wallis test, $p = 6.416 \cdot 10^{-6}$) and control snails (Kruskal-Wallis test, $p < 0.0001$). This result demonstrates the role played by serine protease in activation of the laccase-like enzyme

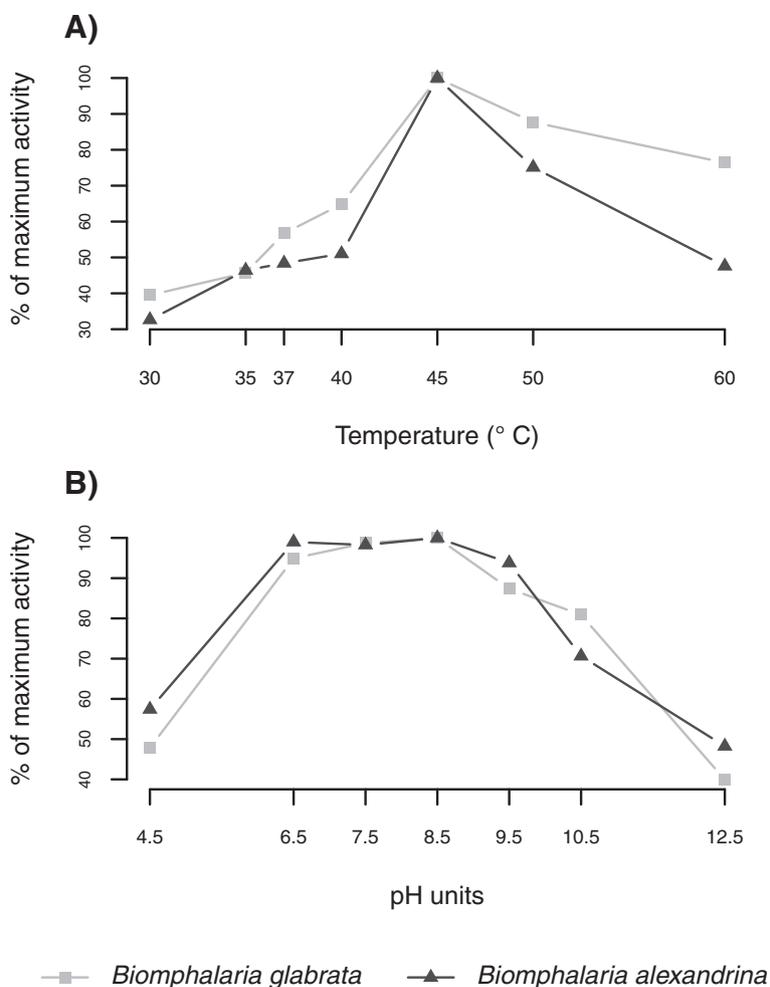


Fig. 2 The effect of temperature and pH on the laccase activity in the hemolymph of *B. glabrata* (n = 70) and *B. alexandrina* (n = 70). Activity was measured as the amount of dopachrome formed from the oxidation of PPD in the hemolymph of both species, at the stated temperatures (a) and pH (b). Values are expressed as a percentage of the maximum activity, obtained at a 45 °C (a) and pH 8.5 (b)

in *B. glabrata*. However in infected snails, this difference is only seen during weeks 1 to 6 (Welsh *t*-test, *p* = 0.0093). From weeks 7 to 9, there is no difference, suggesting that no extra proPO enzyme can be activated.

Discussion

In this study, we demonstrate that the PO activity from both *B. glabrata* and *B. alexandrina* hemolymph is laccase activity, and is located in the plasma. We then examined the impact of *S. mansoni* infection on the PO activity of *B. glabrata*, demonstrating a dramatic reduction from week 7 post-infection.

Characterization of the phenoloxidase activity

We characterized specific PO activity in both *B. glabrata* and *B. alexandrina* using PPD substrate and inhibition using DETC. PPD substrate is specific to laccase and laccase-like enzymes, and has been previously used to

demonstrate laccase activity in the hemolymph of the oyster *Crassostrea gigas* [43], the clam *Venerupis philippinarum* [48] and the abalone *Haliotis tuberculata* [49]. We found no formation of dopachrome with the L-tyrosine substrate, as seen in oyster [43] and abalone [49]. Some PO activity was measured with the L-DOPA substrate but this was significantly lower than with PPD. This result is most likely explained by the non-specific nature of the L-DOPA substrate: this is probably metabolized by the laccase-like enzyme but at a lower rate than the PPD. Hemocyanin, another copper-containing protein present in hemolymph that is known to show catecholase activity in crustaceans [50], does not show any phenoloxidase activity in *B. glabrata* [51] and therefore cannot be responsible for L-DOPA oxidation. PO inhibition by DETC, which chelates copper present in the enzyme [39, 44, 52, 53], confirms that the enzyme responsible for the metabolization of the PPD and the

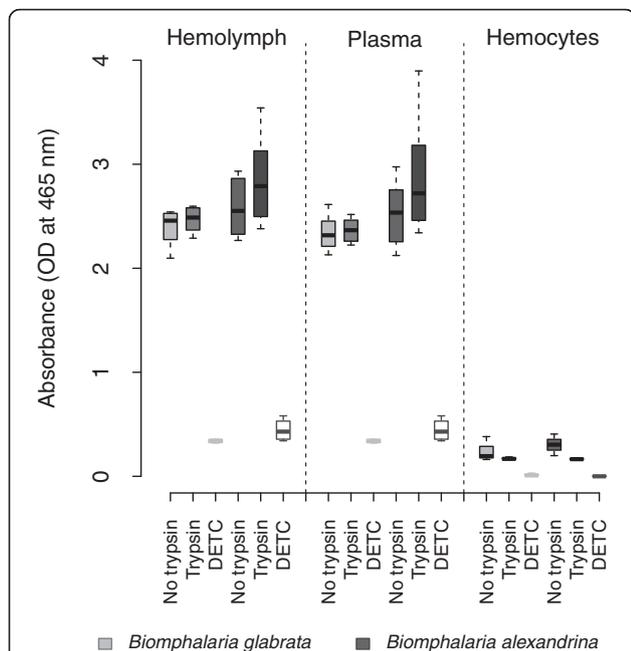


Fig. 3 Location of the laccase activity inside the hemolymph of *B. glabrata* (n = 15) and *B. alexandrina* (n = 15). PO activity was measured in (i) the hemolymph, (ii) the acellular compartment (*i.e.* plasma) and (iii) the cellular compartment (*i.e.* hemocytes) of both snail species, with and without trypsin as protease activator. Diethylthiocarbamate (DETC) was used as inhibition control. Activity was measured as the amount of dopachrome formed from the oxidation of PPD substrate (10 mM), 4 h after the substrate addition. No difference in laccase activity was found between hemolymph and plasma for both species, and minimal activity was detected in the hemocytes

L-DOPA is a copper-containing metalloenzyme [28]. Our results illustrate the importance of specific characterization of PO activity, because this allows development of assays with much greater sensitivity as shown by the higher detection of activity with PPD than with L-DOPA. Specificity is also improved using PPD substrate and DETC inhibitor, as L-DOPA can be oxidized by peroxidase or can auto-oxidize [33, 35] resulting in false positive results for PO.

To further characterize PO activity, we determined reaction optima. The *Biomphalaria* laccase-like enzyme has maximum activity between pH 6.5 to 8.5 (with a maximum at 8.5) and at 45 °C which is consistent with the values reported for other mollusks (Table 1). The explanation for the high optimal temperatures found in many invertebrates is not yet known. One hypothesis is that PO temperature optima may be selected to correspond to the temperature generated by local inflammation at the wound site. Such temperature matching would increase the efficiency of the enzyme reaction while at the same time limit unwanted PO-related tissue damage elsewhere in the organism. These optimal parameters allowed determination of the K_m and V_{max} of the laccase-like enzyme for both snail species. K_m and V_{max} values were similar between *B. glabrata* and *B. alexandrina* but lower than other laccase-like enzyme characterized in gastropods and bivalves (Table 1), which reveals a higher affinity for the PPD substrate in *Biomphalaria* spp.

We localized laccase activity in the plasma (*i.e.* acellular fraction of the hemolymph) in *Biomphalaria* spp. as observed in some other mollusks [48, 54, 55]. In comparison, Bahgat *et al.* [38] reported PO activity in hemocyte lysates, although no data was shown in their manuscript, and PO

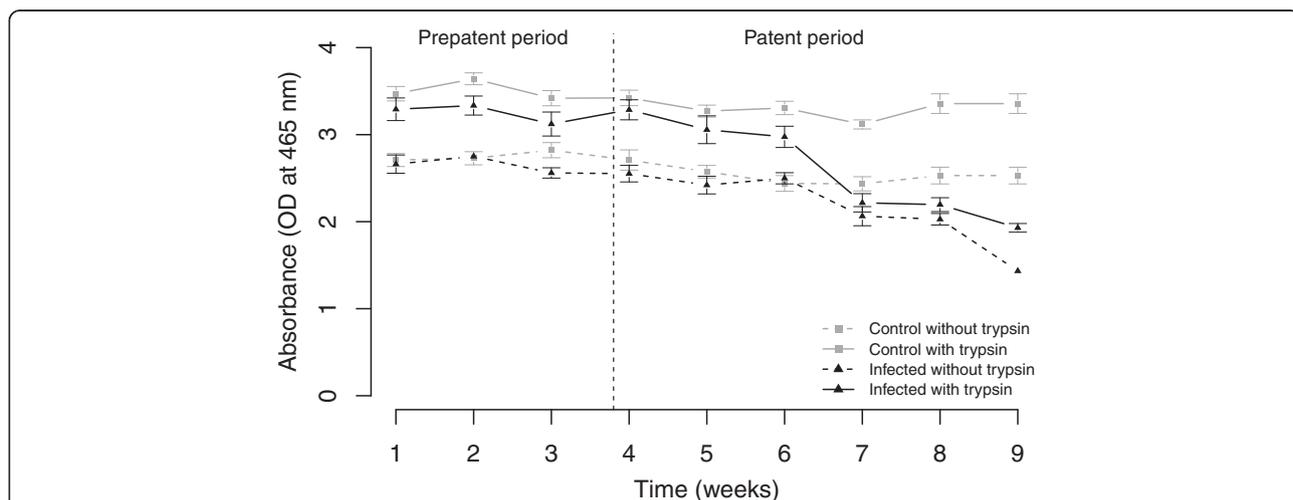


Fig. 4 Impact of *S. mansoni* infection on laccase activity in the hemolymph of *B. glabrata*, across the infection (9 weeks). PO activity was measured as the amount of dopachrome formed from the oxidation of PPD substrate (50 mM), with and without trypsin as protease activator, 2 h after the substrate addition. Total (with trypsin activation) and intrinsic (without trypsin activation) laccase activity are shown in solid and dotted lines respectively. Grey lines refer to uninfected control snails and black lines to infected snails. *S. mansoni* has a strong impact on snail laccase activity (both total and intrinsic). This effect was not immediate but appeared after 6 weeks of infection

Table 1 Summary of the general characteristics of PO enzymes determined from hemolymph of several mollusks

Class	Species	Substrate used for PO assays	PO optimum temperature (°C)	PO optimum pH	Michaelis constant (K_m) of PO enzyme identified (mM) ^a	V_{max} of PO enzyme identified (OD.min ⁻¹) ^a	References
Gastropods	<i>Biomphalaria glabrata</i>	PPD	45	6.5-8.5 Max:8.5	1.45	0.024	Present study
	<i>Biomphalaria alexandrina</i>	PPD	45	6.5-8.5 Max:8.5	1.19	0.025	Present study
	<i>Halitotis tuberculata</i>	PPD	ND	8.2	13.5	0.029	Le Bris <i>et al.</i> , 2014 [49]
Bivalves	<i>Saccostrea glomerata</i>	L-DOPA	37	8	NA	NA	Aladaileh <i>et al.</i> , 2007 [64]
	<i>Crassostrea virginica</i>	L-DOPA	ND	6-7.5	NA	NA	Jordan and Deaton, 2005 [44]
	<i>Crassostera gigas</i>	PPD	ND	ND	45	0.00059	Luna-acosta <i>et al.</i> , 2011 [58]
	<i>Ruditapes philippinarum</i>	L-DOPA	40	7	NA	NA	Cong <i>et al.</i> , 2005 [53]
	<i>Venerupis philippinarum</i>	PPD	40	8.4	14.46	0.23	Le Bris <i>et al.</i> , 2013 [48]
	<i>Chlamys farreri</i>	L-DOPA	45	6	NA	NA	Sun and Li, 1999 [65]

ND Not determined

NA Not applicable

^aMichaelis constant (K_m) and maximum velocity (V_{max}) of PO enzyme are strongly dependent to the substrate used. We mentioned K_m and V_{max} values when they are assessed using PPD substrate only, as in the present study

activity was tested in neither whole hemolymph nor plasma. Furthermore, their PO measurements were performed with the non-specific L-DOPA substrate 6 h after adding the substrate, at 405 nm (a suboptimal wavelength to quantify dopachrome formation which has a maximum of absorbance at 490 nm) and with no control for auto-oxidation of the L-DOPA substrate. No L-DOPA oxidation was identified in the hemocytes of another fresh water snail, *Lymnaea stagnalis* [36]. In our study, we detected very low laccase activity in the hemocytes of *Biomphalaria*. This may be due to the high sensitivity of our assay, allowing detection of residual activity from the cells' membrane, as hypothesized in the oyster *C. virginica* [44]. These results demonstrate the importance of testing both cellular (hemocytes) and acellular (plasma) fractions in order to accurately localize the PO activity.

In other arthropods such as insects or crustaceans, PO activity is mainly localized in the hemocytes and is typically tyrosinase activity, using tyrosine present in the hemolymph [16, 56]. The differences in the type of PO activity (tyrosinase vs laccase) and the enzyme localization (hemocyte vs plasma) in arthropods and mollusks raise intriguing questions about the evolution of PO enzymes in these two phyla.

Addition of trypsin, a serine protease enzyme, demonstrated the presence of circulating prolaccase-like enzyme in the snail hemolymph. One possible alternative explanation, that trypsin makes the active site of the laccase-like enzyme more accessible [57], is unlikely because trypsin had no impact on laccase activity of infected snails (7-9 weeks). Prolaccase-like enzymes may be activated by endogenous serine proteases produced by the snail [38] or by exogenous serine proteases produced by pathogens [17, 58]. Non-activated PO enzymes are common in all PO systems characterized [56]. In arthropods, comparisons of intrinsic PO activity (PO enzymes activated by endogenous proteases) and the total PO activity (intrinsic PO + PO enzymes activated by exogenous proteases) provide an important parameter used to assess immunocompetence and health status in an ecological context [17].

Impact of *S. mansoni* on *B. glabrata* PO activity

We examined the impact of *S. mansoni* infection on *B. glabrata* laccase activity over 9 weeks. Our results demonstrate a strong reduction in laccase activity in the snail hemolymph starting 7 weeks post-infection. Similar negative impacts of parasitic infection on PO activity were found in the oyster *C. virginica* and the mussel *Geukensia demissa* infected by *Perkinsus marinus* [44], while PO activity of the abalone *H. diversicolor* was reduced by infection with *Vibrio parahaemolyticus* [59]. Susceptibility to QX disease (caused by the paramyxean protozoan *Marteilia sydneyi* [60, 61]) in the Sydney rock oyster *Saccostrea glomerata* is also associated with a rapid

decrease in PO activity [62]. During the late stage of infection (7-9 weeks) trypsin had no effect on laccase activity. We interpret this result to mean that that total PO activity drops to the same level as intrinsic PO activity in infected snails, because laccase-like enzyme production has collapsed.

The decrease in PO activity in the snail hemolymph is correlated with the development of the *S. mansoni* secondary sporocysts in these experiments. This intramolluscan parasite stage metabolizes snail tissues, such as the hepatopancreas and the albumen gland, organs that are involved in the protein production [63], potentially including the laccase-like enzyme. However, parasite growth does not have any negative impact on snail PO activity during the first 6 weeks. These results are consistent to those obtained by Seppälä and Leicht [37] in the freshwater snail *Lymnaea stagnalis*. They observed no change in PO activity after the injection of trematode (*Plagiorchis sp.*) infected gonads compared to the injection of uninfected gonad. In contrast, injection of bacteria (*E.coli* or *Micrococcus lysodeikticus*) led to a decrease in PO activity.

Conclusion

We report and characterize laccase activity in the hemolymph of *B. glabrata* and *B. alexandrina*, intermediate hosts of the human blood fluke *S. mansoni*. Based on these findings, we employed a sensitive and specific spectrophotometric assay using PPD as substrate. This paves the way to better understanding of the role of PO enzymes in molluscan biology and host defense. Infection of snails with *S. mansoni* had a severe impact on PO production, but only after 6 weeks of infection. This strong negative effect of the parasite may be explained by the fact that secondary sporocysts of *S. mansoni* metabolize snail tissues involved in protein production, during their development and cercariae production.

Additional file

Additional file 1: Figure S1. Absorption spectrum of the product of PPD oxidation by the laccase-like enzyme in the hemolymph of *Biomphalaria* spp. The absorption spectrum was determined using continuous wavelength scanning from 375 to 675 nm. The maximum absorption, after 2 h of reaction with 50 mM of PPD, corresponds to wavelength of 465 nm. (PDF 4 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WL, TJCA, and FDC designed the experiments. WL performed the experiments and the data analyses. WL, TJCA, and FDC drafted the manuscript. All authors read and approved the final manuscript.

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References

- Morgan JA, Dejong RJ, Snyder SD, Mkoji GM, Loker ES. *Schistosoma mansoni* and *Biomphalaria*: past history and future trends. *Parasitology*. 2001;123(Suppl):S211–28.
- El-Nassery SMF, Abou-El-Naga IF, Allam SR, Shaat EA, Mady RFM. Genetic variation between *Biomphalaria alexandrina* snails susceptible and resistant to *Schistosoma mansoni* infection. *Biomed Res Int*. 2013;2013:160–320.
- Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. *Acta Trop*. 2000;77(1):41–51.
- Hirst SI, Stapley LA. Parasitology: the dawn of a new millennium. *Parasitol Today*. 2000;1(16):1–3.
- van der Werf MJ, de Vlas SJ, Brooker S, Looman CWN, Nagelkerke NJD, Habbema JDF, et al. Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Trop*. 2003;86(2-3):125–39.
- Bayne CJ, Buckley PM, DeWan PC. *Schistosoma mansoni*: cytotoxicity of hemocytes from susceptible snail hosts for sporocysts in plasma from resistant *Biomphalaria glabrata*. *Exp Parasitol*. 1980;50(3):409–16.
- Ratcliffe NA. Invertebrate immunity - a primer for the non-specialist. *Immunol Lett*. 1985;10:253–70.
- Loker ES, Cimino DF, Hertel LA. Excretory-secretory products of *Echinostoma paraensei* sporocysts mediate interference with *Biomphalaria glabrata* hemocyte functions. *J Parasitol*. 1992;78(1):104–15.
- Lockyer AE, Spinks J, Kane RA, Hoffmann KF, Fitzpatrick JM, Rollinson D, et al. *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni*. *BMC Genomics*. 2008;9:634.
- Nacif-Pimenta R, Alves de Mattos AC, da Silva Orfanó A, Barbosa L, Pimenta PFP, Coelho PMZ. *Schistosoma mansoni* in susceptible and resistant snail strains *Biomphalaria tenagophila*: in vivo tissue response and in vitro hemocyte interactions. *PLoS One*. 2012;9(7):e45637.
- Zhang SM, Loker ES. Representation of an immune responsive gene family encoding fibrinogen-related proteins in the freshwater mollusc *Biomphalaria glabrata*, an intermediate host for *Schistosoma mansoni*. *Gene*. 2004;341:255–66.
- Mitta G, Adema CM, Gourbal B, Loker ES, Theron A. Compatibility polymorphism in snail/schistosome interactions: From field to theory to molecular mechanisms. *Dev Comp Immunol*. 2012;37(1):1–8.
- Goodall CP, Bender RC, Brooks JK, Bayne CJ. *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with resistance/susceptibility to *Schistosoma mansoni*. *Mol Biochem Parasitol*. 2006;147(2):207–10.
- Galinier R, Portela J, Moné Y, Allienne JF, Henri H, Delbecq S, et al. Biomphalysin, a new β pore-forming toxin involved in *Biomphalaria glabrata* immune defense against *Schistosoma mansoni*. *PLoS Pathog*. 2013;9(3):e1003216.
- Martins-Souza RL, Pereira CAJ, Martins Filho OA, Coelho PMZ, Corrêa Jr A, Negrão-Corrêa D. Differential lectin labelling of circulating hemocytes from *Biomphalaria glabrata* and *Biomphalaria tenagophila* resistant or susceptible to *Schistosoma mansoni* infection. *Mem Inst Oswaldo Cruz*. 2006;101 Suppl 1:185–92.
- Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol*. 2008;29(6):263–71.
- Cornet S, Biard C, Moret Y. Variation in immune defence among populations of *Gammarus pulex* (Crustacea: Amphipoda). *Oecologia*. 2009;159(2):257–69.
- Mucklow PT, Vizoso DB, Jensen KH, Refardt D, Ebert D. Variation in phenoloxidase activity and its relation to parasite resistance within and between populations of *Daphnia magna*. *Proc Biol Sci*. 2004;271(1544):1175–83.
- Adamo SA. Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J Insect Physiol*. 2004;50(2-3):209–16.
- Hofreiter M, Schöneberg T. The genetic and evolutionary basis of colour variation in vertebrates. *Cell Mol Life Sci*. 2010;67(15):2591–603.
- Cieslak M, Reissmann M, Hofreiter M, Ludwig A. Colours of domestication. *Biol Rev*. 2011;86(4):885–99.
- Kalyani R, Nellaiappan K, Ramalingam K. Phenoloxidase activity of reproductive gland and its role in stabilization of egg envelopes of *Achatina fulica* (Pulmonata: Stylommatophora). *Int J Invert Reprod Dev*. 1985;8(2):73–81.
- Eshete F, LoVerde PT. Characteristics of phenol oxidase of *Schistosoma mansoni* and its functional implications in eggshell synthesis. *J Parasitol*. 1993;79(3):309–17.
- Li J, Christensen BM. Involvement of L-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. *Insect Biochem Mol Biol*. 1993;23(6):739–48.
- Bai G, Li J, Christensen BM, Yoshino TP. Phenoloxidase activity in the reproductive system and egg masses of the pulmonate gastropod, *Biomphalaria glabrata*. *Comp Biochem Physiol B Biochem Mol Biol*. 1996;114(4):353–9.
- Durrant HJ, Ratcliffe NA, Hipkin CR, Aspan A, Söderhäll K. Purification of the pro-phenol oxidase enzyme from haemocytes of the cockroach *Blaberus discoidalis*. *Biochem J*. 1993;289(Pt 1):87–91.
- Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell Mol Life Sci*. 2005;62(15):1707–23.
- Walker J, Ferrar P. Diphenol oxidases, enzyme-catalysed browning and plant disease resistance. *Biotechnol Genet Eng Rev*. 1998;15:457–98.
- Asada N, Fujimoto K, Tanaka M, Ohnishi E. Genetic polymorphism of prophenoloxidase A1 in *Drosophila melanogaster*. *Jpn J Genet*. 1993;68(3):219–27.
- Cárdenas W, Dankert JR. Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish. *Fish Shellfish Immunol*. 2000;10(1):33–46.
- Liu G, Yang L, Fan T, Cong R, Tang Z, Sun W, et al. Purification and characterization of phenoloxidase from crab *Charybdis japonica*. *Fish Shellfish Immunol*. 2006;20(1):47–57.
- Riley PA, Cooksey CJ, Johnson CI, Land EJ, Latter AM, Ramsden CA. Melanogenesis-targeted anti-melanoma pro-drug development: effect of side-chain variations on the cytotoxicity of tyrosinase-generated ortho-quinones in a model screening system. *Eur J Cancer*. 1997;33(1):135–43.
- Puiui M, Babaligea M, Oimazu C, Raducan A, Oancea D. Peroxidase-mediated oxidation of L-DOPA: a kinetic approach. *J Biochem Engineer*. 2010;52:248–54.
- Eastmond DA, Smith MT, Ruzo LO, Ross D. Metabolic activation of phenol by human myeloperoxidase and horseradish peroxidase. *Mol Pharmacol*. 1986;30(6):674–9.
- Parsons PG. Modification of dopa toxicity in human tumour cells. *Biochem Pharmacol*. 1985;34(10):1801–7.
- Vorontsova YL, Slepnevab IA, Yurlova NI, Glupova WV. Do snails *Lymnaea stagnalis* have phenoloxidase activity in hemolymph? *Invert Surviv J*. 2015;12:5–12.
- Seppälä O, Leicht K. Activation of the immune defence of the freshwater snail *Lymnaea stagnalis* by different immune elicitors. *J Exp Biol*. 2013;216(Pt 15):2902–7.
- Bahgat M, Doenhoff M, Kirschfink M, Ruppel A. Serine protease and phenoloxidase activities in hemocytes of *Biomphalaria glabrata* snails with varying susceptibility to infection with the parasite *Schistosoma mansoni*. *Parasitol Res*. 2002;88(6):489–94.
- Bai G, Johnston LA, Watson CO, Yoshino TP. Phenoloxidase activity in the reproductive system of *Biomphalaria glabrata*: role in egg production and effect of schistosome infection. *J Parasitol*. 1997;83(5):852–8.
- Bonner KM, Bayne CJ, Larson MK, Blouin MS. Effects of Cu/Zn superoxide dismutase (SOD1) genotype and genetic background on growth, reproduction and defense in *Biomphalaria glabrata*. *PLoS Negl Trop Dis*. 2012;6(6):e1701.
- Thurston CF. The structure and function of fungal laccases. *Microbiology*. 1994;140:19–26.
- Rescigno A, Zucca P, Flurkey A, Inlow J, Flurkey WH. Identification and discrimination between some contaminant enzyme activities in commercial preparations of mushroom tyrosinase. *Enzyme Microb Technol*. 2007;41(5):620–7.
- Luna-Acosta A, Rosenfeld E, Amari M, Fruitier-Arnaudin I, Bustamante P, Thomas-Guyon H. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. *Fish Shellfish Immunol*. 2010;28(4):719–26.
- Jordan P, Deaton L. Characterization of phenoloxidase from *Crassostrea virginica* hemocytes and the effect of *Perkinsus marinus* on phenoloxidase activity in the hemolymph of *Crassostrea virginica* and *Geukensia demissa*. *J Shellfish Res*. 2005;24:477–82.

45. Ritchie R, Prvan T. Current statistical methods for estimating the K_m and V_{max} of Michaelis-Menten kinetics. *Biochem Educ.* 1996;24(4):196–206.
46. Lewis FA, Stirewalt MA, Souza CP, Gazzinelli G. Large-scale laboratory maintenance of *Schistosoma mansoni*, with observations on three schistosome/snail host combinations. *J Parasitol.* 1986;72(6):813–29.
47. Allienne JF, Théron A, Gourbal B. Recovery of primary sporocysts in vivo in the *Schistosoma mansoni/Biomphalaria glabrata* model using a simple fixation method suitable for extraction of genomic DNA and RNA. *Exp Parasitol.* 2011;129(1):11–6.
48. Le Bris C, Paillard C, Stiger-Pouvreau V, Guérard F. Laccase-like activity in the hemolymph of *Venerupis philippinarum*: characterization and kinetic properties. *Fish Shellfish Immunol.* 2013;35(6):1804–12.
49. Le Bris C, Lepretre M, Paillard C, Guérard F. Characterization of a laccase-like activity in the hemolymph of the abalone *Haliotis tuberculata*. *Aquaculture.* 2014;424-425(0):194 – 200.
50. Terwilliger NB. Oxygen transport proteins in Crustacea: hemocyanin and hemoglobin. In: Chang ES, Thiel M, editors. *Physiology.* vol. 4. Oxford University Press: New York, United States; 2015. p. 359 – 390.
51. Lieb B, Dimitrova K, Kang HS, Braun S, Gebauer W, Martin A, et al. Red blood with blue-blood ancestry: intriguing structure of a snail hemoglobin. *Proc Natl Acad Sci U S A.* 2006;103(32):12011–6.
52. Fan T, Li M, Wang J, Yang L, Cong R. Purification and characterization of phenoloxidase from *Octopus ocellatus*. *Acta Biochim Biophys Sin (Shanghai).* 2009;41(10):865–72.
53. Cong R, Sun W, Liu G, Fan T, Meng X, Yang L, et al. Purification and characterization of phenoloxidase from clam *Ruditapes philippinarum*. *Fish Shellfish Immunol.* 2005;18(1):61–70.
54. Hellio C, Bado-Nilles A, Gagnaire B, Renault T, Thomas-Guyon H. Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) in vitro. *Fish Shellfish Immunol.* 2007;22(4):433–40.
55. Reid HI, Soudant P, Lambert C, Paillard C, Birkbeck TH. Salinity effects on immune parameters of *Ruditapes philippinarum* challenged with *Vibrio tapetis*. *Dis Aquat Organ.* 2003;56(3):249–58.
56. Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. *Immunol Rev.* 2004;198:116–26.
57. Decker H, Rimke T. Tarantula hemocyanin shows phenoloxidase activity. *J Biol Chem.* 1998;273(40):25889–92.
58. Luna-Acosta A, Saulnier D, Pommier M, Haffner P, De Decker S, Renault T, et al. First evidence of a potential antibacterial activity involving a laccase-type enzyme of the phenoloxidase system in Pacific oyster *Crassostrea gigas* haemocytes. *Fish Shellfish Immunol.* 2011;31(6):795–800.
59. Cheng W, Hsiao IS, Chen JC. Effect of nitrite on immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Dis Aquat Organ.* 2004;60(2):157–64.
60. Adlard R, Ogburn D. Development of a Disease Zoning Policy for Marteiliiosis to Support Sustainable Production, Health Certification and Trade in the Sydney Rock Oyster. Final Report - 2001/214. Sydney, NSW: NSW Fisheries; 2003.
61. Anderson TJ, Adlard RD, Lester RJG. Molecular diagnosis of *Marteilia sydneyi* (Paramyxia) in Sydney rock oysters, *Saccostrea commercialis* (Angas). *J Fish Dis.* 1995;18(6):507–10.
62. Peters R, Raftos DA. The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). *Aquaculture.* 2003;223(1-4):29–39.
63. Maldonado JF, Matienzo JA. The development of *Schistosoma mansoni* in the snail intermediate host, *Australorbis glabratus*. *PR J Public Health Trop Med.* 1947;22:331–73.
64. Aladaileh S, Rodney P, Nair SV, Raftos DA. Characterization of phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*). *Comp Biochem Physiol B Biochem Mol Biol.* 2007;148:470–480. <http://dx.doi.org/10.1016/j.cbpb.2007.07.089>.
65. Sun H, Li G. Phenoloxidase and myeloperoxidase activity in the hemocytes and serum of *Chla mys farrieri*. *J Fishery Sci China.* 1999;6:9–13.

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