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The effects of hypoxia and pH on phenoloxidase activity in the Atlantic blue crab, *Callinectes sapidus*

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Abstract

In its natural coastal and estuarine environments, the blue crab, *Callinectes sapidus*, often encounters hypoxia, accompanied by hypercapnia (increased CO_2) and an associated decrease in water pH. Previous studies have shown that exposure to hypercapnic hypoxia (HH) impairs the crab's ability to remove culturable bacteria from its hemolymph. In the present study we demonstrate that the activity of phenoloxidase (PO), an enzyme critical to antibacterial immune defense in crustaceans, is decreased at the low levels of hemolymph O_2 and pH that occur in the tissues of blue crabs exposed to HH. Hemocyte PO activity was measured at tissue O_2 levels that occur in normoxic (5% and 15% O_2, approximate venous and arterial hemolymph, respectively) and hypoxic (1% O_2) crabs and compared to PO activity in air-saturated conditions (21% O_2). PO activity decreased by 33%, 49% and 70% of activity in air at 15%, 5% and 1% O_2, respectively. When O_2 was held at 21% and pH lowered within physiological limits, PO activity decreased with pH, showing a 16% reduction at pH 7.0 as compared with a normoxic pH of 7.8. These results suggest that decreased PO activity at low tissue O_2 and pH compromises the ability of crustaceans in HH to defend themselves against microbial pathogens.

Keywords: Phenoloxidase; *Callinectes sapidus*; Blue crab; Hypoxia; Crustaceans; Innate immunity; Hypercapnia; Acidosis

1. Introduction

The Atlantic blue crab, *Callinectes sapidus*, like many other estuarine species, frequently encounters areas of low dissolved oxygen (hypoxia), high carbon dioxide (hypercapnia) and low pH (acidosis) (Diaz and Rosenberg, 1995; Burnett and Stickle, 2001). These naturally-occurring conditions may be aggravated in coastal zones by high levels of decomposing organic matter from terrestrial runoff (Renaud, 1986; Diaz, 2001), elevated temperatures that encourage phytoplankton growth which consumes more oxygen, and layering of fresh and salt water along coastal areas, which discourages mixing of oxygen-poor and oxygen-rich waters. The Mississippi River Delta and Chesapeake Bay are well-documented examples of this occurrence (Breitburg, 1992; Rabalais et al., 2001).

Exposure to hypoxia and hypercapnia triggers a broad assortment of behavioral, physiological, biochemical and genetic responses in marine organisms, including changes in the tissue concentrations of dissolved gasses and pH (Burnett and Stickle, 2001). Blue crabs held in well-aerated water (normoxia; P_{O_2}=20.7 kPa, 20.95% O_2) have oxygen pressures as high as 16.3 kPa (~16.5% O_2) in arterial hemolymph (Burnett et al., 2006). In hypoxic waters (6.7 kPa, 6.7% O_2), the oxygen pressure of hemolymph drops to approximately 0.9 kPa (1% O_2) (Burnett, 1992). Environmental hypercapnia can depress the pH of blue crab hemolymph from 7.8 (Wood and Cameron, 1985) to as low as 7.2 (Cameron, 1985).

Exposure to hypoxia and hypercapnic hypoxia (HH) also can alter patterns of disease resistance in crustaceans causing, for example, an increase in the rate of lethal infection from bacterial pathogens in shrimp (Le Moullac et al., 1998; Mikulski et al., 2000; Cheng et al., 2002). Blue crabs that were exposed to HH, then injected with sub-lethal doses of *Vibrio campbellii*, had

Abbreviations: L-DOPA, L-3,4 dihydroxyphenylalanine; DETC, sodium diethyldithiocarbamate; HH, hypercapnic hypoxia; HLS, hemocyte lysate supernatant; NC, *N*-nitrocatechol; PTU, *N*-phenylthiourea; PO, phenoloxidase; PPO, prophenoloxidase activating system; SDS, sodium dodecyl sulfate.

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higher levels of culturable bacteria in their hemolymph 30 min after introduction of the bacterial pathogen, as compared to crabs held in normoxia and given the same bacterial pathogen (Holman et al., 2004). Similarly, exposure to hypoxia or HH impaired the ability of penaeid shrimp *Litopenaeus vannamei* to kill *V. campbellii*, as defined by the recovery of culturable bacteria from selected tissues up to four hours after injection of the bacteria (Burgents et al., 2005).

The specific mechanisms underlying the effects of hypoxia and HH on mortality from bacterial pathogens and on tissue levels of culturable bacteria have not been pinpointed. The hemolymph of blue crabs collected from hypoxic estuaries has lower antibacterial activity against shell disease suggesting a decrease in circulating levels of antimicrobial peptides (Noga et al., 1996). Decreased numbers of circulating hemocytes (Le Moullac et al., 1998) and decreased production of reactive oxygen species (ROS) in shrimp (Cheng et al., 2002). In vitro exposure to hypoxia suppressed the production of ROS by the hemocytes of oysters (Boyd and Burnett, 1999) and anterior kidney cells of teleostean fish (Boleza et al., 2001).

The prophenoloxidase activating system (PPO), one of the most important and well-studied mechanisms of immune defense in crustaceans, is another possible target for the effects of low oxygen and pH on disease resistance in the blue crab. The end product of the PPO cascade is melanin, which can seal wounds, harden and darken post-molt carapace, and minimize bacterial and fungal infections through encapsulation (Söderhäll, 1982; Smith and Söderhäll, 1991; Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004). Phenoloxidase (PO) is the terminal enzyme of the PPO cascade. Using oxygen as a proton acceptor, PO catalyzes the conversion of phenols into a variety of end products, including quinones that spontaneously rearrange into the end product melanin (Aspán et al., 1995). PO is activated in situ by an endogenous serine protease and its activity is controlled by proteinase inhibitors (Cerenius and Söderhäll, 2004). In crustaceans the activated enzyme is sensitive to divalent cations and metals, the number and placement of substitutions on the phenolic ring of substrates and changes in pH over a wide range from 6.0 to 9.0 (Sung et al., 1998). In shrimp exposed to hypoxia PO activity has been reported to increase (Le Moullac et al., 1998) and to decrease (Cheng et al., 2002), however, in both of these studies in vitro PO assays were conducted under fully air-saturated conditions. To our knowledge the role of dissolved oxygen concentration in regulating PO activity has not been studied despite the crucial role of oxygen in the reaction. The present study tested the hypothesis that PO activity is suppressed by the decreased levels of oxygen and associated changes in pH that occur in the hemolymph of blue crabs experiencing sub-lethal hypoxia.

2. Materials and methods

2.1. Animals

Male blue crabs were trapped in the creeks of Charleston Harbor, Charleston, South Carolina and transported to the Grice Marine Laboratory where they were held in recirculating seawater systems at 25 ppt salinity and 24–26 °C with a 12 h light cycle. Crabs weighed between 92 and 236 g and were used within 1–5 days of capture. Crabs were fed frozen fish or shrimp every two days.

2.2. Hemocyte lysate preparation

Procedures for preparation of hemocyte lysate supernatants (HLS) were performed at 4 °C. Hemolymph was extracted through the arthrodial membrane of the hind walking leg using an 18 gauge needle and 30 cc syringe. Seven to nine mL of hemolymph were sampled from each animal and mixed with 1 to 3 mL of anticoagulant solution (415 mM NaCl, 100 mM glucose, 30 mM sodium citrate, 26 mM citric acid, 30 mM EDTA, pH 4.6) that was already loaded in the syringe. For each experiment, the hemolymph from three crabs was combined to form a pool of approximately 30 mL hemolymph+anticoagulant.

The pooled hemolymph was centrifuged for 10 min at 400 × *g* to precipitate the hemocytes. The cell-free supernatant was discarded and the cell pellet was gently washed with 10 mL of wash solution (415 mM NaCl, 100 mM glucose, 10 mM cacodylic acid, pH 7.0) per sampled crab to remove the EDTA still present in the cell pellet. The cells were centrifuged again for 10 min at 400×*g*. Cells in the resulting pellet were re-suspended in 3.0 mL lysis buffer (415 mM NaCl, 100 mM glucose, 10 mM cacodylic acid, 5 mM $CaCl_2$, pH 7.0) and sonicated for one minute at 40% power (Branson Sonifier, Danbury, CT). Cell debris was removed by centrifugation for 35 min at 3000×*g*. The resulting HLS was used for all subsequent PO assays.

2.3. Phenoloxidase activity assay

PO activity was quantified by monitoring the rate of formation of dopachrome from L-3,4 dihydroxyphenylalanine (L-DOPA) in a colorimetric assay. In a 1 mL cuvette, 100 μL HLS was combined with 25 μL 10% SDS and allowed to incubate for 15 min at 25 °C. Then, 875 μL of the L-DOPA solution (1 mg of L-DOPA/mL in 100 mM sodium phosphate, at the test pH) was added to the cuvette. The cuvette was immediately capped, inverted, blanked on its own absorbance and monitored for changes in optical density at 475 nm ($OD_{475\ nm}$) in a spectrophotometer over 5 min. PO activity was recorded as the maximum change in absorbance over any one min interval ($\Delta OD_{475\ nm}$/min) during the first 5 min of the assay.

2.4. Inhibition treatments

The sensitivities of the reaction to PO inhibitors sodium diethyldithiocarbamate (DETC), *N*-nitrocatechol (NC), *N*-phenylthiourea (PTU) and to a peroxidase inhibitor, sodium azide (NaN_3), were measured to assure that the observed oxidation of L-DOPA was due to the activity of PO. For each inhibition assay, 100 μL HLS was activated with SDS as described above, then 10 μL of a concentrated stock solution of

each inhibitor in 0.1 mM sodium phosphate pH 7.6 was added to the assay cuvette. One minute later, 865 μL of L-DOPA in 0.1 mM sodium phosphate, pH 7.6, was added to the cuvette. PO activity of each HLS was measured as previously described, and % inhibition calculated against activity of the same HLS without addition of an inhibitor.

2.5. Hypoxia treatments

To measure PO activity under O_2 concentrations that occur in the hemolymph of crabs experiencing hypoxia, the cuvettes, HLS, and L-DOPA required for the PO assay were sealed in a chamber with inlet and outlet tubes through which mixed gasses could be passed. This chamber was kept inside another larger glove box with a clear top for observation. Prior to each low oxygen experiment, the integrity of the gas chambers was verified by a smoke test, then both the interior and exterior chambers were flushed with pure nitrogen gas for 30 min. Subsequently, the interior chamber containing the cuvettes and the reaction mixtures was flushed for 15 min with a mixture of pure nitrogen and oxygen using a Wösthoff gas mixing pump to produce conditions of O_2 that reflect actual tissue/hemolymph levels of this gas that occur in the hemolymph of crabs held in normoxia, hypoxia and HH (Table 1). The assay reagents were combined in cuvettes and held in the low O_2 mixture of the internal chamber for the intervals as described above. After the final addition of substrate, the vials were capped, removed from the chamber and the $OD_{475\ nm}$ monitored as described above (Phenoloxidase Activity Assay). PO activities of each HLS under low O_2 conditions were normalized to the activity of the same HLS at aerial O_2 concentration (20.7 kPa, 20.95%). Normalized PO activities reported for each low oxygen mixture are the average (±SE) of independent measurements on five pooled HLS samples.

2.6. pH treatments

To simulate the acidosis within crab hemolymph during HH, aliquots of the L-DOPA substrate stock solution were dissolved in 100 mM monobasic and dibasic sodium phosphate to achieve the designated pH treatments (6.6, 7.0, 7.4, 7.6, and 7.8). PO activities of each HLS under low pH conditions were normalized to the activity of the same HLS at pH 7.8, which is the hemolymph pH of a normoxic crab (Burnett, 1992).

Table 1
Levels of dissolved oxygen used in the phenoloxidase assays and their physiological occurrence in the hemolymph of *Callinectes sapidus*

Oxygen levels			Physiological occurrence in hemolymph
% O_2	P_{O_2} (Torr)	P_{O_2} (kPa)	
21	155	21	Air
15	111 [a]	15	Normoxia, arterial hemolymph
5	37 [a]	5	Normoxia, venous hemolymph
1	7.4 [b]	1	Hypoxia, venous hemolymph

[a] (Burnett et al., 2006).
[b] (Burnett, 1992).

Table 2
Impacts of selected inhibitors on PO activity of crab HLS

Inhibitor	Final inhibitor concentration (mM)	SDS	Inhibition (% of control)
–	–	+	100 (control)
–	–	−	0
Diethyldithiocarbamate	0.01	+	24.8±11.3
Diethyldithiocarbamate	0.1	+	0
4-nitrocatechol	0.1	+	47.0±2.2
4-nitrocatechol	1.0	+	0
N-phenylthiourea	0.001	+	23.6±5.5
N-phenylthiourea	0.01	+	0
Sodium azide	1.0	+	91.0±5.3
Sodium azide	10.0	+	53.8±3.6

After activation of PO in 100 μL HLS for 10 min by the addition of 25 μL 10% SDS, the inhibitors were added in a volume of 10 μL, followed 1 min later by addition of 865 μL L-DOPA substrate. Inhibition values given above are the average (±SE) of 3 independent HLS preparations.

Normalized PO activities reported for each low pH mixture are the average (±SE) of independent measurements on five pooled HLS samples.

2.7. Statistical analysis

SigmaStat 3.0 software was used to perform all statistical analyses. Values for PO activity at 15%, 5% and 1% O_2 were normalized to PO activity at 21% O_2. The resulting data were not normally distributed within each oxygen treatment, so a Kruskal–Wallis One-way ANOVA on Ranks was performed to determine if there was a significant effect of O_2 on PO activity. Pair-wise multiple comparisons between O_2 treatment groups were performed by Dunn's method. To determine if there was a significant effect of pH on PO activity, a one-way ANOVA was performed on the normalized PO data for all pH treatments.

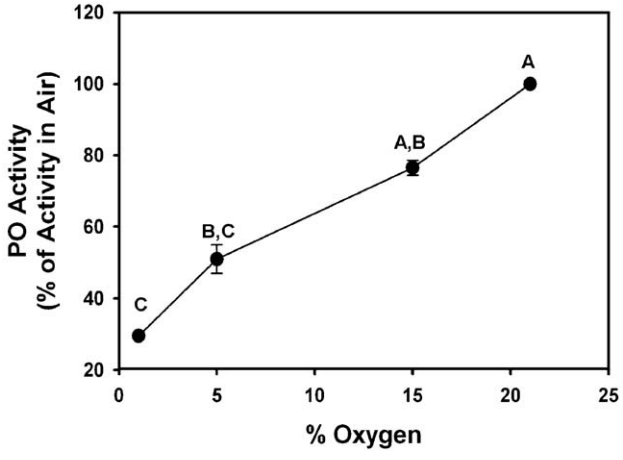

Fig. 1. The effect of oxygen concentration on phenoloxidase (PO) activity. PO activity of each hemocyte lysate was normalized against PO activity of the same lysate in 21% O_2. Indicated values are means±SE (n=5). A Kruskal–Wallis One-way ANOVA on Ranks revealed a significant effect of oxygen on PO activity (p<0.001). Pairwise comparisons using Dunn's Method indicated differences between some, but not all treatments. Values with the same letter (i.e., A, B, C) are *not* significantly different.

Pair-wise multiple comparisons were performed by the Holm–Sidak method.

3. Results

3.1. Controls

In the absence of SDS, blue crab HLS did not catalyze the oxidization of L-DOPA, as detected in this assay. At concentrations of 0.1 and 0.01 mM, respectively, PO inhibitors DETC, NC and PTU completely suppressed the oxidase activity of the HLS (Table 2). At the highest concentration tested (10 mM) peroxidase inhibitor sodium azide reduced the oxidation of L-DOPA by less than 50%.

3.2. Hypoxia and pH treatments

Dissolved O_2 pressures significantly impacted PO activity of *C. sapidus* over the physiologically relevant range tested in this study (Fig. 1; Kruskal–Wallis One-way ANOVA on Ranks, $p<0.001$). Exposure to 15%, 5%, or 1% O_2 reduced PO activity by 23.5%, 49.0%, and 70.5%, respectively, as compared to control enzyme activity at 21% O_2. The effects of 5% and 1% O_2, but not 15% O_2, were significantly different from control enzyme activity at 21% O_2 (Multiple comparison by Dunn's method, $p<0.05$).

pH significantly affected PO activity over the range of physiologically relevant values that occur in crab tissues (one-way ANOVA, $p<0.001$). When compared to its activity at pH 7.8, average PO activity decreased by 6.8% in pH 7.6, 9.4% at pH 7.4, 15.8% at pH 7.0, and 32.7% at pH 6.6 (Fig. 2). In pairwise comparisons (Dunn's Method) PO activity at 7.8 was significantly different from the activities at pH 6.6 ($p<0.001$), 7.0 ($p<0.001$), and 7.4 ($p=0.010$). Some but not all other pairwise comparisons were significantly different among the pH treatment groups (Fig. 2).

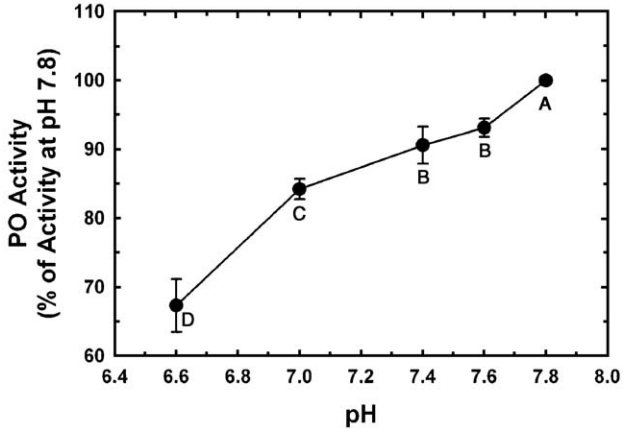

Fig. 2. The effect of pH on phenoloxidase (PO) activity. The PO activity of each hemocyte lysate was normalized against PO activity of the same lysate at pH 7.8. All experiments were performed in aerial conditions (21% O_2). Indicated values are means±SE ($n=5$). A One-way ANOVA on Ranks revealed a significant effect of pH on PO activity ($p<0.001$). Pairwise comparisons using the Holm–Sidak method indicated differences between all but one pair of treatments. Values with the same letter (i.e., A, B, C) are *not* significantly different.

4. Discussion

The results of the present study show that oxygen and pH independently suppress PO activity over the physiological range of these variables in the tissues of the blue crab. The impacts of O_2 are dramatic, with more than 70% reduction in enzyme activity at 1% O_2 (Fig. 1), which is the partial pressure of oxygen in venous hemolymph of blue crabs exposed to hypoxia (Burnett, 1992). Furthermore, hypoxia is often accompanied by elevated concentrations of carbon dioxide (hypercapnia) which in turn lowers the pH of both the water and the hemolymph. The impacts of pH on PO activity are significant, but less dramatic than for O_2. PO activity at pH 7.0, which occurs in the hemolymph of crabs experiencing HH, is 16% lower than the enzyme activity at pH 7.8 which is characteristic of hemolymph in normoxic animals (Fig. 2). The present study did not assess the combined effects of low oxygen and low pH, however, these two water quality parameters independently and additively impact ROS production by hemocytes of the oyster *Crassostrea virginica* (Boyd and Burnett, 1999). Given the central important of PO to the immune defense of crustaceans (Cerenius and Söderhäll, 2004; Iwanaga and Lee, 2005) the striking sensitivity of PO activity to P_{O_2} and pH as reported in the present study may contribute to an increased incidence of infectious disease in natural blue crab populations exposed to hypoxia (Noga et al., 1994), as well as slower elimination of culturable bacteria from the hemolymph of blue crabs held in the laboratory under hypoxic versus normoxic conditions (Holman et al., 2004).

To our knowledge, this is the first report to verify the oxygen sensitivity of PO activity for crustaceans over the physiologically relevant range of oxygen. Other studies have reported significant changes in PO activity in crustaceans following exposure of whole animals to hypoxia. For example Le Moullac et al. (1998) found that PO activity increased in shrimp *Penaeus stylirostris* exposed to hypoxia (1 mg O_2 mL^{-1}, equivalent to approximately 3% O_2) but assays of PO activity in that study were conducted under aerial conditions, not at the concentrations of O_2 found in the tissues of hypoxic shrimp.

As reported in the present study, the activity of *C. sapidus* PO against the *o*-diphenol substrate L-DOPA increased with pH over the physiologically relevant range of pH 6.6–7.8 (Fig. 2). These results are consistent with optimum pH values for PO in the brown shrimp *Penaeus californiensis* (Gollas-Galván et al., 1999) and the red swamp crayfish, *Procambarus clarkia* (Cardenas and Dankert, 2000). pH optima closer to 6.0 are reported for hemocyte PO in other crustaceans, such as *Charybdis japonica* (Liu et al., 2006) and *Penaeus chinensis* (Fan and Wang, 2002). Also, pH optima for PO are lower in terrestrial arthropods, such as 6.0 in the silkworm, *Bombyx mori* (Ashida, 1971) and 4.5 in the flesh fly, *Sarcophaga bullata* (Barrett, 1986). The lower pH optima for PO in insects may correlate with a lower hemolymph pH (Harrison, 2001; Consoli and Vinson, 2002) compared to that of crustaceans.

It is likely that oxygen and pH have many additional impacts on the immune system of invertebrates. For example, Boyd and Burnett (1999) found that production of reactive oxygen species

(ROS) by hemocytes of the oyster *C. virginica*, an important part of the immune defense in bivalves, was depressed by 67% under the low oxygen and pH conditions found in hypoxic oysters. The pathways that invertebrates use to produce ROS, as reported for snails (Adema et al., 1991) and oysters (Anderson, 1996), are remarkably similar to those of the vertebrates, and it is among the vertebrates that impacts of hypoxia have been documented most extensively. For example, ROS production in endothelial cells from bovine pulmonary arteries decreased by 70% after 2 h of exposure to 1% oxygen (Zulueta et al., 1995). ROS production by endothelial cells from the pulmonary arteries of yearling piglets declined by 43% after 2 h of exposure to 5% oxygen (Yang and Block, 1995). Human neutrophils exposed to P_{O_2}<21 kPa also produced very small amounts of ROS (Gabig et al., 1979).

Blue crab PO was very sensitive to inhibitors of *o*-diphenoloxidase activity and relatively insensitive to a peroxidase inhibitor, strongly suggesting that the oxidase activity detected in HLS of blue crabs is an authentic crustacean PO. Inhibition profiles of NC, PTU and the copper chelator DETC against the blue crab enzyme are similar to the PO purified from the freshwater crayfish *Pacifastacus leniusculus* at 0.1 mM (Aspán et al., 1995), greater than 94% of PO activity in the brown shrimp *P. californiensis* (Gollas-Galván et al., 1999), although the latter enzyme was relatively insensitive to 10 mM PTU (Gollas-Galván et al., 1999). In contrast, even at the highest dose tested in the current study (10 mM) the peroxidase inhibitor, sodium azide suppressed less than 50% of the blue crab PO activity, a finding similar to the reported 23.5% inhibition of brown shrimp PO at 10 mM NaN_3 (Gollas-Galván et al., 1999).

The results of the present study show that low O_2 and pH suppress the activity of crustacean PO. The implications of these findings are that when blue crabs are exposed to HH resulting in lower tissue P_{O_2} and acidosis, PO activity will be dramatically impaired, and the animals will be more susceptible to infectious pathogens. As the incidence and severity of hypoxia in coastal waters continue to increase (Diaz and Rosenberg, 1995), crustacean populations inhabiting these waters may become more susceptible to infection and their survival threatened by opportunistic pathogens. Severe economic and ecological losses may be avoided by management strategies that enhance water quality.

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