The Effects of pH on Phenoloxidase Activity in the Brachyuran Crab, *Cancer magister*

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Abstract

In its natural estuarine environment, the Dungeness crab, *Cancer magister*, often experiences low pH. Similar studies done involving other species of crab have shown that low pH suppresses phenoloxidase activity. Phenoloxidase (PO) is responsible for catalyzing the reaction of L-DOPA to melanin. In this study, hemolymph was used to execute a PO assay using four pH treatments, 6.7, 7.0, 7.4, and 7.8. PO activity was lowest at pH of 6.7 and highest at pH of 7.8. The results suggest that low pH suppresses PO activity in *C. magister.*

Introduction

Phenoloxidase (PO) is an enzyme that vitally protects arthropods both internally and externally. PO protects arthropods internally from invasions by infectious microorganisms, and protects them externally by catalyzing the sclerotization of an animal's newly formed or repaired exoskeleton (Terwilliger and Ryan, 2006). Using oxygen as a proton acceptor, PO catalyzes the conversion of phenols, for example L-3,4 dihydroxyphenylalanine (L-DOPA), to quinones which spontaneously rearrange into the end product melanin (Söderhäll and Cerenius, 1998; Tanner *et al*., 2006; Aspán *et al*., 1995). Melanin is responsible for clotting hemolymph at wounds, for darkening and hardening post-molt carapace, and for minimizing bacterial and fungal infections through encapsulation (Söderhäll, 1982; Smith and Söderhäll, 1991; Söderhäll and Cerenius, 1998). PO is stored and synthesized as prophenoloxidase (PPO), the inactive form of PO (Smith and Söderhäll, 1991). In the presence of a bacterial or fungal infection, hemocytes release PPO into the hemolymph, where PPO is converted to PO by a serine

protease (Söderhäll and Cerenius, 1998). In the laboratory, PPO can be activated using detergents, for instance sodium dodecylsulfate (SDS) (Aspán and Söderhäll, 1995).

PO activity is sensitive to a number of environmental factors, such as pH. The hemolymph pH of a normoxic crab is 7.8 (Burnett, 1992). PO activity has been documented to decrease in shrimp that have been exposed to low pH (6.8 and below) (Cheng *et al*., 2002) and has been documented to increase in shrimp exposed to low pH (Le Muollac *et al*., 1998). A recent study found that PO activity is suppressed at low pH in the Atlantic blue crab *Callinectes sapidus* (Tanner *et al.,* 2006). The present study tested the hypothesis that the decreased levels of pH suppress PO activity in the Dungeness crab, *Cancer magister*.

Cancer magister inhabits sandy and muddy bays and estuaries with dense eelgrass (*Zostera*) cover (McGaw, 2004), and like many other estuarine species frequently encounters areas of low dissolved oxygen, high carbon dioxide, and low pH (Tanner *et al.,* 2006). On sandy beaches, crabs that are left stranded by the receding water may burrow for protection, leaving only interstitial water available for ventilation of the gills. Estuarine sediments and the water above them are often extremely hypoxic (Airriess & McMahon, 1994); therefore, crabs in either habitat may face low levels of pH with every tidal cycle.

Materials and Methods

The *Cancer magister* that were used in this experiment were collected off of the Oregon Institute of Marine Biology boat dock in Charleston, Oregon and put in labeled aquariums with flow-through seawater. The crabs were fed mussels every two days. A volume of 200 μL of hemolymph was extracted from each crab using an ice-cold syringe

filled with 800 µL HEPES anticoagulant buffer (HAC) (Terwilliger and Ryan, 2006) and an ice-cold needle. The sample was briefly analyzed in the hemocytometer to ensure that a good draw had been attained. The samples were then transferred to microcentrifuge tubes in a bucket of ice. The samples were centrifuged for 6 minutes at 1600 rpm. The supernatant was put into a labeled microcentrifuge tube and kept on ice, and each pellet was resuspended in 800 µL HAC. A 20 µL aliquot was set aside, and kept cool, for cell count analysis with a hemocytometer.

Each hemolymph sample was then assayed for phenoloxidase (PO) activity at pH treatments of 6.7, 7.0, 7.4, and 7.8 (Tanner *et al*., 2006). In a cuvette, 795 µL of phosphate buffer (pH of 6.7, 7.0, 7.4, or 7.8), 100 µL of the hemocyte sample, and 50 µL of L-DOPA were mixed. The cuvette was placed in the spectrophotometer and run as background, to get an initial absorbance. Ten µL of 10% sodium dodecylsulfate (SDS) was added, and the sample was inverted and placed back in the spectrophotometer. The sample was read at a wavelength of 475 nm over a time span of six minutes, with data points taken every half-minute. The slope was calculated for the line of accumulation of product over time. Absorbance was normalized using the concentration of cells per mL according to the hemocyte count.

Two negative controls were run. One control consisted of 795 µL of phosphate buffer, 100 µL of distilled water, and 50 µL of L-DOPA. The second control contained 100 µL of supernatant instead of distilled water. Both controls ran for 6 minutes at 475 nm and both controls were run for each of the five phosphate buffers.

The 20 µL aliquot of the hemocyte sample was then analyzed in the hemocytometer, in order to normalize the PO activity between the two crabs. Ten µL of

the gently agitated hemocyte sample was pipetted in the groove between the coverslip and the hemocytometer, a precise distance of 0.1 mm. A hemocyte count was taken in one of the 0.25 mm x 0.25 mm regions that contain 16 squares. The number of hemocytes was counted in 10 squares so that an average number of cells per square could be calculated. The volume of fluid that was analyzed was calculated, and from there the concentration of cells was calculated using the average number of cells divided by the volume of fluid.

Results

Assays were successfully run on two individual crabs at 4 pH levels, as shown by figures 1 and 2. The trend in figure 1 and 2 is that absorbance, which increases over time as product (melanin) accumulates, is highest when the pH is at the normoxic pH of 7.8 and absorbance is lowest when the pH is well below 7.8, at 6.7. Both figures illustrate that PO activity is greatest at pH 7.8 and lowest at 6.7. Also, there is a gradient of PO activity increasing from 6.7 to 7.8. Figure 3 shows the averaged normalized values for crab 1 and 2. The values were normalized using the calculated concentration of cells per mL. Then the values for crab 1 and 2 were averaged together to attain a single line for each pH. The slopes were calculated to be 0.076, 0.446, 0.486, and 0.542 for pH 6.7 through 7.8, respectively.

The hemocyte count yielded a high variance. Crab 2 had a higher average number of cells per square, in the 0.25 mm x 0.25 mm region that contains 16 squares, with 31.8, compared to 21.2 for crab 1. Crab 2 also had a higher concentration of cells per mL with 5.09 x 10^6, and crab 1had 3.40 x 10^6.

Discussion

The results of the present study show that low pH suppresses PO activity. The low absorbance at pH 6.7 means that PO was unable to catalyze the reaction of L-DOPA to quinones to melanin. The high absorbance at pH 7.8 indicates that substantial quantities of the end product melanin were being made. This makes sense because 7.8 is the normoxic pH of crab hemolymph, and melanin production is therefore going to be optimal. The striking sensitivity of PO activity to pH demonstrates that *C. magister,* if exposed to water with low pH, would not be able to defend itself against biological or fungal infections. Furthermore, this animal would not be able to harden its post-molt carapace, thus making it susceptible to increased predation. Tanner *et al.* (2006) found similar results with *Callinectes sapidus*. *C. sapidus* is highly sensitive to changes in pH, perhaps contributing to the increased incidence of infectious disease in natural *C. sapidus* populations.

The second crab had distinctly more cells per square and a significantly higher concentration of cells per mL. The second crab was observed to have had hemolymph with a milky-yellow tint to it, while the first crab had clear hemolymph. The milky-yellow tint could be attributed to the crab having a bacterial or fungal infection, and its hemolymph is flooded with hemocytes as a result. Briggs and McAliskey, 2002, found that when the lobster *Nephrops norvegicus* was infected with the parasite *Hematodinium*, the color of its hemolymph changed from transparent to milky–yellow. No increase in hemocytes was documented in this study.

It is clear that PO activity is sensitive to low pH in *Cancer magister*. Well, *C. magister* will encounter low pH when temperatures rise, causing increased phytoplankton

growth. This phytoplankton growth will result in lower dissolved oxygen, high carbon dioxide, and as a result low pH (Tanner *et al.,* 2006). Nutrient run-off is another cause of low pH because excessive nutrient loading leads to phytoplankton blooms, which cause hypoxic conditions, i.e. low pH.

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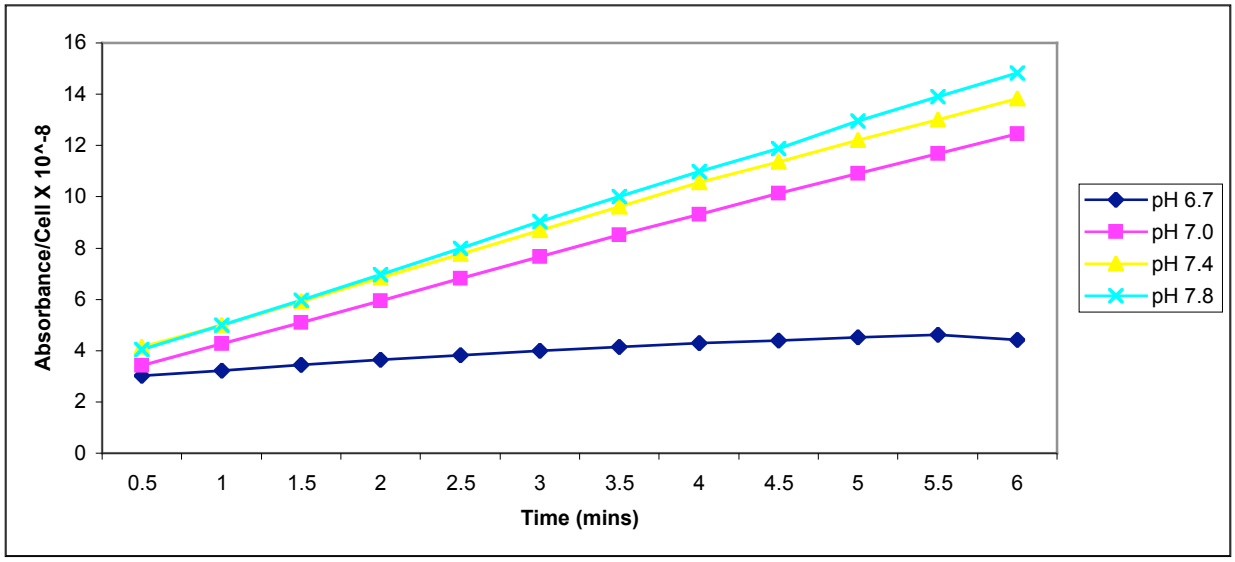

Figure 1 – Normalized absorbance versus time at various pH values for crab #1.

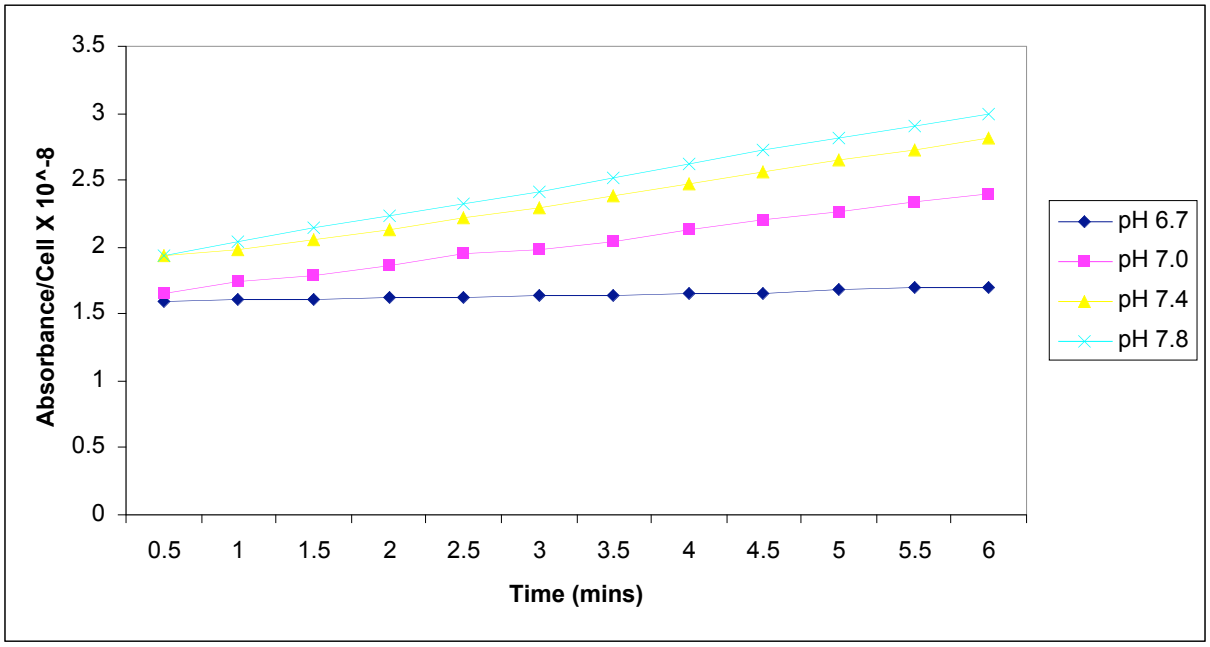

Figure 2 – Normalized absorbance versus time for various pH values for crab #2.

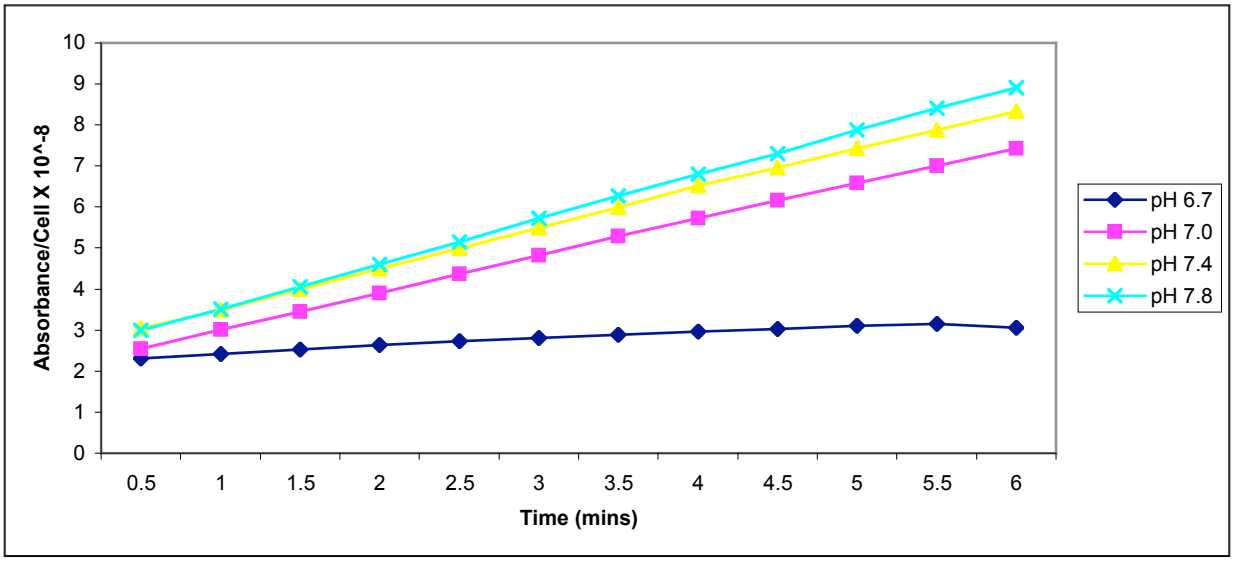

Figure 3 – Normalized absorbance versus time for the averaged pH values for both crabs.

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Final Protocol
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I. Preparing the Hemolymph Samples

- Fill a bucket of ice and chill 2-3 (depending on how many crabs you are drawing from) 1 mL syringes with 22 gauge needles attached, and chill labeled (hemocyte, cell count, and supernatant) microcentrifuge tubes, three for each crab. Also fill a bag with ice (double bag it) and put it in the centrifuge to chill it.
- Fill a 1 mL syringe with 800 µL of already prepared (see below for preparation directions) and chilled hemocyte anticoagulant (HAC) and keep on ice.
- Have someone hold the crab down on its back, making sure its claws are held down. Draw 200 µL of hemolymph from one of the back two legs of the crab, holding the leg you are drawing from with your free hand to secure it. Aim toward the joint pocket, not the muscle, so point the needle away from the body and at a 45° (roughly).
- After drawing the blood, take the needle off and empty the syringe into the hemocyte labeled tube. Gently agitate sample with the syringe.
- Centrifuge the sample for 6 min at 1600 rpm, perhaps longer if you cannot see a pellet forming.
- Save supernatant, without disturbing the pellet, and leave a small amount with the pellet. Put the supernatant in the chilled tube labeled supernatant and keep on ice.
- Put the hemocyte tube on ice and add 800 µL of chilled HAC and gently resuspend the pellet by pipetting gently up and down with a P 1000 pipette and blue tip. Keep the sample on ice until it is needed.
- Aliquot 20 µL of hemocyte sample into the chilled and labeled cell count tube, and keep on ice until it is needed.

II. Hemocytometer Counts of Blood Cells

- Place the special coverslip on the hemocytometer, but be very careful since the coverslip is very delicate and expensive.
- Put the slide on the stage and gently agitate the cell count sample before putting on the slide. Pipette 10 µL of the sample onto the slide, slowly.
- Determine an area to count, one of the four corners with 16 squares or the middle area. Count 10 squares and calculate the average. Calculate the volume of fluid by taking the area of the square. Lastly calculate the number of cells per mL by taking the average number of cells divided by the volume of fluid.

III. Spectrophotometric Assay of Phenoloxidase Activity

- Set the spectrophotometer to timed drive at a wavelength of 475 nm, 10/min, for 6 minutes, and set the parameters of the graph at +1.0 and -0.2. Calibrate by hitting start and with no cuvette in it.
- It is best to have a pipette for each of the solutions so that you do not have to waste time changing the value. So, in a cuvette add:
    - 795 µL of Phosphate buffer (either pH 6.7, 7.0, 7.4, or 7.8)

    - 100 µL hemocyte sample
    - 50 µL substrate – L-DOPA
- Put the sample in the spec to get an initial absorbance. Take the sample out and add 10 µL 10% SDS to the sample, invert, put in the spec and hit run. Record the absorbance every .5 min and calculate the greatest slope over a one-minute time interval. Repeat for each pH and each hemocyte sample (if applicable).
- For the negative control (no cells), add to a cuvette:
    - 795 µL of Phosphate buffer (either pH 6.7, 7.0, 7.4, or 7.8)
    - 100 µL supernatant sample that has been on ice
    - 50 µL substrate – L-DOPA
    - Run for 6 minutes on timed drive.
    - Repeat for each pH and each hemocyte sample.
- For the negative control (reagents), add to a cuvette:
    - 795 µL of Phosphate buffer (either pH 6.7, 7.0, 7.4, or 7.8)
    - 100 µL distilled water
    - Run for 6 minutes on timed drive. If there is any activity, you are just testing L-DOPA, not hemolymph.
    - 50 µL substrate – L-DOPA

IV. Chemical Preparation

- Mixing L-DOPA Solution in a 1.0 mL tube:
    - Wrap the 10 mL tube with foil
    - 1.5 mL distilled water
    - 6 mg L-DOPA (be careful as this is very light sensitive)
    - Vortex the tube until L-DOPA completely dissolves
    - Have to make a new solution at the start of each day you use it.
- Making HAC (500 mL):
    - 1.30 g of HEPES – final concentration 10mM
    - 1.86 g of EDTA – final concentration 10mM
    - 0.373 g of KCl – final concentration 10mM
    - 13.15 g of NaCl – final concentration 450 mM
    - Adjust to pH 7.4 with NaOH using a pH meter
    - Store in the fridge
- 10% Sodium Dodecylsulfate (SDS)
    - 1 g SDS
    - 10 mL distilled water
    - Store at room temperature
    - Can use this solution until it is gone.
- 200 mM Monobasic sodium phosphate
    - Use a 200+ mL bottle
    - 2.4 g NaH PO (monbasic, anhydrous)
    - 100 mL distilled water
    - Autoclave, then store in the fridge
- 200 mM Dibasic sodium phosphate
    - Use a 200+ mL bottle

    - 2.84 g NaH PO (dibasic, anhydrous)
    - 100 mL distilled water
    - Autoclave and store in the fridge
- Making Phosphate buffer – pH 7.8
    - Use 200+ mL bottle
    - 4.25 mL stock 200 mM Monobasic sodium phosphate
    - 45.75 mL stock 200 mM Dibasic sodium phosphate
    - 50 mL of distilled water
    - Adjust to pH 7.8 with either 200 mM Mono- or Dibasic sodium phosphate
    - Autoclave, cool and store in fridge
- Making Phosphate buffer – pH 7.0
    - Use 200+ mL bottle
    - 19.5 mL 200 mM Monobasic sodium phosphate
    - 30.5 mL 200 mM Dibasic sodium phosphate
    - 50 mL distilled water
    - Autoclave, cool and store in fridge
- To make the other two phosphate buffers, 6.7 and 7.4, use the 7.0 pH and pH it up or down with the 200 mM Dibasic or Monobasic sodium phosphate