

Carbonic Anhydrase Activity in the Epidermis of the Fiddler crab, *Uca pugilator*, during the Molting Cycle

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ABSTRACT

Periodic shedding of the crustacean exoskeleton is necessary for growth, a process known as ecdysis. The crustacean exoskeleton is composed of three major components, chitin, proteins, and largely, an inorganic matrix primarily consisting of CaCO_3 . Previous studies have revealed the chitin and protein components to be degraded by chitinolytic enzymes and collagenases, respectively, but the factor responsible for degradation of the inorganics has yet to be elucidated. Decomposition of CaCO_3 requires an acidic condition, a requirement fulfilled in most biological systems by the enzyme carbonic anhydrase's catalysis of CO_2 and H_2O to H_2CO_3 .

This project investigated the extent and degree of carbonic anhydrase's activity during the molting cycle of *Uca pugilator*. Epidermal tissue from fiddler crabs in various molt stages was assayed for enzymatic activity using fluorospectrophotometry. In the process, a methodology for translation of fluorescence readings into changes in acidity was developed.

Enzyme activity peaked during intermolt stage C and showed progressive decline in premolt stages D_0 , D_1 , and D_{3-4} followed by an increase in activity post-molt stage AB. Comparison of enzyme activity patterns to hemolymph titer patterns indicate high levels of molting hormones present at low levels of enzyme activity. However, injection of intermolt stage C crabs with the exogenous molting hormone resulted in a four-fold increase of enzyme activity. Overall results suggest the existence of an unaccounted for hormonal factor that co-regulates carbonic anhydrase activity in the epidermis with the molting hormone.

INTRODUCTION

Crustaceans are limited in the extent of their growth by the rigid exoskeleton, requiring the periodic shedding called ecdysis. The molting cycle consists of four periods: postmolt, intermolt, premolt, and ecdysis. In the premolt stage, calcium is released from the inorganic matrix of the exoskeleton and deposited in the blood system of the crustacean. New setae begin to form and the beginning of a new exoskeleton is laid down when a new cuticle is secreted (Chang, 1993). The old exoskeleton is shed during ecdysis, at which point the crab becomes soft-shelled. After molting, the exoskeleton hardens by deposition of calcium, at which point it is most vulnerable to cannibalism (Bliss, 1982). Postmolt period is followed by the intermolt period during which the crab is in normal conditions with a stable exoskeleton. Endocuticle formation continues throughout intermolt stage while the membranous layer that is the innermost layer of the exoskeleton is also laid down (Chang, 1993; Green, 1961; Lachaise et al., 1993). The molting cycle in *Uca pugilator*, or sand fiddler crab, can subsequently be defined by molt stages: A-B (postmolt), C (intermolt), D_0 (premolting), D_1 (premolting), D_{3-4} (premolting), and E (ecdysis), according to the setogenic method defined by Vigh and Fingerman (1985).

The molting cycle is controlled by ecdysteroid hormones collectively called molting hormones, of which 20-hydroxyecdysone is the predominant form. Produced and secreted by the Y-organs in crustacean, ecdysteroid hemolymph titers follow differential patterns of increasing immediately before ecdysis during premolt stages D_0 through D_4 . In postmolt,

ecdysteroid levels decrease rapidly and remained relatively low through intermolt stage C (Soumoff and Skinner, 1983; Hopkins, 1983).

Upon removal of the Y-organs during pre-molt stages D₀ and D₁, progression in molt stages towards ecdysis is halted altogether while those with glands removed in late pre-molt stages continued to undergo physiological processes preparing for ecdysis. In addition, progression towards ecdysis can be halted in fiddler crabs as late in the molt cycle as D₁ (Hopkins, 1983). Removal of eyestalks in a study by Abramowitz and Abramowitz (1940) resulted in more frequent molting, indicating that there is an inhibitory factor from the eyestalks. Molt-inhibiting hormone, responsible for the suppression of molting hormone, is secreted by the X-organ-sinus gland complex located in the eyestalks of crustacean (Hopkins, 1983). Nucleation of calcium carbonate crystals in invertebrate hard tissues involves acidic macromolecules, or proteins (Dodgson et al., 1991). Furthermore, carbonic anhydrase has been shown to be involved in invertebrate exoskeleton calcification (Endo et al., 2004).

The crustacean exoskeleton is composed of polysaccharides, lipids, and proteins encased in an inorganic matrix. In the decomposition of the exoskeleton, it has been found that the polysaccharides, consisting of mostly chitin, are broken down by chitinolytic enzymes chitinase and chitinase (Zou and Bonvillain, 2004). These enzymes are regulated by molting hormones secreted by the Y-organs. On the other hand, the proteinaceous portion of the exoskeleton is catabolized by collagenases, their activities are also affected by the molting hormone (Toups and Zou, 2009). However, it is unknown how the other major composing unit of the exoskeleton, the inorganic matrix, is broken down. Calcium carbonate is the principle compound making up the exoskeleton. Degradation of calcium carbonate requires an acidic environment. In many biological systems, the reaction catalyzed by carbonic anhydrase is the common source of hydrogen ions (Randall, 2002). The reaction involves conversion of carbon dioxide and water molecules into proton ions and bicarbonate:



The reaction is reversible and direction of catalysis based upon pH. Kinetics of the reaction carried out by carbonic anhydrase can be best explained by the Henderson-Hasselbach equation (Ed, 1991; Shingles and Moroney, 1997). This study investigated whether the activity of carbonic anhydrase in the epidermis of the fiddler crab is under control of the molting hormone, with an ultimate goal of gaining insight into the role of this acid-producing enzyme in the degradation of the old exoskeleton in premolt stage.

The project investigated the extent and degree of carbonic anhydrase activity during the molting cycle of *Uca pugilator*. Epidermal tissue extracted from fiddler crabs in various molt stages was assayed for its enzymatic activity and compared to its protein concentration. In the process of establishing the role of carbonic anhydrase in *Uca pugilator* molting cycle, a method for measuring the activity of carbonic anhydrase using fluorescence in relation to pH changes was also established.

METHODS AND MATERIALS

Female *Uca pugilator* were purchased from the Gulf Specimen Marine Laboratories (Panacea, FL). Crabs were stored at 19-20 ° C in tanks containing artificial seawater prepared with Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH), submitted to natural light cycles of

14 hours light followed by 10 hours darkness a day, fed raw oatmeal once a week, and allowed to acclimate to their new environment for at least 5 days before experimental study.

Determination of an individual fiddler crab's molt stage was accomplished using the setogenic staging technique of Vigh and Fingerman (1985). The method involves examination of pleopods using light microscopy whereby the extent of epidermis withdrawal from the cutaneous layer distinguishes crabs in postmolt stage A-B, Intermolt stage C, and Premolt stage D₀, D₁, and D₃₋₄. For the collection of epidermal tissue samples, epidermis was removed from the carapace of each crab, homogenized with 600 μ L 0.5 mM pH 6 HEPES-KOH, then centrifuged at 10 000 g for a period of 10 minutes. Supernatant tissue samples were isolated into tubes, snap-frozen using liquid nitrogen, and then stored at -80°C to await analysis.

Calibration

Eight solutions of 0.5 mM HEPES-KOH buffers with pH values ranging from 6.8-8.0 were prepared for the establishment of a calibration curve using the RF-5301 PC Spectrofluorophotometer. A volume of 500 μ L 200 nM pyranine followed by 1500 μ L 0.5 mM HEPES-KOH was treated to excitation wavelengths ranging from 350 nm to 550 nm and emission wavelengths collected at 512 nm.

Enzyme Analysis and Protein Assay

Epidermal tissue from 5-10 fiddler crabs representing each molting stage was collected into 600 μ L 0.5 mM pH 6 HEPES-KOH buffer. The epidermal tissue was homogenized and then centrifuged at 10 000 g for 10 min. The supernatant was isolated into tubes, snap-frozen using liquid nitrogen, and then stored at -80°C to await analysis.

Solutions, including 5mM KCO₃, 0.5 mM bicine-KOH and 1 mM HEPES-KOH containing proteinase-inhibitor, were allowed to rest to room temperature before use while samples were thawed and remained on ice throughout the enzyme and protein assay. All components for sample enzyme analysis; 500 μ L 200 nM pyranine, 350 μ L 1 mM HEPES-KOH with proteinase inhibitor at a pH of 6.0, 150 μ L epidermis sample, were added to and mixed in glass tubes. Contents of tubes were individually poured into the sample cuvette, placed in the spectrofluorometer, zeroed, 1000 μ L of 5 mM KHCO₃ 0.5 mM bicine-KOH at a pH of 8.0 added to the cuvette, and finally fluoresced at 466 nm. A duplicate set of samples were prepared in the same way and fluoresced at 413 nm. A protein assay was carried out for all samples using the Bradford method.

Hormone Injection

Forty intermolt fiddler crabs were selected and allowed to rest at room temperature to acclimate for a period of 24 hours. Experimental injection fluid was prepared by first dissolving 20-hydroxyecdysone in ethanol for a concentration of 2 mg/mL to form a stock hormone solution. One aliquot of the stock solution was mixed with nine aliquots of Pantin's crustacean saline for a final injection solution of 0.2 mg/mL 20-hydroxyecdysone with 10% v/v ethanol. After the initial waiting period was over, crabs were injected with either a solution of 10% v/v ethanol in Pantin's crustacean saline or 0.2 mg/mL 20-hydroxyecdysone and 10% v/v ethanol. Crabs injected with its appropriate injection solution at a dosage of 5 μ g/g through the arthroal membrane at the base of a walking leg. Crabs were then allowed to mature for a period of 48 hours before sacrifice and collection of epidermal samples. Epidermal samples were collected in 600 μ L 0.5 mM pH 6 HEPES-KOH, homogenized, centrifuged, supernatant isolated, and stored at -80°C to

await enzyme analysis. Enzyme analysis was carried out in the same fashion as reported in the last section. Enzyme activity between control and experimental crabs were then compared.

RESULTS

The Henderson-Hasselbach equation describes carbonic anhydrase activity equilibrium:

$$\text{pH} = \text{pK}_a + \log \left[\frac{(F_s/F_{is}) / (F_s/F_{is})_m}{1 - (F_s/F_{is}) / (F_s/F_{is})_m} \right] \quad (1)$$

where F_s is fluorescence sensitive (466 nm), and F_{is} is fluorescence insensitive (413 nm), and the subscript "m" indicates the maximum value. Equation 1 above was used to calculate changes in acidity throughout various stages of the molting cycle by manipulation into a modified form. The first step in modification of the Henderson-Hasselbach equation involved the conversion of the ratio F_s / F_{is} to F . PH and pK_a were also converted to their negative logarithm values so that the equation would appear as so:

$$-\log[\text{H}^+] = -\log K_a + \log \left[\frac{F/F_m}{1 - F/F_m} \right] \quad (2)$$

Condensing equation 2 results in:

$$-\log [\text{H}^+] = \log \left[\frac{F/F_m}{1 - F/F_m} \right] / K_a \quad (3)$$

A number of simplifications lead us to the final product, which was used in this project:

$$[\text{H}^+] = K_a F_m (1/F) - K_a \quad (4)$$

Fluorescence values of samples at wavelength 466 nm and 413 nm were used in the calculation of hydrogen ion concentration by combining the manipulated Henderson-Hasselbach equation (see equation 4) and the linear equation earlier established. The resulting linear regression in Fig. 1B, shows a graph of hydrogen ion concentration as a function of reciprocal fluorescence values taken from the results found in Fig. 1A where the fluorescence in relation to changes in pH were found when a series of buffers ranging in acidity were excited at 466 nm. Enzymatic activity in the unit of unit/ μg protein/s can be determined by the formula:

$$\text{Enzyme activity} = \Delta\text{pH}/[\text{protein}]/\text{time} \quad (5)$$

where ΔpH is the change in pH between time zero and time t , or 3.2 s; $[\text{protein}]$ is the concentration of protein of the sample; and time is the amount of time during which the enzyme was active. The Bradford method was used to assay for protein concentrations in epidermal tissue extracts.

RESULTS

Two distinctions in the calibration curve shown in Fig. 1A are noteworthy. First, all 0.5 mM Hepes-KOH solutions fluoresced at the same point when excited with wavelengths measuring at 413 nm. Secondly, less acidic 0.5 mM Hepes-KOH solutions fluoresced at a greater and proportional rate to more acidic solutions when excited with wavelengths measuring at about 458 nm. Literature research confirms 466 nm to be the excitation wavelength at which pyranine is most sensitive when emission wavelengths were collected at 512 nm (Shingles and Moroney

1997). Therefore, reciprocal fluorescence values for each 0.5 mM Hepes-KOH solution obtained when excited at 466 nm were graphed according to its respective solution's hydrogen ion concentration to achieve Fig. 1B, serving as calibration curve for this project. The resulting linear regression was $y = 4 \times 10^{-8}x - 2 \times 10^{-8}$ with an R^2 -value of 0.9954.

Varying supernatant volumes in solution mixtures for analysis resulted in proportional rate changes in fluorescence as shown in Fig. 2A and Fig. 2B where doubling supernatant volumes resulted in two-fold fluorescence value changes. Curved lines in Fig. 2A represent analysis of samples by the F_s value, or 466 nm, whereas lines following the straight trend were excited by light at the F_{is} value, 413 nm. Fluorescence changes were considered only up to 3.2 s. Rates of enzyme activity were calculated for each supernatant volume. Differences in fluorescence between F_s and F_{is} at times 3.2 s and 0 s were compared to their protein concentrations, as shown in Fig. 2B.

The previously found linear regression from Fig. 1B, $y = 4 \times 10^{-8}x - 2 \times 10^{-8}$, was used to correlate fluorescence values obtained through spectrofluorophotometer readings to changes in acidity by the substitution with Equation 4. Where hydrogen ion concentration is substituted for y , $K_a F_m$ replaces the linear regression's slope value of 4×10^{-8} , and K_a is substituted with 2×10^{-8} so that x is $1/F$.

A comparison of enzyme activity throughout the various molt stages, A-B, C, D_0 , D_1 , and D_{3-4} can be found in Fig. 3. Intermolt stage C-crabs exhibited the greatest enzyme activity while pre-molt stage D-crabs showed progressively lower rates of enzyme activity with D_{3-4} crabs being the lowest in activity.

When crabs were treated to an injection solution containing either 10% v/v ethanol or molting hormone 20-hydroxyecdysone in 10% v/v ethanol, those injected with the exogenous ecdysteroid exhibited a quadruple-fold change in enzyme activity (Fig. 4).

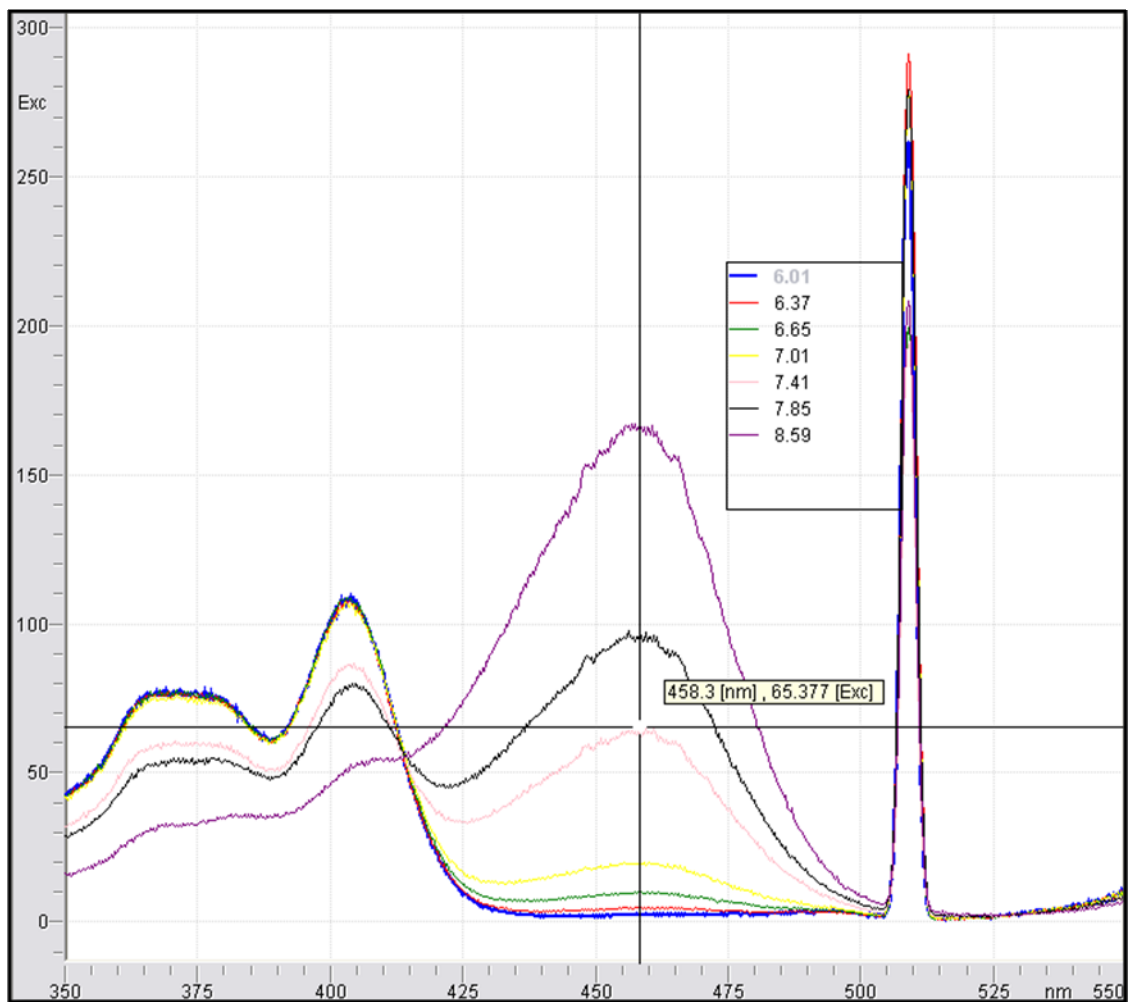


Figure 1A. Fluorescent curve used for calibration resulting from analysis of a series of Hepes buffer solutions ranging in pH.

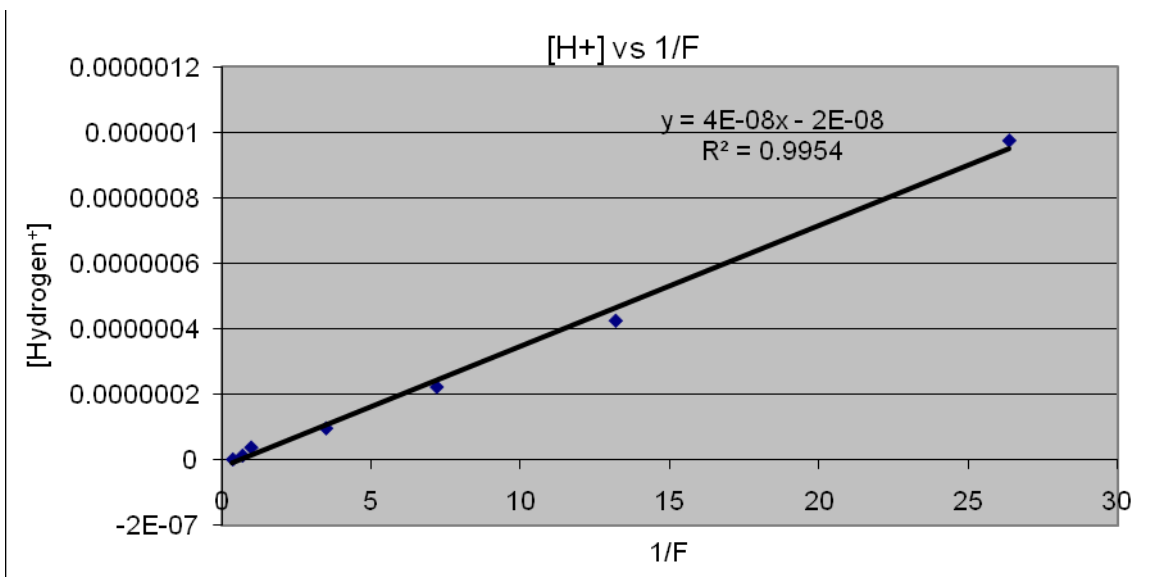


Figure 1B. Linear regression of calibration curve shown in Fig. 1 obtained by graphing reciprocal each solution's fluorescent values at 466 nm against its hydrogen ion concentration determined using the aid of a pH meter.

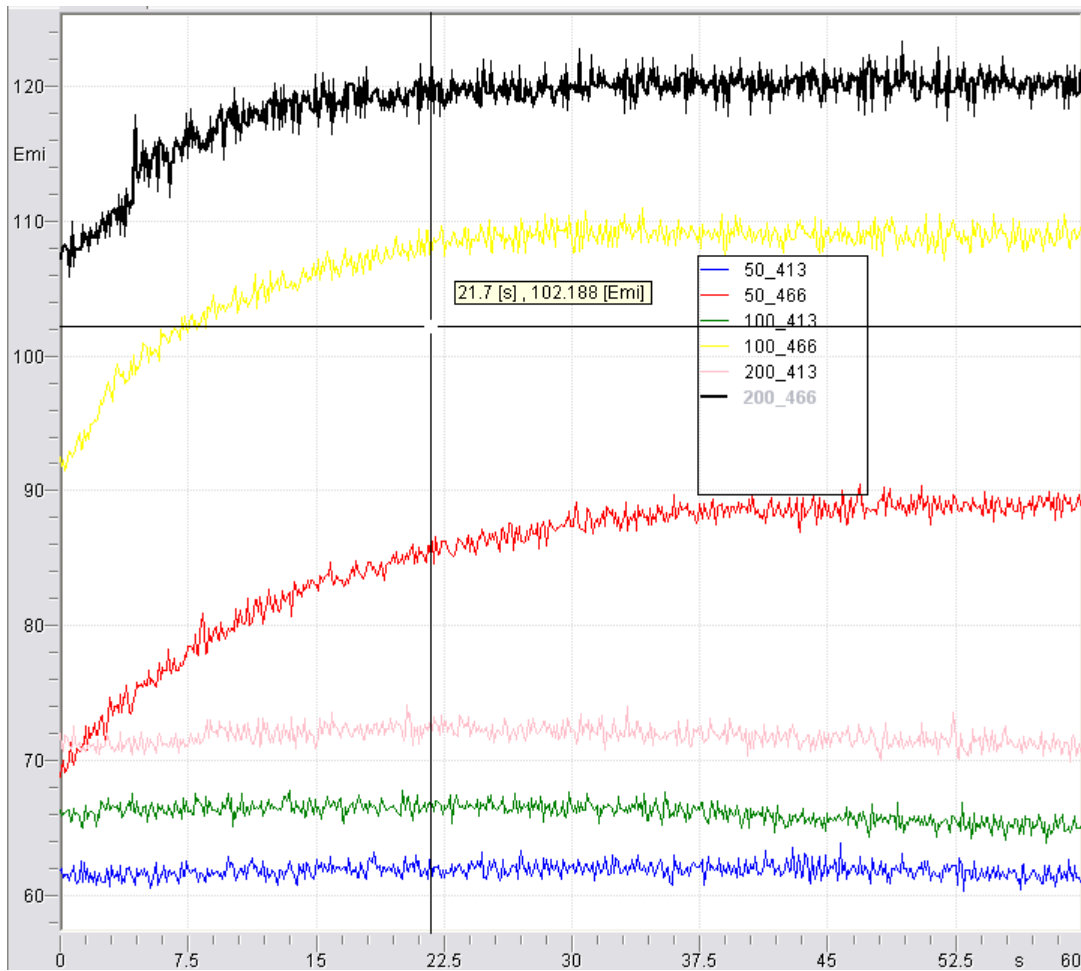


Figure 2A. Fluorespectrophotometer results showing changes in enzyme activity proportional to changes in supernatant.

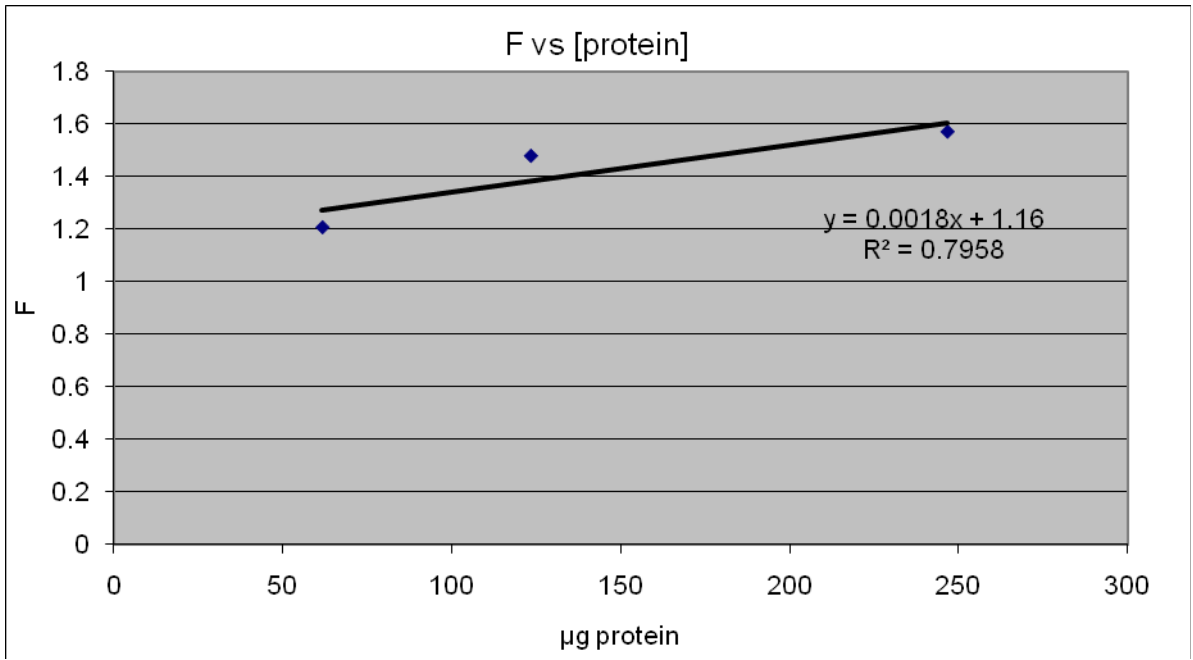


Figure 2B. Proportional changes in fluorescence as protein concentration is changed.

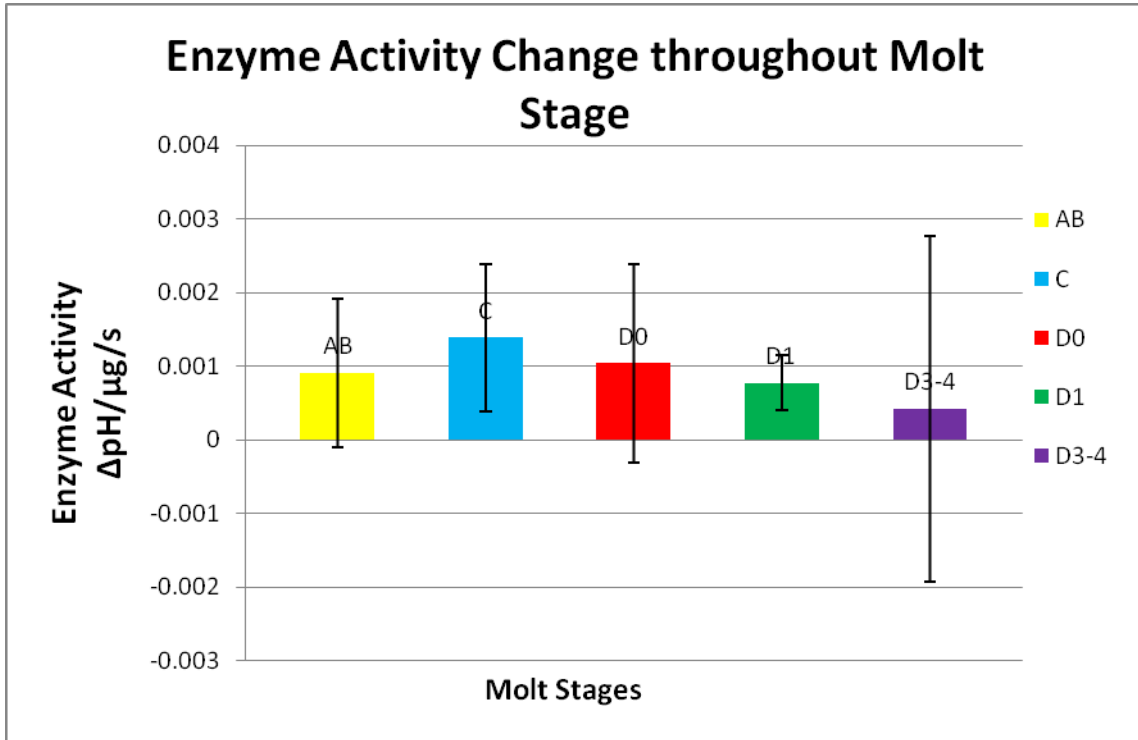


Figure 3. Enzyme Activity changes with standard deviations throughout post-molt stage AB, intermolt stage C, and pre-molt stages D₀, D₁, and D₃₋₄.

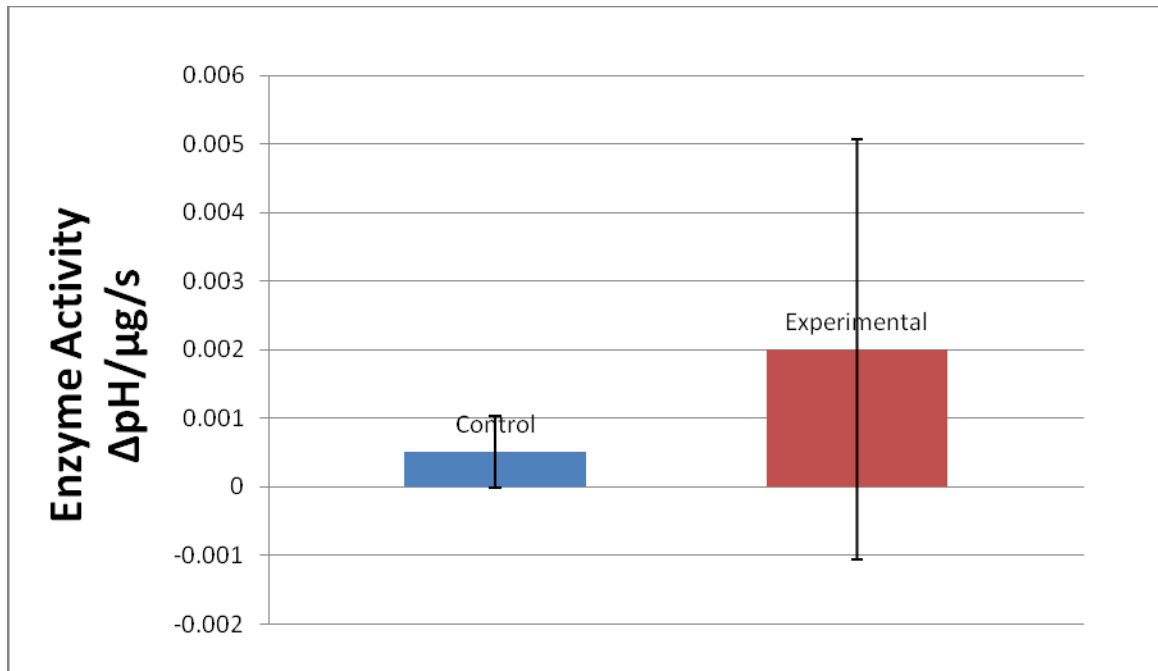


Figure 4. Enzyme activity with standard deviation in crabs injected with control-injection fluid versus injection with molting hormone 20-hydroxyecdysone.

DISCUSSION

The method used for the measurement of carbonic anhydrase activity in this project is fluorometric. The combined use of a fluorophore with fluorescence spectroscopy allowed direct measurement of bicarbonate's dehydration reaction. Shingles and Moroney (1997) showed pyranine to have no toxicity towards carbonic anhydrase activity while exhibiting great sensitivity towards pH changes within a range of 6.0 to 8.0. Usefulness of pyranine exists within a fluorescence range (Shingles et al., 1997). Clement and Gould (1981) reported pyranine to have fluorescence intensity at 510 nm. Pyranine has a 7.2 pKa, near neutrality, which is ideal for carbonic anhydrase catalysis. Fluorescence readings were only valuable in the project with the successful modification of the Henderson-Hasselbach equation as previously adapted by Shingles and McCarty (1994) and further transformed for translation into enzyme activity, given in pH changes over time with respect to protein concentration.

Initial stages of the project included verification of methodology parameters including optimal pyranine fluorescence, wavelengths at which pyranine was most sensitive and insensitive to readings, as well as establishment of a calibration curve. Fis and Fs were confirmed to be 413 nm and 466 nm, respectively. The calibration curve was established by a

linear regression between each buffer solution's calculated hydrogen ion concentration and its reciprocal fluorescence value obtained at the determined F_s value. The acquired linear equation was then used in modification of the Henderson-Hasselbach equation for the translation of fluorescence readings into changes in pH. A simple test involving the variation of supernatant volumes analyzed through the fluorospectrophotometer resulted in proportional changes in fluorescence readings, thereby confirming sample fluorescence to be dependent upon the amount of sample proteins.

Carbonic anhydrase activity in the epidermis was found to vary during the molting cycle, with the peak activity occurring in intermolt stage. This pattern of changes in enzymatic activity does not appear to correlate with the hemolymph titer of ecdysteroids during the molting cycle of *Uca pugilator*. Hopkins (1983) found that ecdysteroid levels in the hemolymph of *Uca pugilator* are low in postmolt stage AB and intermolt stage C and peak twice in premolt stage. Yet, the injection results of the present study show that the activity of epidermal carbonic anhydrase was inducible by 20-hydroxyecdysone, suggesting that the enzymatic activity is impacted by the molting hormone. The lack of correlation between the profiles of epidermal carbonic anhydrase activity and ecdysteroid levels during the molting cycle may indicate the involvement of an additional hormonal factor in the regulation of carbonic anhydrase activity in the epidermis. Presumably, this unknown factor controls the enzymatic activity in intermolt stage while the molting hormone regulates the enzymatic activity in premolt stage. The future studies should be directed toward the unraveling of this unknown factor.

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