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Ca^{2+}ATPase, Carbonic Anhydrase and Alkaline Phosphatase Activities in Red Sternum Mud Crabs (*Scylla serrata*)

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ABSTRACT

Red sternum is a poorly understood symptom affecting mud crab aquaculture in Thailand. This work presents the results of enzymatic activities in mud crab exhibiting the red sternum symptom. The enzymatic activities of Ca^{2+}ATPase, carbonic anhydrase and alkaline phosphatase were analyzed in five tissues (muscle, gill, hepatopancreas, integument and hemolymph) from symptomatic and asymptomatic mud crabs. The main features of the symptom include reddish colored stripes on the abdominal thorax, sternum and chelae, swollen hepatopancreas, milky hemolymph and opaque and whitish muscle. The most distinctive change could be observed in the hemolymph which enabled classification of its severity into three groups—group I: translucent brown hemolymph, group II: cloudy orange hemolymph and group III: milky unclotted hemolymph. Ca^{2+}ATPase levels were highest in the muscle of asymptomatic crabs. This enzyme activity tended to increase in all tissues of symptomatic crabs in groups I and II, especially in the gill of group II where it was fivefold higher than in asymptomatic crabs. Carbonic anhydrase and alkaline phosphatase values were highest in the hepatopancreas of asymptomatic crabs. Alkaline phosphatase decreased in all tissues of the three groups of symptomatic crabs. Carbonic anhydrase levels were highly variable with a decreasing trend in most tissues of groups I and II.

Keywords: Ca^{2+}ATPase, carbonic anhydrase, alkaline phosphatase, red sternum crab, *Scylla serrata*

INTRODUCTION

The mud crab, *Scylla serrata*, is a commercially important crab especially for the production of "soft-shell crab". Soft-shell crabs are the post-molt crab and at this stage, the whole animal can be consumed because of its soft exoskeleton. With the increasing demand in both the domestic and international markets, aquaculture of soft-shell crab has been developed along the coastline of Thailand. In these aquaculture systems, red sternum symptom is often found. This symptom causes a severe decrease in the production of soft-shell crabs (Salaenoi *et al*., 2006). The external features of the symptom include reddish colored stripes on the abdominal thorax, sternum and chelae, swollen hepatopancreas, milky hemolymph and opaque and whitish muscle. The shells of red sternum crabs are softer than those of normal crabs while the muscle is loose and has slow motility (Salaenoi *et al*., 2006). Ca^{2+}ATPase, carbonic anhydrase

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and alkaline phosphatase are essential enzymes that play various physiological roles such as ion transport, gas exchange, acid-base balance, osmoregulation and biomineralization (Ahearn *et al.*, 2004; Wheatly *et al.*, 2007; Gaume *et al.*, 2011). The changes in these enzymatic activities could be interesting biomarkers that reflect the actual physiological status of the organisms.

Alkaline phosphatase is an enzyme that maintains the orthophosphate pool, the transfer of phosphoryl groups and the hydrolysis and esterification of metabolites moving across the membrane (Sandhu and Jande, 1982). Alkaline phosphatase is involved in the absorption of phosphatase and calcium for the shell formation of *Scylla serrata* (Park *et al.*, 2001). The alkaline phosphatase activities in the hemolymph of mud crab infected with white spot syndrome virus was reported to be higher than in the control group (Liu *et al.*, 2011). A correlation between the enzyme activities of alkaline phosphatase and carbonic anhydrase was seen during shell formation in European abalone (Gaume *et al.*, 2011). Shell biomineralization requires several enzymes to accelerate the interaction of bicarbonate and calcium to form $CaCO_3$ (Fabritius and Ziegler, 2003; Gaume *et al.*, 2011). Carbonic anhydrase is an enzyme involved in both the respiration and biomineralization process which catalyzes the hydration of carbon dioxide to form carbonic acid and then dissociates to H^+ and HCO_3^- ions; it maintains an ionic and electrical gradient which is involved in physiological processes such as gas exchange, acid-base balance, osmoregulation and formation of calcium skeletons (Kupriyanova and Pronina, 2011). Ca^{2+}ATPase is a membrane-bound enzyme that regulates the intracellular calcium within a narrow range by transporting calcium against the concentration gradient (Wheatly,1999). Calcium performs a role as an intracellular messenger of hormonal action and is also involved in the biomineralization of crustaceans (Ahearn *et al.*, 2004). Ca^{2+}ATPase can be located on either apical or basolateral membranes depending upon the direction of transport at different stages in the molting cycle of the crustacean (Wheatly, 1999).

A current literature search has found no report on these enzymatic activities in symptomatic crab. Therefore informative correction of the enzyme activities of Ca^{2+}ATPase, carbonic anhydrase and alkaline phosphatase will provide a better understanding on the deteriorative effect of this symptom.

MATERIALS AND METHODS

Animal preparation

Normal and red sternum mud crabs (*Scylla serrata*) having 6.5–8.5 cm carapace width were collected from a soft-shell crab farm in Chantaburi province, Thailand. The crabs were transferred to the laboratory and kept in individual aquariums containing 20–25 ppt water salinity. The crabs were handled in a humane manner and prior to experimentation, the crabs were anaesthetized in cold water at 4 ^oC for 1 min. Hemolymph samples were withdrawn from the sinus at the base of the pereiopods and 10% tri-sodium citrate was used as anticoagulant at the ratio of 5:1. The hepatopancreas, integument, gills and muscle were dissected and kept at -20 ^oC for further analysis.

Crude enzymes preparation

Tissues were homogenized in 100 mM Tris-HCl buffer (pH 8.0) and then centrifuged at 4,500×*g* for 30 min at 4 ^oC. The clear supernatant was kept and stored at -20 ^oC for the analysis of alkaline phosphatase and carbonic anhydrase. To collect Ca^{2+}ATPase, the tissues were homogenized in 0.25 M sucrose, 6 mM EDTA, 20 mM imidazole (pH 6.8) and 0.1% deoxycholate was added prior to use. The homogenate was centrifuged at 4,500×*g* for 30 min at 4 ^oC. The supernatant was further centrifuged at 7,500×*g* for 60 min at 4 ^oC. The supernatant was then used for the analysis of Ca^{2+}ATPase activity.

Analysis of enzyme activities

Ca^{2+}ATPase

Ca^{2+}ATPase activity was determined according to the method of Cameron (1989). A sample of 100 µL of crude extract was added to 1 mL of 20 mM imidazole buffer (pH 8.0), 5 mM ATP, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. Blanks of ATP were replaced by 5 mM EGTA (ethylene-glycol-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid). The reaction mixture was incubated at 30 °C for 30 min and the reaction was stopped with 1 mL of 10% trichloroacetic acid (w/v). After centrifuging at 3,500×*g* for 15 min, the supernatant was brought to 10 mL with distilled water, and 1.0 mL of 2.5% ammonium molybdate (in 3N H_2SO_4) was added. The mixture was shaken and then 0.4 mL of 0.25% aminonapthosulfonic acid (in 0.5N NaOH) was added and mixed. After 10 min, the absorbance was measured at 660 nm with a spectrophotometer (V-550; JASCO Inc; Easton, MD, USA). The Ca^{2+}ATPase specific activity was determined from the difference of inorganic phosphate (Pi) formation in the presence and absence of Ca^{2+} and calculated as µmoles Pi.min^{-1} per milligram protein.

Carbonic anhydrase

The carbonic anhydrase content was determined using a modified method of Armstrong *et al*. (1966) and Pocker and Stone (1967). The assay system contained 1mL of 3 mM *p*-nitrophenyl acetate with 40 µL of crude extract dissolved in 1 mM phosphate buffer at pH 7.2. The measurement was carried out at 348 nm.

Alkaline phosphatase

The alkaline phosphatase activity was determined according to the method described by Messer *et al.* (1975) and Villanueva *et al.* (1997). A sample of 40 µL of crude extract was added to a 2.2 mL buffer-substrate mixture consisting of 50 mM Glycine-NaOH buffer (pH 10.0), 0.05 mM $ZnCl_2$, 0.23 mM $MgCl_2$ and 4.5 mM *p*-nitrophenyl phosphate. The reaction mixtures were incubated at 30 °C for 30 min. The reaction was stopped by adding 0.5 mL 0.1 N NaOH and then centrifuged at 3,500×*g* for 10 min and the supernatant was collected. The amount of *p*-nitrophenol was measured spectrophotometrically at 405 nm. Enzyme activity was expressed as µmoles *p*-nitrophenol.min^{-1} per milligram protein.

Protein measurement

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Morphology of red sternum mud crab

The characteristic changes in the red sternum mud crab from the normal crab are the thin and brittle carapace, reddish colored stripes on the abdominal thorax, sternum and chelae joint, the deformation of gills and the hepatopancreas, a milky hemolymph and loose muscle (Figure 1). Additionally, the red thoracic-sternum crabs are inactive and slow moving with a low feeding rate, incomplete withdrawal during ecdysis, and eventual mortality. However, the most distinctive change could be observed in the hemolymph which enabled classification into three levels of deterioration—group I: translucent brown hemolymph, group II: cloudy orange hemolymph and group III: milky unclotted hemolymph (Table 1).

Ca^{2+}ATPase activity

The activities of Ca^{2+}ATPase in the hemolymph, integument, gill, hepatopancreas and muscle of normal crabs ranged from 34.48 ± 7.51 to 323.88 ± 8.27 µmol.min^{-1} per milligram protein and from 25.91 ± 1.17 to 1,018.36 ± 20.36 µmol. min^{-1} per milligram protein in red sternum crabs. The Ca^{2+}ATPase activities of red sternum groups I and II increased in all tissues especially in the gill of group II and was fivefold higher than in the normal crabs while the red sternum group III showed lower activities of Ca^{2+}ATPase than the normal crab except in the gill and hepatopancreas (Figure 2).

Figure 1 Morphology shown in each column of images for: (A) Normal crab; (B) Red sternum crab, group I; (C) Red sternum crab, group II; (D) Red sternum crab, group III.

Table 1 Characteristics and behavior of normal and red sternum mud crabs.

Organ, tissue and observed behavior	Normal mud crab	Red sternum mud crab		
		Group I	Group II	Group III
Carapace	Hard	Brittle, thin	Brittle, thin	Brittle, thin
Abdomen	White to pale yellow	Pink stripes	Red stripes	Red stripes
Sternum	White to pale yellow	Red stripes	Red stripes	Red stripes
Chelae and joint	White to pale yellow	Red	Red	Red
Integument	Fresh	Pale	Pale	Soft, pale
Hepatopancreas	Fresh and rigid	Swollen	Swollen	Swollen
Gills	Fresh and rigid	Pale and soft	Pale and soft	Pale and soft
Muscle	Fresh and rigid	Loose	Loose	Loose
Hemolymph color	Colorless to pale blue	Translucent brown	Cloudy orange	Milky
Blood clotting	Clotted after leaving at room temp. for 1 min	Clotted after leaving at room temp. for 5 min	Unclotted	Unclotted
Locomotive activity	Highly active	Slow movement	Very slow movement	Paralyzed

Alkaline phosphatase activity

Alkaline phosphatase levels in the hemolymph, integument, gill, hepatopancreas and muscle ranged from 0.88 ± 0.04 to 49.17 ± 2.21 μmol.min^{-1} per milligram protein in normal crabs and from 0.19 ± 0.01 to 9.07 ± 0.59 μmol. min^{-1} per milligram protein in red sternum crabs. A decrease in the activity of this enzyme was found in the hemolymph of groups II and III as well as in the integument, gill, hepatopancreas and muscle of all three groups. In contrast, the activity in the hemolymph of group I was 0.44-fold higher than in the normal crab (Figure 2).

Carbonic anhydrase activity

Carbonic anhydrase levels in the hemolymph, integument, gill, hepatopancreas and muscle ranged from 106.02 ± 0.17 to 659.87 ± 0.36 μmol.min^{-1} per milligram protein in normal crabs and from 33.31 ± 0.46 to 302.54 ± 0.32 μmol. min^{-1} per milligram protein in red sternum crabs. A decrease in the activity of carbonic anhydrase was observed in the hemolymph and muscle of all three groups. However, the activities of this enzyme were varied in the integument, hepatopancreas and gill (Figure 2).

DISCUSSION

The studies on the red sternum crabs revealed symptoms which included thin and brittle carapace, reddish colored stripes on the abdominal thorax, sternum and chelae joint, the deformation of gills and the hepatopancreas, a milky hemolymph and loose muscle, while red sternum crabs were characterized by inactivity, slow movement, a low feeding rate, incomplete withdrawal during ecdysis and eventual mortality. Andersen *et al.* (2000) reported a new shell disease of non-infectious *Scylla serrata* from Australia,

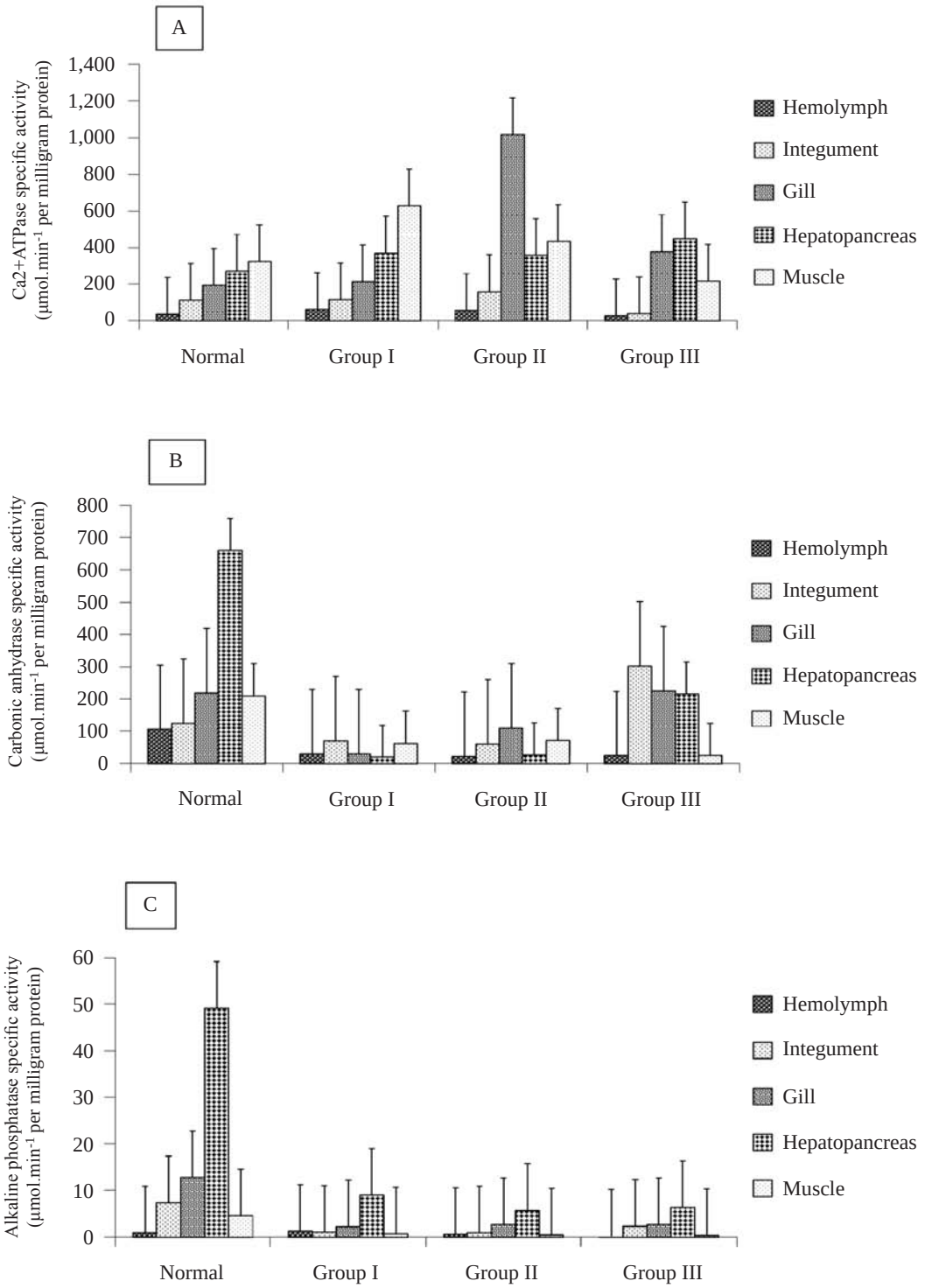

Figure 2 $Ca2^{+}$ATPase (A), carbonic anhydrase (B) and alkaline phosphatase (C) specific activity in hemolymph, integument, gill, hepatopancreas and muscle of normal and red sternum crabs. Values are expressed as mean + SD error bar for nine observations (three animals × three replications).

commonly called rust spot. It initially appeared as well-circumscribed orange-colored areas on the dorsal carapace due to a defect in the formation of the endocuticular layer rather than pathogenic cuticular erosion.

Another noticeable change in red sternum crabs was the hemolymph which could be classified into three consecutive groups—group I: translucent brown hemolymph, group II: cloudy orange hemolymph and group III: milky unclotted hemolymph. A milky hemolymph has been reported in bitter crab disease (Meyers *et al.*, 1996; Stentiford and Shields, 2005) and milky hemolymph syndrome (MHS) in snow crab *Chionoecetes opilio* (Kon *et al.*, 2011). MHS showed as a milky or opaque coloration of the hemolymph which resembled that of red sternum crabs. The yellow or ivory discoloration on the ventral shell and uncalcification of the walking legs arthrodial membranes were apparent in severely affected MHS crabs. It was found that MHS was associated with an intranuclear bacilliform virus (Kon *et al.*, 2011). A previous report of protein in the hemolymph analyzed by SDS-PAGE showed an intense band of oxyhemocyanin (approximately 75 kD) in normal crabs but none in red sternum crabs (Salaenoi *et al.*, 2006). An increase in calcium, magnesium and iron and a decrease in copper, manganese and zinc were reported in red sternum crabs (Salaenoi *et al.*, 2006). Hemocyanin and phenoloxidase belong to the family of copper proteins; they play significant roles in the crustacean immune response (Sritunyalucksana and Söderhäll, 2000; Terwilliger, 2007; Fredrick and Ravichandran, 2012). Phenoloxidase was also important in the hardening of the new exoskeleton after molting (Terwilliger, 1999). Crab hemocyanin may be converted to phenoloxidase during rapid sclerotinization at the molting stage (Terwilliger, 2007). Thus, the soft shell of red sternum crab, unclotting and colorization of the hemolymph are possibly due to the depletion of oxyhemocyanin.

The enzymatic activities of red sternum crabs showed that alkaline phosphatase in the integument, gill, hepatopancreas and muscle sunbstantially decreased in all three groups. In contrast, in the hemolymph of group I, this enzyme remained 0.44-fold higher than in normal crab and decreased in groups II and III. Gaume *et al*. (2011) reported a role of alkaline phosphatase in the initiation of mineralization during early shell formation in the European abalone *Haliotis tuberculata*. The alkaline phosphatase was also important in the absorption of phosphatase and calcium for the formation of shell in the crab *Scylla serrata* (Park *et al.*, 2001). The increase in alkaline phosphatase in red sternum crab group I indicated the initiation of shell formation after ecdysis, the decreasing activities in groups II and III were possibly caused by the interruption of mineralization.

Increased activity of Ca^{2+}ATPase was found in red sternum crabs especially in the gill of group II at fivefold higher levels than in the normal crabs. Conversely, the carbonic anhydrase activities of all three groups were lower than in the normal crabs except in the integument and gill of group III that were higher than in normal crabs. Ca^{2+}ATPase performs a role in regulating intracellular calcium which is not only a messenger of hormonal actions but also is involved in the biomineralization of crustaceans (Ahearn *et al.*, 2004). Ca^{2+} and carbonate are necessary for the calcification of the fresh water common pond snail *Lymnaea stagnalis*, in which the embryos take up Ca^{2+} from the media and utilize endogenous HCO_3^-/CO_3^{2-} produced by carbonic anhydrase that catalyzes the hydration of CO_2 (Ebanks *et al.*, 2010 a, b). A role of carbonic anhydrase is in the enhancement of the bicarbonate ion when rapid $CaCO_3$ precipitation is required during shell formation in the European abalone *Haliotis tuberculata* (Gaume *et al.*, 2001). Red sternum crabs are post-molt crabs which require a rapid uptake of calcium from the environment. The current observations showed a decrease in carbonic anhydrase, which should thus interrupt the formation of $CaCO_3$ and lead to the accumulation of Ca^{2+}. Therefore, Ca^{2+}ATPase

activity in the gill of red sternum crabs is increased to regulate the intracellular calcium. This suggestion is supported by Wheatly *et al.* (1999) who reported that activity of Ca^{2+}ATPase at the brachial epithelium of crayfish is correlated with unidirectional Ca^{2+} influx that is taken up from external water. Interruption of the calcium regulatory process can become a potential hazard to the neuromuscular performance as well as the molting process (Ahearn *et al.*, 2004).

Although there is no clue as to the cause of red sternum in mud crab, the changes in the three enzyme activities help to gain an understanding of the progression of this symptom and how it affects the biomolecular mechanisms which maintain its normal condition.

CONCLUSION

Five tissues of red sternum crabs were analyzed for Ca^{2+}ATPase, carbonic anhydrase and alkaline phosphatase. The study showed elevated levels of Ca^{2+}ATPase in the gill hepatopancreas and muscle while carbonic anhydrase and alkaline phosphatase activities were decreased in red sternum crabs. The decrease in alkaline phosphatase and carbonic anhydrase should interrupt the formation of $CaCO_3$ thus leading to the accumulation of Ca^{2+} and elevation of the level of Ca^{2+}ATPase activity. The changes in the activity levels of these enzymes implied the disturbance of the mechanisms which maintain its normal condition.

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