Activities of Alkaline Phosphatase and Ca^{2+}ATPase over the Molting Cycle of mud Crab (*Scylla serrata*)

J. Salaenoi, A. Thongpan, M. Mingmuang

Abstract—The activities of alkaline phosphatase and Ca^{2+}ATPase in mud crab (*Scylla serrata*) collected from a soft-shell crab farm in Chantaburi Province, Thailand, in several stages of molting cycle were observed. The results showed that the activity of alkaline phosphatase in gill after molting was highly significant ($p<0.05$) comparing to those at intermolt and premolt stages. The activity profiles of alkaline phosphatase in integument and haemolymph were similar showing a decrease from intermolt to 2-week premolt stage and increased during 2-day premolt to 6-h postmolt stage before dropping at 7-day postmolt stage, while this enzyme in the gill was quite low at intermolt and premolt stages. For Ca^{2+}ATPase, the activity profiles in gill and integument corresponded to the molting variation, especially the activities increased during 5-7 day postmolt stage were at highly significant levels ($p<0.05$) comparing to those at premolt and early postmolt stages. The highest activity of Ca^{2+}ATPase in haemolymph was found at 2-week premolt stage ($p<0.05$). Changes in alkaline phosphatase and Ca^{2+}ATPase activities over the molting cycle clearly indicated their active functions on calcification.

Keywords—*Scylla serrata*, molting cycle, alkaline phosphatase, Ca^{2+}ATPase

I. INTRODUCTION

MUD crab, an economic aquatic animal, increases the body size through molting. The time interval of molting depends largely on their ages and sizes. It takes a longer interval time for older and larger ones. There are several factors contributed to structural changes and softening of shells during molting cycle. One of the major factors involved is enzyme. Proteinases involve indigestion of protein and chitinolytic enzymes cleave chitin into *N*-acetylglucosamine [1]. Carbonic anhydrase also plays an important role in molting cycle because the animals mobilize CaCO$_3$ layer during the postmolt formation of the new exoskeleton. Apart from these enzymes, alkaline phosphatase has been informed to associate with the maintenance of orthophosphate pool, transfer of phosphoryl groups, hydrolysis and esterification of metabolites [2]. This enzyme is also shown to connect with absorption and transport mechanisms in several organs, and has a close relationship with Ca^{2+}ATPase [3]. Alkaline phosphatase promotes precipitation of calcium phosphate complexes within the vesicles [4] and joins with Ca^{2+} ATPase in calcification [5].

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Calcification also requires the active transport of calcium while ATP acts as a possible source of energy for calcium pump. However, calcium resorption and deposition directly involve Ca^{2+}ATPase while the changes of epithelial cell size and shape during molting is believed to depend on the net movement of calcium ion into and out of the carapace where the reactions of chitin degradation and chitin biosynthesis occur.

Therefore, knowing the activities of alkaline phosphatase and Ca^{2+}ATPase in the target organs of interest, i.e., gill, integument and haemolymph could enable us to understand the mechanism of molting and could be used as a guideline to synchronize the molting time in mud crab for better management of mud crab farming and harvesting, especially for soft-shell crab production which is increasingly popular as delicacies and is highly demanded in the world market.

II. MATERIALS AND METHODS

A. Animal preparation

Mud crabs (Scylla serrata) having 65-90 mm in carapace width (about 95-130 g in weight) were collected from a soft-shell crab farm at Chantaburi Province, Thailand. Each crab was transferred to an individual closed system aquarium having 20-25 ppt water salinity at Kasetsart University and fed ad libitum with freshly chopped fish until the experiments were started. The dactylopodite and propodus were examined to identify the stages in molting cycle. The interest stages of molting cycle are divided into 8 stages [6]: C (intermolt), D1 (2-week premolt), D2 (1-week premolt), D3 (2-day premolt), A1 (6-hour postmolt), A2 (24-hour postmolt), B1 (5-day postmolt) and B2 (7-day postmolt). Three replicates were done in each stage and 9 crabs were used for each stage.

B. Enzyme assay

The animals were anaesthetized in cold water at 4 ^{o}C for 1 min, then sacrificed. Gills and integument tissues were dissected and weighed immediately. Haemolymph samples were withdrawn from the sinus at the base of the pereiopods and transferred to an anti-coagulant solution (10% trisodium citrate) at the ratio of 5:1. To determine alkaline phosphatase activities, tissues were homogenized in 100 mM Tris-HCl buffer (pH 8.0) then centrifuged at 4,500 g for 30 minutes at 4 ^{o}C. The clear supernatant was collected for analysis. For Ca^{2+}ATPase, the tissues were homogenized in the medium containing 0.25 M sucrose, 6 mM EDTA, 20 mM imidazole (pH 6.8). Just before use, 0.1% deoxycholate was added. The homogenate was centrifuged at 4,500 g for 30 min at 4 ^{o}C. The supernatant was collected and further centrifuged at 7,500 g for 60 minutes at 4 ^{o}C. The clear supernatant containing microsomal pellets was used for analysis.

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Alkaline phosphatase activity was determined according to Villanueva *et al* [7]. The reaction mixture composed of 50 mM phosphate buffer 0.05 mM $ZnCl_2$, 0.23 mM $MgCl_2$, crude extract and 4.5 mM *p*-nitrophenyl phosphate. The mixtures were incubated for 30 min at 30 °C. The reaction was stopped by adding 0.1N NaOH, then centrifuged at 3,500 g for 10 minutes 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} mg $protein^{-1}$.

Ca^{2+}ATPase activity was determined according to Cameron [8]. The reaction mixture contained 20 mM imidazole buffer, 5 mM ATP, 10 mM $CaCl_2$ and 10 mM $MgCl_2$. The mixture was incubated at 30 °C for 30 minutes, then 10% (w/v) trichloroacetic acid was added. After centrifuging at 3,500 g for 15 minutes, 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 0.4 ml of 0.25% aminonaphthosulfonic acid solution was added. After 10 minutes, the absorbance was measured at 660 nm. Ca^{2+}ATPase specific activity was determined from the difference of Pi formation in the presence and absence of Ca^{2+} and calculated as *u*moles Pi min^{-1}mg $protein^{-1}$. Blanks containing the reaction mixture without ATP but with EGTA (ethylene-glycol-bis (2-aminoethyl)-*N,N,N',N'*-tetraacetic acid) was used. The optimum temperatures of the two enzymes were determined (at the optimum pH) at 30, 40, 50, 60, 70 and 80 °C.

C. Protein determination

Protein concentration in the samples was determined by the method of Bradford [9] using bovine serum albumin as a standard.Copyright Form

D. Statistical analysis

One-way analysis of variance (ANOVA) was used to test the significant difference between enzyme specific activities at various molting stages. Differences among treatments were considered significant at *p*<0.05. LSD was employed to determine multiple comparison between enzyme specific activities in gill, haemolymph and integument. The results were expressed as means $\pm$ SD of the data.

III. RESULTS

A. Optimum conditions for alkaline phosphatase and Ca^{2+}ATPase in mud crab

The optimum conditions for alkaline phosphatase and Ca^{2+} ATPase activity in mud crab were studied and the results showed that the maximum activity of alkaline phosphatase in both integument and haemolymph were observed at pH 7.5 but at pH 10 for the gill. For optimum temperature, maximum alkaline phosphatase activities were observed at 50 °C for gill and haemolymph but at 60 °C for integument. For Ca^{2+}ATPase, the highest activity for gill was observed at pH 8.5 while those of integument and haemolymph were at pH 9.0 and 8.0, respectively.

For optimum temperature, the highest Ca^{2+}ATPase activity was found at 60 °C in all observed tissues of mud crab.

B. Variation of alkaline phosphatase activities during molting cycle

Alkaline phosphatase specific activities during the molting cycle were found in the wide range of 4.13 $\pm$ 0.49 to 90.98$\pm$11.29, 1.48$\pm$0.31 to 20.73$\pm$1.42 and 2.07$\pm$0.1 to 10.42$\pm$1.04 µmol min^{-1}mg protein $^{-1}$ in gill, integument and haemolymph, respectively (Table I). During intermolt stage (C), the activity of this enzyme was mainly found in the integument (20.73$\pm$1.42 µmol min^{-1}mg protein $^{-1}$) which is three times the amounts found in gill and haemolymph of 5.60$\pm$1.99 and 6.17$\pm$1.74 µmol min^{-1}mg protein^{-1}, respectively.

Considering each organ separately, alkaline phosphatase activity in gill was maintained at low level of 4-9 µmol min^{-1}mg protein^{-1} during intermolt (C) and premolt (D1-D3) stages (Table I). The activity increased sharply from 6-h postmolt (A1) to the maximum level of 90.98$\pm$11.29 µmol min^{-1}mg protein^{-1} at 5-day postmolt (B1) and then declined thereafter back to the intermolt level. The highly significant changes of alkaline phosphatase in gill after molting (A2-B2) from those at intermolt (C) and premolt stages (D1-D3) were clearly observed. Opposite to those in gill, alkaline phosphatase specific activity in integument abruptly decreased from intermolt (C) at 20.73$\pm$1.42 µmol min^{-1}mg protein^{-1} to the low level of 1.92$\pm$0.61 µmol min^{-1}mg protein^{-1} at early premolt (D1) and remained stable throughout the premolt (D1-D3) and postmolt (A1-B2) stages with only a slight increase during 24-h postmolt (A2) to the level of 8.19$\pm$1.17 µmol min^{-1}mg protein^{-1}. After molting, alkaline phosphatase specific activity returned to the intermolt level (C) which was the maximum level and this change was highly significant comparing to all other stages of molting. Although the enzyme activity at postmolt stages were higher than that of premolt but they were not significantly different.

In haemolymph, alkaline phosphatase activity decreased from intermolt (C) of approximately 6.17$\pm$1.74 µmol min^{-1}mg protein^{-1} to the low level of 2.07$\pm$0.10 µmol min^{-1}mg protein^{-1} at early premolt stage (D1-D2) with highly significant change (*p*<0.05). The activity then drastically increased to 7.51$\pm$1.11 µmol min^{-1}mg protein^{-1} at the late premolt (D3) and reaching the highest level of 10.42$\pm$1.04 µmol min^{-1}mg protein^{-1} at 24-h postmolt (A2) before dropping back during 5 to 7 days postmolt (B1-B2) and rose again at the intermolt level (C). The changes in specific activity of alkaline phosphatase at postmolts (A1-B1) were highly significant (*p*<0.05) comparing to those of early premolts (D1-D2) but was not as much as those of intermolt (C) and late premolt (D3).

TABLE I
COMPARISON OF ALKALINE PHOSPHATASE SPECIFIC ACTIVITY AT DIFFERENT MOLTING STAGES OF *SCYLLA SERRATA*

Stage	Alkaline phosphatase specific activity (µmol min^{-1}mg protein $^{-1}$)

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	Gill	Integument	Haemolymph
Intermolt (C)	$5.60^a\pm1.99$	$20.73^c\pm1.42$	$6.17^b\pm1.74$
2- week premolt (D1)	$4.13^a\pm0.49$	$1.92^a\pm0.61$	$2.07^a\pm0.10$
1- week premolt (D2)	$9.04^a\pm0.65$	$1.48^a\pm0.31$	$2.38^a\pm0.43$
2-day premolt (D3)	$5.40^a\pm1.14$	$3.05^{ab}\pm0.61$	$7.51^b\pm1.11$
6-hour postmolt (A1)	$25.36^b\pm3.90$	$4.54^b\pm1.17$	$9.22^c\pm0.92$
24-hour postmolt(A2)	$49.30^c\pm6.39$	$8.19^b\pm1.17$	$10.42^c\pm1.04$
5-day postmolt (B1)	$90.98^d\pm11.29$	$5.28^b\pm1.09$	$8.06^{bc}\pm0.99$
7-day postmolt (B2)	$60.19^c\pm11.72$	$3.78^{ab}\pm0.11$	$3.36^a\pm0.18$

Note: Variation of alphabets in the same column showed the highly significant differences ($p<0.05$) of the activity.

C. Variation of Ca^{2+}ATPase activities during molting cycle

The Ca^{2+}ATPase activity profiles during the molting cycle of both gill and integument were similar but opposite to that of the haemolymph. The activities were in the ranges of 0.28 ± 0.04 to 4.33 ± 0.5, 0.17 ± 0.02 to 2.5 ± 0.09 and 0.08 ± 0.02 to 1.03 ± 0.31 μmol min^{-1}mg protein^{-1} in gill, integument and haemolymph, respectively (Table II).

In gill and integument, Ca^{2+}ATPase specific activities gradually decreased from the low level of 1.35 ± 0.29 μmol min^{-1}mg protein^{-1} intermolt (C) to early premolt stage (D1) and were quite stable during premolt stages (D1-D2). After molting, the enzyme activities increased slightly from 6-h to 24-h postmolt and rose abruptly to the maximum level of 4.33 ± 0.50 μmol min^{-1}mg protein^{-1} at 7-day postmolt .

The changes in specific activities of Ca^{2+}ATPase during 5-7 day postmolt (B1-B2) in these two tissues were highly significant ($p<0.05$) comparing to those of premolts (D1-D3) and postmolts (A1-A2).

For haemolymph, however, the specific activities increased from intermolt to the highest peak of 1.03 ± 0.31 μmol min^{-1}mg protein^{-1} at 2-weeks before molting (D1) then decreased sharply until reaching the low range of 0.08-0.20 μmol min^{-1}mg protein^{-1} at the postmolt stages (A1-B2) and rose thereafter back to the high level at intermolt (C). The change in Ca^{2+}ATPase activity at early premolt (D1) was highly significant ($p<0.05$) comparing to those at late premolt (D3) and postmolt stages (A1-B2).

TABLE II
COMPARISON OF CA^{2+} ATPASE SPECIFIC ACTIVITY AT DIFFERENT MOLTING STAGES OF *SCYLLA SERRATA*

Stage	Ca^{2+}ATPase specific activity (μmol min^{-1}mg protein $^{-1}$)		
	Gill	Integument	Haemolymph
Intermolt (C)	$1.35^{ab}\pm0.29$	$0.72^{ab}\pm0.09$	$0.82^c\pm0.18$
2- wk premolt (D1)	$0.44^a\pm0.13$	$0.17^a\pm0.02$	$1.03^c\pm0.31$
1- wk premolt (D2)	$0.76^a\pm0.19$	$0.17^a\pm0.03$	$0.76^c\pm0.07$
2-day premolt (D3)	$0.28^a\pm0.04$	$0.52^a\pm0.06$	$0.38^b\pm0.02$
6-h postmolt (A1)	$0.71^a\pm0.11$	$0.71^{ab}\pm0.15$	$0.12^a\pm0.03$
24- h postmolt (A2)	$0.86^a\pm0.06$	$1.32^{bc}\pm0.22$	$0.20^a\pm0.06$
5-day postmolt (B1)	$2.18^{bc}\pm0.32$	$2.24^c\pm0.30$	$0.08^a\pm0.02$
7-day postmolt (B2)	$4.33^d\pm0.50$	$2.50^c\pm0.09$	$0.15^a\pm0.02$

Note: Variation of alphabets in the same column showed the highly significant differences ($p<0.05$) of the activity.

For haemolymph, however, the specific activities increased from intermolt to the highest peak of 1.03 ± 0.31 μmol min^{-1}mg protein^{-1} at 2-weeks before molting (D1) then decreased sharply until reaching the low range of 0.08-0.20 μmol min^{-1}mg protein^{-1} at the postmolt stages (A1-B2) and rose thereafter back to the high level at intermolt (C). The change in Ca^{2+}ATPase activity at early premolt (D1) was highly significant ($p<0.05$) comparing to those at late premolt (D3) and postmolt stages (A1-B2).

IV. CONCLUSIONS AND DISCUSSION

The optimum pH of alkaline phosphatase in mud crab (pH 7.5 to 10) is similar to those found in tissues of other organisms where phosphate metabolism occurs, i.e., hepatopancreas of crayfish (pH 6.8-7.3 and pH 8.0-8.5) [10], kidney, liver and intestine of trout, carp and eel (pH 10.0) [11]. Moreover, in gills of the blue crab *Callinectes sapidus*, pH optima for alkaline phosphatase inhibitor (as levamisole-sensitive and levamisole-insensitive) were found at pH 9.1 and 7.1 [12]. However, these differences of pH optimum for alkaline phosphatase are often due to the types of substrate and substrate concentrations used [13]. The optimum temperature of alkaline phosphatase activity of mud crab in this study (50-60 °C) is also in the same range as those of tropical fish, *Aphanopus carbo* and *Epigonus telescopus* (60 °C) and is comparable to that of silver carp (40 °C) [14]. (Gelman *et al*. 1992).

The pH ranges of Ca^{2+}ATPase activity in mud crab (pH 8.0 to 9.0) are similar to those found in different tissues of other organisms in which active transport of calcium occurs, i.e., in the shell gland of Japanese quail (pH 7.8) [15], Japanese eel (pH 8.1-8.3) [16], fish gill (pH 7.9-8.1) [17] and hepatopancreas of blue crab (pH 7.5) [18]. The optimum temperature of Ca^{2+} ATPase in all observed tissues of mud crab was found at 60 °C which is comparable to that in hepatopancreas of blue crab (45-50 °C) [18]. The optimum conditions (pH and temperature) were, therefore, used to determine the specific activities of each tissue of mud crab.

Since the alkaline phosphatase activity profiles in integument and haemolymph were similar showing high level at intermolt and during late premolt (D3) to early postmolt (A1) but that in gill was high only after molting, indicating that calcification and chitin biosynthesis should occur concomitantly with the higher activity of alkaline phosphatase found in these organs.

The role of phosphatase in chitin synthesis is to convert Dol-PP (Dolichol pyrophosphate) to Dol-P (Dolichol phosphate) or Dol-P to dolichol [19] (Figure 1). Dol-P, therefore, acts as the carrier in the assembly of pyrophosphate-linked oligosaccharides, and is also the acceptor in the synthesis of the sugar donors Dol-P-Glc from UDP-Glc. The availability of Dol-P represents a key factor in the assembly of lipid-linked oligosaccharide.
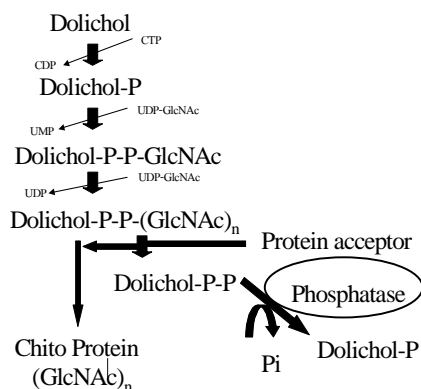

Fig. 1 Partial dolichol pathway of *N*-linked glycosylation [19]

As for calcification which occurs in all three tissues, calcium ion from seawater passively transports through cuticle via gill and haemolymph and reacts with bicarbonate before precipitating in the cuticle layer. Alkaline phosphatase would promote precipitation of calcium phosphate complexes within the vesicles [20]. This enzyme also participates in the formation of hydroxyapatite (HA) crystals. It is associated with Pi homeostasis and can hydrolyse a variety of phosphate compounds [21]. Mornet *et al* [22] reported the link between calcium homeostasis maintained by annexins and Pi supply provided by alkaline phosphatase in calcification.

Tissues that exhibit high alkaline phosphatase activity generally function in either secretion or absorption of mineral by active transport [13]. The variation of alkaline phosphatase activity in gill, integument and haemolymph of mud crab during premolt and postmolt stages seem to correspond well to the time of chitin degradation, chitin biosynthesis and calcification. The activity of alkaline phosphatase in integument and haemolymph decreased from intermolt (C) to early premolt (D1) when the old cuticle is in the process of deterioration. At premolt stage (D3), the alkaline phosphatase activities in integument and haemolymph increased supposedly due to the formation and rearrangement of the new cuticle. Alkaline phosphatase was also found to associate with the integument of the amphipod *Orchestia cavimana* during the molting [23]. Distribution of alkaline phosphatase in *Scylla serrata* as reported by Monin and Rangneker [24]. confirmed that the activity is confined to the brush borders of the hepatopancreas. The activity is increased after bilateral removal of eyestalks and also after the injection of eyestalk extract into eyestalk-less and normal animals. The blood sugar level after 24-h postmolt is accompanied by a restoration of the enzymatic activity.

The changes clearly indicate that the alkaline phosphatase has a role in the transport of glucose across the cell membrane. However, the physiological role of alkaline phosphatase remains inconclusive except for a role in bone mineralization [25]. which involves various transport processes, such as intestinal phosphate and calcium transfer [26].

Ca^{2+}ATPase activity profiles in gill and integument, on the other hand, were found somewhat similar but opposite to that of the haemolymph. The variation patterns of Ca^{2+}ATPase activity correspond to the change of calcium content in gill, integument and haemolymph [27]. Calcium was presumably transferred from gill and integument via haemolymph to store at hepatopancreas during premolt stages, so both calcium content and Ca^{2+}ATPase in these tissues were reduced while they were found at high level in haemolymph. After molting, calcium from seawater could enter the gill for calcification to take place in the integument. This support our results of higher calcium content and Ca^{2+}ATPase activities found in gill and integument but less in haemolymph.

Variation in the level of Ca^{2+}ATPase activity in gill, integument and haemolymph of mud crab during premolt and postmolt stages is suspected to correspond to the time of mineral resorption and deposition from the old carapace to the new body. At premolt stage, the function of Ca^{2+}ATPase should be predominant in haemolymph due to the transport of calcium and other ions from the old carapace to restore at the hepatopancreas, via haemolymph, where the exchange of ions and gases are taking place. This also confirms the activity of the enzyme in haemolymph at premolt to be greater, by several folds, than that found at postmolt stages. After ecdysis, the high level of Ca^{2+}ATPase activity in gill and integument corresponded well to the time of calcium deposition in the new carapace. Anderson [4]. indicated that calcification presumably requires the active transport of calcium, with ATP as a possible source of energy for the calcium pump and Chen *et al* [28] also suggested that Ca^{2+}ATPase in the gills plays a role in calcium transport. Normally, the calcium used in calcification comes from three different sources, the food intake, water absorption and the body accumulation. After molting, the crab absorbs a large amount of water in order to support the new soft structure, and causing it to stop eating, so most calcium for calcification would come from the water uptake. Calcium ions passively enter the gills before being actively transported to the cuticle, so the activity of Ca^{2+}ATPase in gill and integument needs to be increased. However, accumulated calcium in hepatopancreas was later transferred back to the cuticle for calcification via the haemolymph, and, thus causing the increase of Ca^{2+}ATPase activity in the integument to the high level at 7 days postmolt (B2 stage). Other research groups also reported that the gills of crab, *Leptograpsus variegatus* contain a high-affinity Ca^{2+}ATPase, probably located in the basolateral plasma membrane of the gill epithelia. The enzyme is believed to function as a selective extrusion mechanism involved in the maintenance of a low cytosolic Ca^{2+} level in the epithelial cells [29]. Ca^{2+}ATPase specific activity in gill of the Japanese eel, *Anguilla japonica* was highest in the microsomal fraction and was activated by either Ca^{2+} or Mg^{2+} [16].

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It is also known that in the normal condition calcium transportation in the cells is regulated by the high affinity calcium ATPase in the form of plasma membrane Ca^{2+}ATPase (PMCA). This PMCA is responsible for calcium homeostasis, signal transduction, and also dephosphorylation reaction [30].

In the conclusion, it was found that Alkaline phosphatase activity was drastically high in the integument only at intermolt stage possibly due to the need in phosphate absorption at this stage via haemolymph to react with calcium and precipitate into calcium phosphate complex at epidermal cells of cuticle. As for haemolymph, the high range of this enzyme occurred at late premolt and extended to 24-h postmolt. In gill, however, this enzyme showed its high activity after molting but maintained at high level until 7-day postmolt. The need for long time transportation of phosphate and phosphoryl group between cells and/or from seawater into the tissues for phosphate metabolism and biomineralization undoubtedly keep this enzyme to maintain at high level. For Ca^{2+}ATPase, the activity was relatively high in the haemolymph before molting but distinctively high after molting in both gill and integument. This enzyme is active just before molting to transfer calcium and ions from the degrading carapace to accumulate at hepatopancreas. The high Ca^{2+}ATPase at postmolt is responsible for transferring calcium ions from seawater through the basolateral epithelial located at the gill to store at the integument for calcification of new carapace. Activity profiles of alkaline phosphatase and Ca^{2+}ATPase were similar in gill of mud crab indicating their significant co-ordination of these two enzymes for chitin synthesis, chitin degradation and calcification in the molting cycle of mud crab.

ACKNOWLEDGMENT

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