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Chitinase and Carbonic Anhydrase Activities during Molting Cycle of Mud Crab (*Scylla serrata* Forskal 1775)

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ABSTRACT

Optimum conditions for the activities of chitinase and carbonic anhydrase in gill, integument and haemolymph of mud crab (*Scylla serrata* Forskal 1775) were determined. The maximum activity of chitinase in all 3 types of tissue was observed at pH 11 and 40°C. However, optimum conditions for carbonic anhydrase in gill, integument and haemolymph were at pH 7.6 at 50°C, pH 7.2 at 50°C and pH 7.2 at 60°C, respectively. Both chitinase and carbonic anhydrase activities varied during various stages of molting. For chitinase, activity in gill at 2-day premolt was significantly lower ($P<0.01$) than that at 6 hr to 5-day postmolt but not significantly ($P<0.01$) different from that at intermolt. Maximum chitinase activity in integument observed at 24 hr postmolt was significantly higher ($P<0.01$) than that at intermolt and early premolt. Chitinase activity in haemolymph was quite stable during premolt and postmolt but significantly lower than that at intermolt ($P<0.01$). Observation of carbonic anhydrase activities in gill and integument revealed significantly higher activity after molting than during intermolt and premolt stages. Maximum activity of carbonic anhydrase in gill and integument was evident at 6 hr and 5-day postmolt, respectively. For haemolymph, the level of carbonic anhydrase activities during various stages of molting cycle were highly significantly different ($P<0.01$). The results indicated important roles of chitinase and carbonic anhydrase at every stage in molting cycle of mud crab.

Key words : chitinase, carbonic anhydrase, mud crab, molting

INTRODUCTION

The rigid exoskeleton provides a limited space for growth. To increase their size, crustaceans must break down the old exoskeleton, shedding it and enlarging the body mass before accumulating substances to build the new hard shell (Skinner *et al*., 1985). Since protein and chitin are major components of the exoskeleton, digestion of the old chitinous cuticle must occur, by the molting fluid containing proteinases and chitinolytic enzymes, at the beginning of the molting cycle (Stevenson, 1985; Samuels and Reynolds, 1993). One of the chitinolytic enzymes, chitinase, is known to decompose chitin into oligomers of *N*-acetylglucosamine. Chitinase is generally found in various organisms including fish (Lindsay, 1984; Matsumiya and Mochizuki, 1996), shellfish (Smucker and Wright, 1984), squid and seaweed (Sekiguchi *et al.*, 1995). In addition to chitinolytic

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enzymes, carbonic anhydrase may also play a role in molting cycle. The enzyme provides the bicarbonate required for the formation of calcium carbonate crystals for the mineralization of the shell (Kingsley and Watabe, 1987). Carbonic anhydrase has been identified in tissues associated with calcium carbonate formation in crustacean cuticle and bivalve mantle (Henry and Kormanik, 1985). The mechanisms of chitinase and carbonic anhydrase in molting process are not known. This study is conducted to measure the activities of chitinase and carbonic anhydrase at various stages during molting process in mud crab (*Scylla serrata* Forskal 1775), in order to understand the roles of these enzymes in the mechanism of morphological changes during this transition.

MATERIALS AND METHODS

Animal preparation

Mud crabs, *Scylla serrata* of 65-90 mm in carapace width (about 95-130 g in weight) at various stages were collected from a soft-shell crab farm at Bang-Khun-Tian District, Bangkok. The animals were transferred to an individual closed system aquarium containing 5 litre seawater of 20-26 ppt salinity at 21-26°C. The water had been passed through the clam shell filter system to adjust the pH to 7.8. Three-fourths of the total volume of water was changed twice a week. Crabs were fed *ad libitum* with freshly chopped fish daily. The dactylopodite and propodus were examined to identify the stages in molting cycle according to the criteria described by Warner (1977). The molting cycle was divided into 8 stages : A1 stage (softshell, 6-hr postmolt), A2 stage (softshell, 24-hr postmolt), B1 stage (papershell, 5-day postmolt), B2 stage(7-day postmolt), C stage (hardshell, intermolt), D1 stage (white sign, 2-week premolt), D2 stage (pink sign, 1-week premolt), D3 stage (red sign , 2-day premolt).

Preparation of crude enzyme extracts

Gills and integument tissues were homogenized in 100 mM Tris-HCl buffer (pH 8.0), then centrifuged at 3,500 g for 30 min at 4°C. The clear supernatant was kept at -20°C until analysis. Haemolymph samples were drawn from the sinus at the base of the pereiopods and transferred to sample tubes containing an anti-coaggulant (10% tri-sodium citrate) at the volume ratio of 5:1.

Enzyme assay

To find the optimum pH, chitinase activity was assayed according to the method described by Imoto and Yagishita (1971). The reaction mixture consisted of 100 µl crude enzyme extract, 50 mM buffer and 5% colloidal chitin to make the final volume of 2 ml. Three different buffers used in the experiment were 50 mM McIlvaine (pH 3-7), 50mM Tris-HCl (pH 7-10), and 50 mM Glycine-NaOH (pH 10-12). The incubation was allowed to proceed for 30 min at 30°C and 1.5 ml of Schales' reagent (0.5M sodium carbonate solution containing 0.05% potassium ferricyanide) was added to stop the reaction. The solution was then heated in boiling water for 15 min. After cooling, the absorbance was measured at 420 nm. The specific activity was expressed as µmoles *N*-acetyl-D-glucosamine produced min^{-1}mg $protein^{-1}$.

Carbonic anhydrase activity was determined using a modified method of Armstrong *et al.* (1966) and Pocker and Stone (1967). The assay system contained 50 mM phosphate buffer (pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8), 40 µl of crude enzyme extract and 5 mM *p*-nitrophenyl acetate to make the final volume of 2 ml. Incubation was done for 30 min at 30°C, and absorbance was measured at 348 nm. The specific activity was expressed as µmoles *p*-nitrophenol produced per min per mg protein.

Optimum temperature for the activities of chitinase and carbonic anhydrase were determined

at 30, 40, 50, 60, 70 and 80°C, at their respective optimal pH's. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the significance of differences between enzyme specific activities at various molting stages. LSD was employed to determine multiple comparisons between enzyme specific activities in gill, haemolymph and integument at different molting stages (SPSS programme).

RESULTS AND DISCUSSION

The optimum conditions for chitinase and carbonic anhydrase in *Scylla serrata* are shown in Figure 1-4. Chitinase in gill, integument and haemolymph exhibited two pH optima (6 and 11) with higher specific activity at pH 11 (Figure 1). Optimum pH for carbonic anhydrase in both integument and haemolymph was seen at pH 7.2 but at pH 7.6 for the gill (Figure 2).

Optimum temperature for chitinase activity was at 40°C in all three types of tissue while that of carbonic anhydrase activity was observed at 50°C for gill and integument but at 60°C for haemolymph (Figure 3-4).

During intermolt (C), chitinase specific activity was present mainly in the haemolymph (16.93 ± 2.12 µmol/min/mg protein) (Figure 7) at approximately twice the amount found in gill (7.41 ± 1.06 µmol/min/mg protein) (Figure 5) and integument (8.29 ± 0.37 µmol/min/mg protein) (Figure 6). Variations in chitinase specific activity over the molting cycle ranged from 5.03 ± 0.39 to10.18 ± 0.42, 5.14 ± 0.71 to 28.39 ± 2.53, and 0 – 16.93 ± 2.12 µmol/min/mg protein in gill, integument, and haemolymph, respectively. Activity in the gill showed the least variation, increasing slightly during early premolt (D1-D2) then declining slowly to the minimum level at late premolt (D3). The activity regained after molting (A1) and decreased slowly thereafter back to the intermolt level (A2-C)(Figure 5). Chitinase activity at 2-day premolt (D3) was significantly lower ($P<0.01$) than those of the 6 hr to 5-day postmolt (A1-B1) but not significantly different from that at the intermolt (Table1). In integument, chitinase specific activity decreased slightly during early premolt (D1-D2) then abruptly increased to the maximum level at the late premolt (D3) before dropping off as molting took place (A1) (Figure 6). Fluctuation in chitinase activity was observed again after molting , being highest at 24 hr postmolt (A2) then leveled off during 5 to 7-day postmolt (B1-B2) before returning to the intermolt level (C). The peak activity at 24 hr postmolt (A2) differed significantly ($P<0.01$) from that at intermolt (C) and early premolt (D1-D2) (Table1). Chitinase activity in haemolymph was virtually absent during early premolt (D1-D2) but recovered near molting time (D3) and remained quite stable during postmolt stages (A1-B2) before rising thereafter back to the intermolt level (C) (Figure 7). The activity at intermolt stage was highly significantly different ($P<0.01$) from that at premolt (D1-D3) and postmolt stages (A1-B1) ($P<0.05$).

Optimum conditions for chitinase activity in other types of organism were found in both acid and alkaline ranges at different temperatures. Dual pH optima were also observed at 3.5 and 9.0 in seaweed, *Chondrus giganteus* (Sekiguchi *et al.*, 1995), and at 3.5 and 8.5 in yam (Tsukamoto *et al.*, 1984), while one pH optimum of 2.5 was found in yeast, *Saccharomyces cerevisiae* and at pH 6.5 in *Candida albicans* (Correa *et al.*, 1982). Optimum temperature for chitinase activity was reported at 45°C in bacteria, *Aeromonas hydrophila* (Yabuki *et al.*, 1986), at 70-80°C in *Bacillus licheniformis* (Takayanagi *et al.*,1991), at 50°C in the stomach of red sea bream and at 55°C in the stomach of Japanese sea–bass (Sekiguchi *et al.*,1995). Ohtakara *et al.* (1979) reported that optimum condition for chitinase activity of bacteria, *Vibrio*

sp. and *Streptomyces thermoviolaceae* was at pH 10.5 and 8.0-10.0 at 40°C, which was close to the present finding in mud crab.

Carbonic anhydrase specific activity was observed predominantly in the haemolymph while the levels in gill and integument were very low throughout the molting cycle (Figure 8-10). Variations of carbonic anhydrase specific activity over the molting cycle were in the range of 37.8 ± 2.95 to 86.17 ± 2.17, 9.11 ± 1.4 to 29.72 ± 4.71, and 133.61 ± 7.02 to 604.36 ± 12.52 µmol/min/mg protein in gill, integument and haemolymph, respectively. In the gill, the enzyme activity was stable from intermolt (C) to late premolt (D3) and rose to the maximum at 6 hr postmolt (A1) then decreased gradually reaching the intermolt level at 7-day postmolt (B2)(Figure 8). The activity in integument fluctuated in a very limited range, being highest at 5 days after molting (B1)(Figure 9). Specific activities of carbonic anhydrase during intermolt and premolt stages in the gill and integument were highly significantly different ($P<0.01$) comparing to that at postmolt (Table 1) with maximum activity at 6 hr postmolt and 7-day

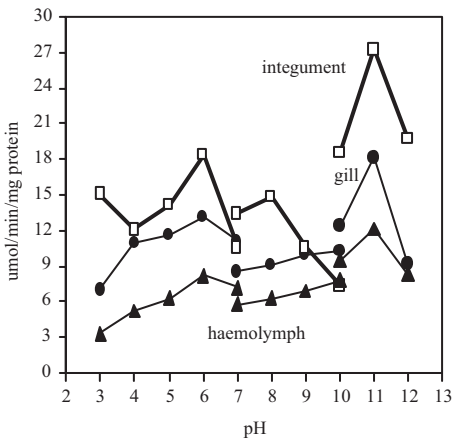


Figure 1 Optimum pH of chitinase in *Scylla serrata.*

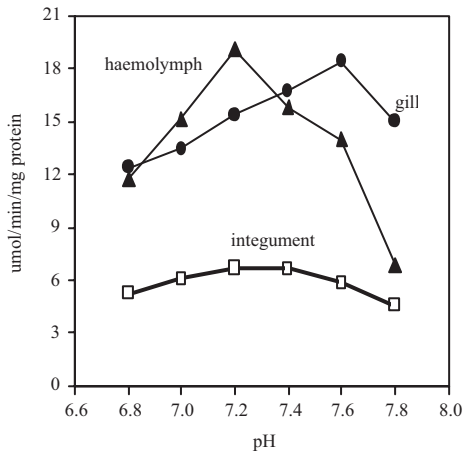


Figure 2 Optimum pH of carbonic anhydrase in *Scylla serrata.*

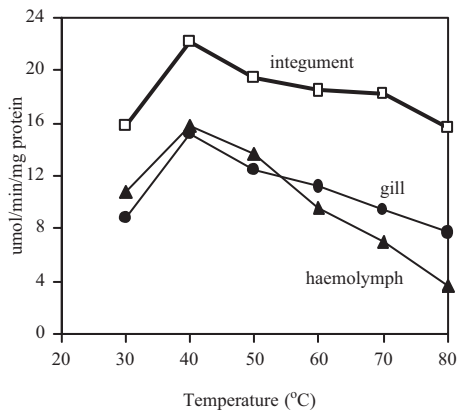


Figure 3 Optimum temperature of chitinase in *Scylla serrata.*

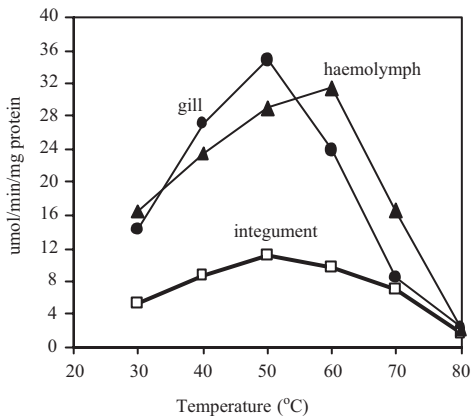


Figure 4 Optimum temperature of carbonic anhydrase in *Scylla serrata* .

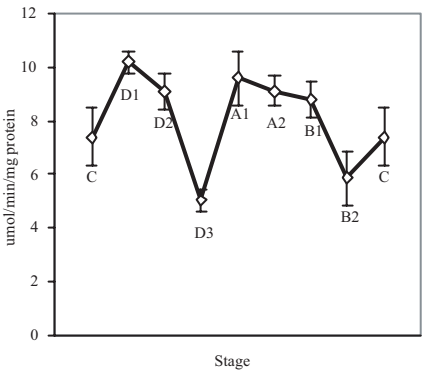


Figure 5 Chitinase specific activity in gill of *Scylla serrata* at various stages of molting cycle.

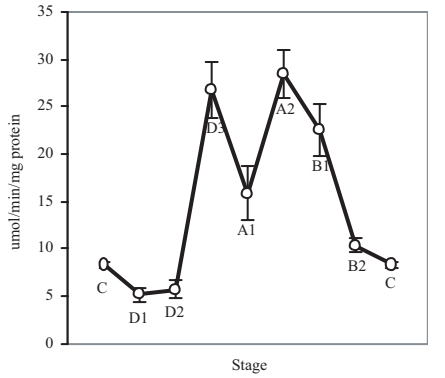


Figure 6 Chitinase specific activity in integument of *Scylla serrata* at various stages of molting cycle.

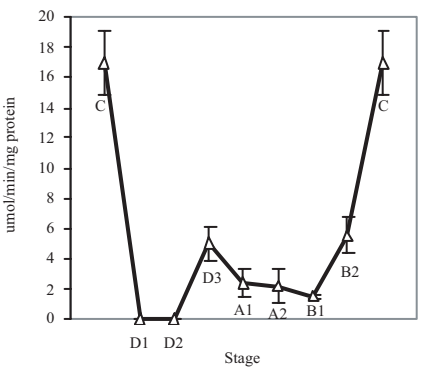


Figure 7 Chitinase specific activity in haemolymph of *Scylla serrata* at various stages of molting cycle.

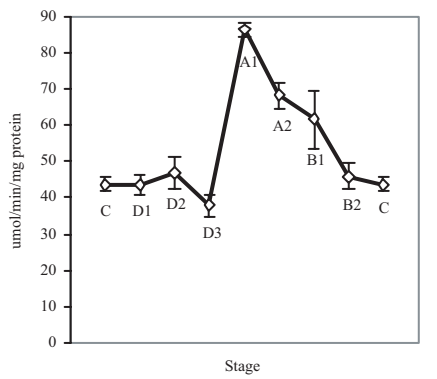


Figure 8 Carbonic anhydrase specific activity in gill of *Scylla serrata* at various stages of molting cycle.

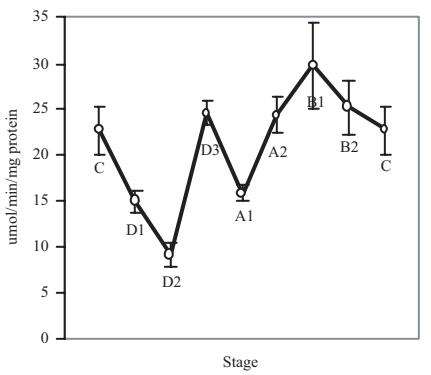


Figure 9 Carbonic anhydrase specific activity in integument of *Scylla serrata* at various stages of molting cycle.

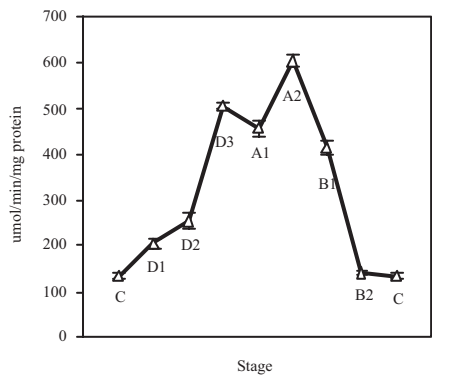


Figure 10 Carbonic anhydrase specific activity in haemolymph of *Scylla serrata* at various stages of molting cycle.

Table 1 Multiple comparison between stages in molting cycle of *Scylla serrata* (P<0.01).

Stage	Chitinase			Carbonic anhydrase		
	Gill	Integument	Haemolymph	Gill	Integument	Haemolymph
Intermolt (C)	7.41±1.06^{BCD}	8.29±0.37^{A}	16.93±2.12^{CD}	43.61±1.82^{A}	22.72±2.62^{CD}	133.61±7.02^{A}
2 wk premolt (D1)	10.18±0.42^{E}	5.14±0.71^{A}	0^{A}	43.7±2.75^{AB}	14.94±1.21^{B}	206.99±11.27^{A}
1 wk premolt (D2)	9.1±0.66^{DE}	5.7±0.91^{A}	0^{A}	47.01±4.45^{AB}	9.11±1.40^{A}	254.02±16.72^{B}
2-day premolt (D3)	5.03±0.39^{AB}	26.79±2.99^{DE}	4.99±1.08^{B}	37.8±2.95^{A}	24.58±1.33^{CD}	505.57±8.88^{D}
6 hr postmolt (A1)	9.6±1.00^{DE}	15.86±2.86^{BC}	2.39±0.88^{A}	86.17±2.17^{E}	15.8±0.83^{B}	455.07±18.64^{D}
24 hr postmolt (A2)	9.11±0.58^{DE}	28.39±2.53^{E}	2.21±1.14^{A}	68.08±3.70^{CD}	24.31±1.93^{CD}	604.36±12.52^{E}
5-day postmolt (B1)	8.81±0.67^{CDE}	22.61±2.77^{CD}	1.5±0.09^{A}	61.43±8.14^{BCD}	29.72±4.71^{DE}	413.81±14.82^{C}
7-day postmolt (B2)	5.86±1.00^{AB}	10.38±0.66^{AB}	5.53±1.17^{B}	45.86±3.50^{AB}	25.21±30^{CD}	140.55±5.20^{A}

Note : Variations of alphabets in the same column indicate the differences are highly significant (P<0.01) by LSD.

postmolt for gill and integument, respectively. In haemolymph, variations of carbonic anhydrase specific activity over the molting cycle followed a normal curve with a slight drop after molting (Figure 10). The activity increased slowly from the intermolt level to the first peak at 2-day premolt, then rose sharply to the second peak at 24 hr postmolt after a short pause near the molting period. The differences in haemolymph carbonic anhydrase specific activities among various stages of molting cycle were highly significant (P<0.01) except at the intermolt and 7-day postmolt (Table1).

The high level of chitinase activity in integument during late premolt corresponded to the time when the epithelial cells began to separate from the old exoskeleton and high activity of chitinase was required to dissolve the hard chitinous shell. Separation of the epidermis was triggered by an increased output of the steroid molting hormones, ecdysteroids, from Y-organ. The activity of these glands was no longer controlled by the molt-inhibiting hormone which was produced by the sinus gland-X-organ complex (Fingerman, 1997). A significant drop of chitinase activity at 6 hr postmolt could probably result from the pulling away of the new body from the old one. Within 6 hr of molting, chitinase activity in integument increased again indicating the period of active synthesis of the new chitinous layer. After the formation of new exoskeleton was accomplished, the activity of chitinase returned to the basal intermolt level, thereby completing the molting cycle. The disappearance of chitinase activity from haemolymph during early premolt, prior to the rise of chitinase activity in integument, could reflect the transfer of enzyme from haemolymph to the epidermal site where active digestion of chitinous structure took place. Fluctuation of chitinase activity in the gill during early premolt and postmolt stages also corresponded to the changes in chitinase activity in integument. It could be visualized that during premolt stages chitinase from haemolymph and

integument, via the haemolymph, was transferred to the integument for chitinous digestion after which chitinase activity returned, via haemolymph, to their respective origins and was ready for the next molting cycle.

Chitinase is generally working together with β-*N*-acetylhexosaminidase in the digesting process of chitin. Chitinase in higher plants destroy the chitin and pathogenic bacterial cells in the cuticles of insects which come in contact with plant tissue and produced *N*- acetylchitooligosaccharides. It was reported that this substance became an elicitor which reacted with plant cells to promote the generation of pathogenic-resistant protein (Sekiguchi *et al.*, 1995). In fish and squid, chitinase activity was high in the stomach and was also present in the liver. Chitinase was found in the blood of fish as well and presumably played a role in the defense toward fungus and parasites (Manson *et al.*, 1992). Matsumiya *et al.* (1998) reported that the liver of squid and cuttlefish secreted digestive juice to the stomach and caecum to aid the intragastric enzymes for digesting the chitin of prey. It is possible that the role of chitinase in gill and integument of mud crab during moltimg cycle might be similar. When the skeleton is still soft and the nutrients enter through the gill, chitinase would help the digestive enzyme to degrade complex carbohydrates and transport to the integument to build the hard shell.

Carbonic anhydrase has been found to be associated with calcification in invertebrates such as barnacles (Yule *et al.*, 1982), crabs (Henry and Kormanik, 1985) and calcareous sponges (Jones and Ledger, 1986). The enzyme catalyzes the conversion of CO_2 to HCO_3^- (equation 1) which would be available for $CaCO_3$ formation (equation 2).

$$CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+ \quad \text{------- (1)}$$

$$Ca^{2+} + HCO_3^- \longleftrightarrow CaCO_3 + H^+ \quad \text{------- (2)}$$

The highest levels of carbonic anhydrase were observed in respiratory and ion transporting epithelial, such as gill (Henry and Saintsing, 1983). The gill performs a number of physiological processes, i.e., gas exchange, ion transport and the regulation of haemolymph acid-base balance (Henry, 1988). Therefore, both gill and haemolymph have relating physiological functions of carbonic anhydrase. The results showed that the activity of carbonic anhydrase in gill, integument and haemolymph after molting was higher than that at premolt stage. The basal level of activity at intermolt was, however, higher in haemolymph than in gill and integument, which agreed with its role in gas exchange and regulation of acid-base balance in haemolymph. According to Cameron (1989), approximately 95 % of total CO_2 in the haemolymph is in the form of HCO_3^-. Therefore, branchial epithelial carbonic anhydrase should have a role in CO_2 excretion and it must have an access to HCO_3^-. Gutknecht *et al.* (1977) showed that carbonic anhydrase was effective in CO_2 transport across membrane and it must be localized at the "upstream" side of membrane where high HCO_3^- concentration also existed. This situation was similar to the body condition of crab after molting, with a lot of water swallowed. The function of carbonic anhydrase in that location is to maintain a high pCO_2 in the boundary layer, which will drive CO_2 across the membrane. After diffusion, HCO_3^- would react with Ca^{2+} and produce $CaCO_3$ which would be accumulated at the new soft cuticle. The H^+ produced during calcium carbonate formation must be removed before calcification could proceed (Kingley and Watabe, 1987). The mechanism for removal of H^+ from the area of calcification may be a simple diffusion. Hydrogen ion could also be removed via Ca^{2+}/H^+ antiport exchange like in human erythrocytes (Niggli *et al.*, 1982). Price *et al.* (1985) suggested that H^+-ATPase may also be involved in H^+ removal. In conclusion, chitinase and carbonic anhydrase showed distinctive roles in molting of crab both at premolt and postmolt stages but their specific physiological functions are yet to be determined.

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