ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (1): 153-156, 2008
ISSN 1682-296X

Changes of Copper and Protein Profiles in Hepatopancreas and Hemolymph Tissues During Different Molt Stages of White Shrimp, *Litopenaeus vannamei* (Boone, 1931)

M. Sreenivasa Rao, B. Rajitha, E. Pavitra and N. Anjaneyulu
Department of Chemistry, Chundi Ranganayakulu Post Graduate College,
Chilakaluripet, Andhra Pradesh, 522 616, India

Abstract: The objective of the present study was to investigate the levels of copper in hepatopancreas and hemolymph during the molt cycle of the white shrimp, *Litopenaeus vannamei*. Molting of *L. vannamei* is classified into four main stages. The postmolt stage, intermolt stage, premolt stage and ecdysis. We observed the increase of copper and protein levels in the hepatopancreas and hemolymph from postmolt to premolt stages. Similarly, the hemocyanin concentration also increased significantly in hemolymph from postmolt to premolt stages. These results suggest that copper is an essential element in the development (molting) of crustaceans.

Key words: Copper, protein, hemocyanin, molt, *Litopenaeus vannamei*

INTRODUCTION

Information on the occurrence and duration of the molt stage is essential for effective management of the commercially important crustaceans. Some of the crustaceans, especially some shrimps, were unacceptable to commercial processors during early postmolt because they are soft-shelled and the tissues are fluid-filled. Catching ability has been found to vary with molt stage in other crustaceans as susceptibility to trapping decreased during premolt and increased markedly during postmolt (Chittleborough, 1975; Vijayan *et al*., 1997). Hence a clear knowledge about the molt stage of the shrimp and prawn will be useful in the hatchery and farming operations of crustaceans. After the classic study by Drach and Tchernigovtzeff (1967), the schedule of molt staging on the basis of setal development has been described for many species of crustaceans (Huner and Colvin, 1979; Longmuir, 1983; Smith and Dall, 1984; Chan *et al*., 1988; Nagaraju *et al*., 2004, 2006).

Penaeid shrimps are among the most economically important crustaceans and are abundant in tropical and subtropical coasts. In order for these applications to come to fruition, however, a great deal of basic information about the nutritional control of molting in crustaceans must be obtained. Copper is an essential trace metal (micro nutrient) required in small doses by organisms for metabolic functions, but it is potentially very toxic if the internal available concentration exceeds the capacity of physiological/biochemical detoxification processes (Sunda and Hanson, 1987). There are at least 12 major proteins that require copper as an integral part of their structure, including the respiratory enzymes. Copper is essential for the proper functioning of these copper-dependent enzymes, including cytochrome C oxidase (energy production), superoxide dismutase (antioxidant protection), tyrosinase (pigmentation) and dopamine hydroxylase (catecholamine production) (Hassall and Dangerfield, 1990). Most crustaceans possess hemocyanin containing much copper as their main oxygen-carrying blood protein (Dallinger, 1977). The limit between the requirement and toxicity of copper is delicate and dependent on a variety of endogenous and exogenous factors (Weber *et al*., 1992).

The objectives of this study were to observe the different molt stages of shrimp *Litopenaeus vannamei* to determine the concentration of copper, protein (in hepatopancreas and hemolymph) and hemocyanin (in hemolymph) in the different molt stages of shrimp.

MATERIALS AND METHODS

The male shrimp (*L. vannamei*) were collected from fresh market at Chirala (Andhra Pradesh, South India). The shrimps were brought to the laboratory and maintained in the laboratory at 28±1°C in tubs partially filled with aged seawater. They were acclimatized to laboratory conditions (12:12 L:D) for at least 5 days before being used in experiments. The water in the tubs was changed daily.

Corresponding Author: M. Sreenivasa Rao, Department of Chemistry, Chundi Ranganayakulu Post Graduate College, Chilakaluripet, Andhra Pradesh, 522 616, India Tel: +91-8647-253984 Fax: +91-8647-253859

In all experiments, only intact, uninjured male shrimps (body weight 17±2 g; carapace length between 21.0 and 23.0 mm) were selected. During their sojourn, the shrimps were fed on shrimp pellet *ad libitum*. Feeding was stopped one day before the commencement of experiment to avoid changes due to prandial activity.

Identification of molt stages: There are three molt stages: postmolt (A, B), intermolt (C) and premolt (D) which can be distinguished by the degree of hardness of the exoskeleton. The molt stages were observed using setal development in the endopodites of the pleopods as described for this shrimp (Chan *et al*., 1988). Microscopic examination of the setae gives a clear indication of the molting stages of *L. vannamei*.

Isolation of organs: After hemolymph collection, the shrimps were immobilized by chilling on ice for 10 min. The hepatopancreas was isolated, immediately placed in ice-cold crustacean physiological saline (Van Harreveld, 1936) to scrap off adhesive tissue. The hepatopancreas was removed from the saline and lightly blotted with the paper towels, weighed wet on an electronic balance.

Measurement of total proteins: Total protein levels in the hepatopancreas and hemolymph source were estimated following the method of Bradford (1976) using bovine serum albumin as standard.

Estimation of hemocyanin: Hemolymph samples were collected in micro tubes, placed on ice, allowed to clot and frozen at -70°C. The clotted hemolymph was homogenized with a polytron homogenizer and then centrifuged at 10,000 g for 50 min and the resulting supernatant decanted and kept at 0°C. Hemocyanin was measured by a method based on that of Johnson *et al*. (1984). In brief, the hemolymph serum samples were diluted with buffer, 50 mM Tris/10 mM $CaCl_2$ pH 8.0 and readings were taken at 280 and 334 nm. The concentration of hemocyanin was calculated with E280 nm = 13.5 and E334 nm + 2.30 as determined for intact undissociated hemocyanin.

Estimation of copper concentration: For copper analysis, hemolymph samples were diluted 4 folds with deionized water; hepatopancreas was wet-digested with 4 mL of concentrated HNO_3 and the residue was sand dried at 100°C, wet ashed and concentrations determined using flame atomic absorption spectrophotometry (Model 2380, Perkin-Elmer, Norwalk, CT; Engel and Brouwer, 1987).

Statistical analysis: The data were analyzed using one-way ANOVA followed by Student-Newman-Keul's test to determine the level of significance.

RESULTS AND DISCUSSION

Copper and protein concentrations were determined in hepatopancreas and hemolymph during molting and shown to significantly increase during development from the postmolt to the premolt stage ($p<0.0001$) (Table 1). The hemocyanin concentrations in hemolymph were also significantly increased during molting. The concentration of hemocyanin was significantly increased in hemolymph as the shrimp approached premolt stage (D_4 stage) (Table 2). The concentrations of copper and hemocyanin from these shrimps were closely related to the stage of molting.

In order to grow, crustaceans must shed (or molt) the old exoskeleton and replace it with a new and larger one. The time between two successive molts is called the molt cycle. In *Litopenaeus vannamei*, the molt cycle is generally divided into four stages. At stage E, ecdysis occurs, which is the actual shedding of the old exoskeleton. Stages A and B immediately follow ecdysis and are called post-molt stages. This is the time for expansion and hardening of the new exoskeleton, which has been synthesized underneath the old exoskeleton. Stage C is anecdysis, which is the time of feeding,

Table 1: Changes of copper, protein and hemocyanin concentrations in different tissues during different molt stages of the white shrimp, *Litopenæus vannamei*

	Copper concentration		Protein concentration		
Molt stage	HP* (μg g^{-1} tissue)	Hemolymph (g dL^{-1})	HP (mg g^{-1} tissue)	Hemolymph (mg mL^{-1})	Hemocyanin (mg mL^{-1})
Postmolt stage (A) (n = 5)	23.2±1.3	3.2±0.8	63.3±6.3	40.3±3.4	10.6±3.2
Postmolt stage (B) (n = 5)	34.6±3.2	4.6±1.2	86.1±4.7	52.2±4.2	17.4±2.4
Intermolt stage (C) (n = 5)	57.4±2.7	9.4±2.3	130.6±5.9	77.3±3.2	37.3±5.2
Premolt stage (D_0) (n = 5)	68.4±3.3	12.8±1.2	156.5±6.4	107.1±5.5	48.6±4.4
Premolt stage (D_1) (n = 5)	82.5±2.1	16.6±0.9	174.1±4.5	119.4±5.3	56.2±3.3
Premolt stage (D_2) (n = 5)	95.2±5.6	21.3±1.4	189.3±4.2	129.2±4.3	60.2±1.1
Premolt stage (D_3) (n = 5)	119.4±6.3	26.1±1.1	214.4±7.3	142.4±2.8	68.1±1.8
Premolt stage (D_4) (n = 5)	132.2±5.4	29.3±1.3	238.7±6.3	161.0±6.3	77.1±3.7
F-ratio	439.2	257.4	549.4	455.5	244.2
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*HP = Hepatopancreas; Values are mean±SD

Table 2: Description of molt stages of white shrimp, *Litopenaeus vannamei*

Molt stage	Molt duration	Characteristics
Postmolt stage (A)	1-2 h	Very soft exoskeleton, epidermis transparent, feeding none, activity weak. matrix fills new spines Branchiostegites flexible, bend under slight pressure.
Postmolt stage (B)	2-5 h	Soft (little hard) exoskeleton, epidermis granular, feeding restored, activity minimal, conical base of spines absent or partially formed.
Intermolt stage (C)	8-10 days	Exoskeleton hard, feeding maximal, activity maximal, conical base of spines complete.
Premolt stage (D_0)	1-2 days	No setal development, feeding maximal, activity maximal, no new cuticle yet, Epidermis starts apolysis.
Premolt stage (D_1)	1-2 days	New pigmented layer is appeared, feeding decreasing, activity maximal, New spine formation begins, Morphological details of new spines become visible.
Premolt stage (D_2)	2 days	Formation of pre-exuvial layers of new skeleton.
Premolt stage (D_3)	1 day	Resorption of old exoskeleton, feeding decreasing, activity minimal, new setae(spine) developed.
Premolt stage (D_4)	1 day	No feeding, water is absorbed, old exoskeleton splits in preparation for ecdysis, spine well developed.
Ecydsis stage (E)	15 min	Old cuticle is shed, body expanded, no feeding.

reproduction and storage of organic reserves. Stage D is premolt, which is the time for preparation for shedding of the exoskeleton and the synthesis of new exoskeleton (Table 2).

The hepatopancreas periodically accumulates and releases copper during molting and starvation (Arumugam, 1989) and it has been shown to be the site of hemocyanin synthesis (Spindler *et al*., 1992). Hemocyanin is a copper containing, multi-subunit protein; it has evolved to carry out the specialized functions of oxygen transport in arthropods and molluscans. The O_2 molecule is bound to two copper ions, each of which is coordinated by three histidines. Crustacean hemocyanins are highly variable in quaternary structures (Burmester, 2004). As the main protein component of hemolymph, hemocyanin classically represents up to 95% of the total amount of protein (Sellos *et al*., 1997). Hemocyanins also gave rise to non-respiratory proteins, which most likely have storage functions (Burmester, 2004).

During the molt cycle, trace metal concentrations in the hepatopancreas and hemocyanin concentration in the hemolymph of blue crabs changes significantly (Engel and Brouwer, 1991). Similarly, the results presented here showed changes of copper and hemocyanin concentration in the shrimp during molt stages. Hemocyanin is synthesized in the hepatopancreas confirming the results obtained in the crayfish using [^{35}S] methionine incorporation (Gellissen *et al*., 1991), in the blue crab using hybridization of hepatopancreas mRNA with an oligonucleotide based on the amino acid sequence encompassing the copper B binding site (Rainer and Brouwer, 1993) and in prawns (Khayat *et al*., 1995). In the present study, the copper profiles of hemocyanin (only hemolymph) and protein were significantly increased from postmolt stage to premolt stage in two tissues (hepatopancreas and hemolymph) of shrimp. These observations agree well with changes of hemocyanin and protein concentrations observed in the white shrimp *Litopenaeus vannamei* in relation to size and molt stage (Cheng *et al*., 2002). We hypothesized that the copper thus accumulated from the medium and released back in to hemolymph in the form of hemocyanin (copper-bound protein). During premolt, hemocyanins give higher rise to respiratory rather than non-respiratory protein (vitellogenin). On the basis of these results on molting in shrimps, we suggest that trace amount of copper may control, at least in part, molting. Furthermore, this work provides strong supporting evidence to the hypothesis that the hepatopancreas is the source of the respiratory and non-respiratory proteins.

ACKNOWLEDGMENTS

We are special thankful to Correspondent, Sree Chundi Subbarao, Chundi Ranganayakulu Post Graduate College, Chilakaluripet for providing specialties and encouragement. This research was generously supported by the University Grant Commission (UGC) grant, India.

REFERENCES

Arumugam, M., 1989. Mobilization of copper among tissues in the estuarine crab *Scylla serrata* (Forskal) under imposed starvation. Arch. Int. Physiol. Biochem., 97: 247-257.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Burmester, T., 2004. Evolutionary history and diversity of arthropod hemocyanins. Micron, 35: 121-122.

Chan, S., S.M. Rankin and L.L. Keely, 1988. Characterization of the molt stages in *Penaeus vannamei*. Setogenesis and hemolymph levels of total protein, ecdysteroids and glucose. Biol. Bull., 175: 185-192.

Cheng, W., C.H. Liu and D.F. Yan, 2002. Hemolymph oxyhemocyanin, protein, osmolarity and electrolyte levels of white leg shrimp *Litopenaeus vannamei* in relation to size and molt stage. Aquaculture, 211: 325-339.

Chittleborough, R.G., 1975. Environmental factors affecting growth and survival of juvenile Western rock lobsters *Panulirus longipes* (Milne Edwards). Aust. J. Mar. Freshwater. Res., 26: 177-196.

Dallinger, R., 1977. The flow of copper through a terrestrial food chain. III. Selection of an optimum copper diet by isopods. Oecologia (Berlin), 30: 273-277.

Drach, P. and C. Tchernigovtzeff, 1967. Sur la méthode détermination des stades d'intermue et son application générale aux Crustacés. Vie Milieu, 18: 595-610.

Engel, D.W. and M. Brouwer, 1987. Metal regulation and molting in the blue crab, *Callinectes sapidus*, metallothionein function in metal metabolism. Biol. Bull., 173: 251-339.

Engel, D.W. and M. Brouwer, 1991. Short-term metallothionein and copper changes in blue crabs at ecdysis. Biol. Bull., 180: 447-452.

Gellissen, G., R. Henneke and K.D. Spindler, 1991. The site of synthesis of hemocyanin in the crayfish, *Astacus leptodactylus*. Experientia, 47: 194-195.

Hassall, M. and J.M. Dangerfield, 1990. Density dependent processes in the population dynamics of *Armadillidium vulgare* (Isopoda: Oniscidae). J. Anim. Ecol., 59: 941.

Huner, J.V. and L.B. Colvin, 1979. Observation on the moult cycles of two species of juvenile shrimp, *Penaeus californiensis* and *Penaeus stylirostris* (Decapod; Crustacea). Proc. Nat. Shellfish. Assoc., 69: 77-84.

Johnson, B.A., C. Bonaventura and J. Bonaventura, 1984. Allosteric modulation of *Callinectes sapidus* hemocyanin by binding L-lactaate. Biochemistry, 23: 872-878.

Khayat, M., B. Funkestein and A. Tiez, 1995. *In vivo*, *in vitro* and cell-free synthesis of hemocyanin in the shrimp *Penaeus semisulcatus* (de Haan). Comp. Biochem. Physiol., 112: 31-38.

Longmuir, E., 1983. Setal development, moult staging and ecdysis in the banana prawn *Penaeus merguiensis*. Mar. Biol., 77: 183-190.

Nagaraju, G.P.C., P.R. Reddy and P.S. Reddy, 2004. Mandibular organ, it's relation to body weight, sex, molt and reproduction in the crab, *Oziotelphusa senex senex* Fabricius (1791). Aquaculture, 232: 603-612.

Nagaraju, G.P.C., P.R. Reddy and P.S. Reddy, 2006. *In vitro* methyl farnesoate secretion by mandibular organs isolated from different molt and reproductive stages of the crab, *Oziotelphusa senex senex*. Fish. Sci., 71: 410-414.

Rainer, J. and M. Brouwer, 1993. Hemocyanin synthesis in the blue crab *Callinectes sapidus*. Comp. Biochem. Physiol., 104: 69-73.

Sellos, D., S. Lemoine and A.V. Wormhoudt, 1997. Molecular cloning of hemocyanin cDNA from *Litopenaeus vannamei* (Crustacea: Decapoda), structure, evolution and physiological aspects. Febs. Lett., 407: 153-158.

Smith, D.M. and W. Dall, 1984. Moult staging in the tiger prawn, *Penaeus esculentus*. Nat. Prawn. Sem. NPSQ, QLD, pp: 85-93.

Spindler, K.D., R. Hennecke and G. Gellissen, 1992. Protein production and the molting cycle in the crayfish *Astacus leptodactylus* (Nordmann, 1842). In: II. Hemocyanin and protein synthesis in the midgut gland. Gen. Comp. Endocrinol., 85: 248-253.

Sunda, W.G. and A.K. Hanson, 1987. Measurement of free cupric ion concentration in seawater by a ligand competition technique involving copper absorption onto C_{18} SEP-PAK cartridg. Limnol. Oceano. Org., 32: 537-551.

Van Harreveld, A., 1936. A physiological solution for freshwater crustaceans. Proc. Soc. Exp. Biol. Med., 34: 428-432.

Vijayan, K.K., K.M. Sunilkumar and A.D. Diwan, 1997. Studies on moult staging, moulting duration and moulting behaviour in Indian white shrimp *Penaeus indicus* Milne Edwards (Decapoda; Penaidae). J. Aqua. Trop., 12: 53-64.

Weber, R.E., A. de Zwaan and A. Bang, 1992. Interactive effects of ambient copper and anoxic, temperature and salinity stress on survival and hemolymph and muscle tissue osmotic in *Mytilus edulis*. J. Exp. Mar. Biol. Ecol., 159: 131-156.