

## Original Article

# Effect of copper on the characterization of proteins in the Spiny lobster, *Panulirus homarus homarus* (Linnaeus, 1758)

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**Abstract:** Copper is most toxic metal in marine organisms. Characterization of protein occurring in the metabolically active tissues of muscle (MU), hepatopancreas (HP) and gills (GL) of the spiny lobster, *Panulirus homarus homarus* on exposure to two sub-lethal doses (9.55 and 19.1 µg/l) of copper were studied for 28 days of exposure (DoE). The electrophoretic pattern of muscle, hepatopancreas and gill proteins revealed 12, 8 and 8 slow moving bands (control). The number of bands decreased to 8 and 7, 6 and 5, 6 and 4 after 7 days of exposure to 9.55 µg/l and 19.1 µg/l concentrations of copper, respectively. After 28 days, the protein bands decreased to 7 and 6, 5 and 4, 4 and 4 at 9.55 µg/l and 19.1 µg/l concentrations of copper, respectively. Present study to indicate that to avoid the Cupro-Nickel coil in lobster holding centers in chiller plants used for cooling of water was found to be responsible for the mortality of lobsters during live transportation.

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## Introduction

The electrophoretic techniques are promising tools for identifying the protein profile in response to stressful and sublethal level of heavy metals. Heavy metal binding proteins have been found to be associated with copper and the lower molecular weight protein in the lake fauna (Dutta et al., 1984). Chang et al. (1987) isolated and purified the aminoacids from the lobster, *Homarus americanus* exposed to copper. Similarly, Canli et al. (1997) analyzed the effect of copper in the gill and hepatopancreas tissues of the Norway lobster, *Nephrops norvegicus*. He also suggested presence of copper metallothionein in the gill and hepatopancreas. Brouwer et al. (1989) studied the structural and functional diversity of copper metallothioneins from the American lobster, *Homarus americanus* and sequenced 56 aminoacids of copper binding proteins. Brouwer et al. (1991)

studied a crucial role of copper binding proteins in the regulation of reactive ions in the same species. Lobster metallothioneins share a number of similarities with mammalian metallothioneins with respect to the presence of copper and cadmium, apparent molecular weights and amino acids composition, but differ substantially in their electrophoretic behaviour (Chou et al., 1991). Copper toxicity of spiny lobster, *Panulirus homarus homarus* in muscle conductivity, bioaccumulation, chromosomal aberrations and histopathological changes also has been reported (Maharajan et al., 2010a; Maharajan et al., 2010b; Maharajan et al., 2011; Maharajan et al., 2012). Krishnamoorthy and Subramanian (1997) reported intensities of major polypeptide bands in the freshwater prawn, *Macrobrachium lamerrei lamerrei*, exposed to copper. Similarly, the reduction in the number of protein fraction in *Scylla serrata* treated with copper

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was reported by Ramanibai (1986).

The present study intended to evaluate the lethal and sublethal effect of copper in the spiny lobster, *P. homarus homarus*, the major spiny lobster exported live from India. Since mortalities due to copper toxicity have been reported in lobster holding centers. It is hoped that the study will provide a useful blueprint for live transportation and packing agencies in designing an effective programme for avoiding toxicity of copper during live transport of lobsters.

### Materials and methods

**Experimental animals and acclimation:** Actively moving juveniles of spiny lobster, *P. homarus homarus* (weight 150-200 g) with no visible signs of disease or morbidity were collected from Kovalam using bottom set gill net at a depth of 5-8 meters located in Tamil Nadu. Immediately after the collection, juvenile lobsters were transferred to the laboratory conditions in two large FRP tanks (cap. 200 L) for more than 2 weeks before the initiation of experiments. The seawater used in acclimation tanks was treated by rapid sand filtration, bio-filtration and passed through ultraviolet radiation. Adequate aeration was provided using air blowers, and optimum water quality parameters were maintained during the acclimation period: temperature, ( $29 \pm 1^\circ\text{C}$ ), salinity ( $33 \pm 1$  ppt), dissolved oxygen ( $6.4 \pm 0.8$  mg l<sup>-1</sup>), pH ( $7.9 \pm 0.4$ ), NO<sub>2</sub>-N ( $< 0.02$  mg l<sup>-1</sup>) and NH<sub>3</sub>/NH<sub>4</sub> ( $0$  mg l<sup>-1</sup>). The seawater used for acclimation and exposure experiments was free from residues of copper. A photoperiod of 12 L (0700 h-1900 h):12 D (1900 h-0700 h) was maintained. Lobsters were fed ad libitum twice a day (0800 h and 1600 h) with live marine clam *Donax cuneatus*. Faeces and uneaten feed was siphoned out twice a day (1000 h and 1730 h) and 50% of water exchanged daily (07.30 h). In order to reduce the amount of excreted products in the test tanks, feeding was stopped 48 h prior to the commencement of acute bioassay tests.

**Test chemical:** Stock solution of copper were prepared by dissolving 3.963 g copper sulphate

pentahydrate (CuSO<sub>4</sub> 5 H<sub>2</sub>O, Merck, Germany) in 100 ml of 2% sulphuric acid solution and making upto 1000 ml with double deionised water. It was stored in a clean standard flask at room temperature in the laboratory.

**Acute bioassay tests:** Acute toxicity test, 10 active animals each were exposed to various concentrations of the copper (80, 100, 120, 140, 180 and 200 µg/l) using filtered sea water as control. Experimental animals were starved for one week. The experiments were conducted in three replicates at room temperature. No feed was given during the test period. Mortality of lobsters was recorded continuously for 12, 24, 36, 48, 72 and 96 hrs. Percent mortality was calculated and the values were transformed into the probit scale. Probit analysis was carried out based on Finney (1971).

**Test solutions and sub-acute tests:** It has been hypothesised that sublethal concentrations of copper offer an excellent scope for observing the behavioural and physiological changes in animals. Two sublethal doses corresponding to 10% and 20% of 96-h LC<sub>50</sub> were selected for sub-acute toxicity experiments. Concentrations of active ingredient of copper present in two sublethal concentrations computed from commercial-grade composition were found to be 9.55 and 19.1 µg/l, respectively. The exact amount of active ingredient of copper present in each sublethal solution however, was not quantified. For evaluation of effects of sublethal concentrations, 90 randomly sampled lobsters of similar size (weight 150-200 g; n=10) were divided into 3 groups, each group comprising of 30 intermoult juveniles (stage.c) of *P. homarus homarus*. One group served as control, while two other groups were exposed to two sublethal doses of copper (one group to one sublethal dose). A total of three replicate aquaria (500 L capacity) were maintained for each dose and the control group (10 lobsters per concentration per replicate). During the exposure period, a mild aeration was provided using air pumps (BOYU, Japan) in order to maintain DO levels not less than 5 mg l<sup>-1</sup>. Juveniles were fed with live marine clam *Donax cuneatus*, ad libitum and

were deprived of feed 24 h before exposure experiments. In toxicological studies, chronic tests of shorter duration (~28 days) have been recommended as an alternative to longer chronic tests (Maki, 1979). The experiment was run for a period of 28 DoE as the estimated intermoult period of *P. homarus homarus* under the laboratory conditions was estimated to be  $21 \pm 1$  days (personal observation). In order to maintain constant concentration of copper in test solutions, the entire toxic medium in each aquarium was gently siphoned out daily (09.00 h) and renewed with freshly prepared solution of respective sublethal concentrations of copper. Aeration was suspended temporarily during water exchange and feeding, and care was taken that the disturbance caused to the lobsters was minimal.

**Characterization of protein:** The lobsters were exposed to 9.55  $\mu\text{g/l}$  and 19.1  $\mu\text{g/l}$  concentrations of copper for 28 days. After 0, 7 and 28 days, the lobsters were sacrificed. Muscle, gills and hepatopancreas were excised out and analyzed for characterization of protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the protocol of Weber and Osborn (1969). The gel was immersed in 5 ml of staining solution (200 mg Coomassie brilliant blue R250+50 ml MeOH (Methyl hydroxide) + 7 ml acetic acid + 43 ml distilled water and was allowed to stain for 4 hours at room temperature. The stain was removed and the gel destained with acetic acid and methyl hydroxide solution (7 ml acetic acid + 30 ml Methyl hydroxide + 63 ml distilled water). The gel was stored in 7% acetic acid, the bands were visualized under UV trans-illuminator.

## Results

**Protein pattern in muscle:** Twelve distinct protein fractions, eight with high molecular weights and four with low molecular weights, have been detected in the muscle tissue of control lobster (*P. homarus homarus*) after 7 days of experiment using standard marker proteins (Fig. 1). The high molecular weight

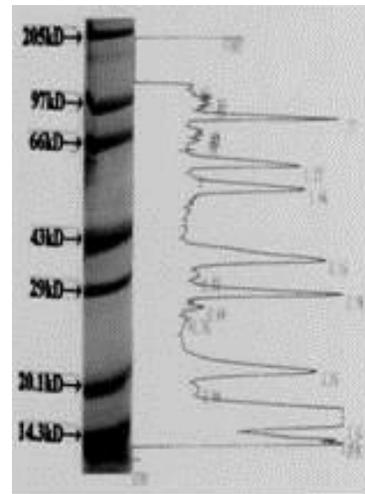


Figure 1. Electrophorogram and densitometric scan image of Marker protein.

fractions ranged from 190 kD to 77 kD. The low molecular weight fractions ranged from 42 kD to 18 kD. The high molecular weight polypeptide constituted 20.642 mg/g protein at 77 kD at an area of 10.646% and 2.25 seconds retention time. The low molecular weight polypeptide constituted 41.103 mg/g protein at 38 kD at an area of 21.198% and 3.38 seconds retention time (Fig. 2A). After 7 days of exposure to 9.55  $\mu\text{g/l}$  concentration of copper, the number of protein fractions declined and only eight numbers of distinct protein fractions were observed. Seven were with high molecular weights and one with low molecular weight. The high molecular weight fractions ranged from 198 kD to 73 kD. The low molecular weight fraction was found to be at 29 kD. The high molecular weight polypeptide constituted 39.323 mg/g protein at 73 kD at an area of 20.502% and 2.11 seconds retention time. The low molecular weight polypeptide constituted 32.788 mg/g protein at 29 kD at an area of 17.098% and 3.42 seconds retention time (Fig. 2B). Similarly lobsters exposed to 19.1  $\mu\text{g/l}$  concentration of copper had seven distinct fractions six with high molecular weights and one with low molecular weight. The high molecular weight proteins ranged from 167 kD to 88 kD. The low molecular weight protein was of 32 kD. The high molecular weight protein was maximum at 88 kD (24.556 mg/g) at an area of 13.353% and 2.32 seconds retention time. The low molecular weight polypeptide constituted 20.233

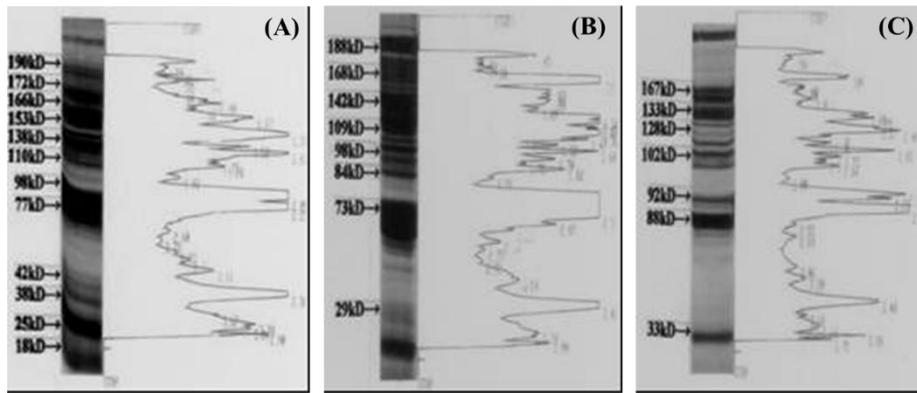


Figure 2. Electrophorogram and densitometric scan image of total proteins in the muscle of *P. homarus homarus* after 7 days of the experiment (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

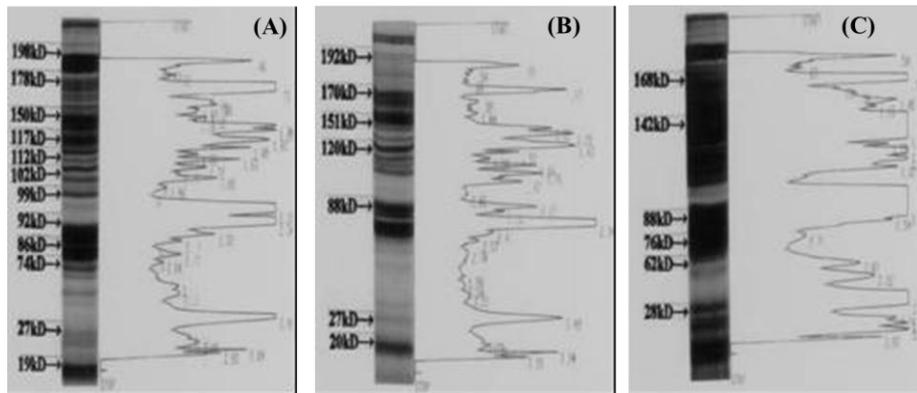


Figure 3. Electrophorogram and densitometric scan image of total proteins in the muscle of *P. homarus homarus* after 28 days of the experiment (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

mg/g protein at 32 kD at an area of 11.002% and 3.48 seconds retention time (Fig. 2C).

Muscle tissue of control lobster after 28 days (closure of the experiment) revealed a broad spectrum of polypeptides with twelve fractions. Among them ten were with high molecular weights and two with low molecular weights. The high molecular weight fractions ranged from 198 kD to 74 kD and the low molecular weight ranged fractions from 27 kD to 19 kD. The high molecular weight protein fraction with maximum quantity was at 92 kD (24.656 mg/g) at an area of 13.032% and 2.18 seconds retention time. The low molecular weight protein with maximum quantity was observed at 27 kD (30.397 mg/g) at an area of 16.066% and 3.49 seconds retention time (Fig. 3A). After 28 days of exposure to 9.55 µg/l concentration of copper the number of protein fractions reduced to seven, five with high molecular weights and 2 with low molecular weights. The high molecular weight

proteins ranged from 88 kD to 192 kD and the low molecular weight protein ranged from 27 kD to 20 kD. Among the high molecular weight fractions, the maximum was found to be 24.492 mg/g protein at 88 kD at an area of 14.484% and 2.34 seconds retention time. The maximum value for low molecular weight polypeptide was found to be 17.191 mg/g protein at 27 kD at an area of 10.1665 and 3.49 seconds retention time (Fig. 3B). Similarly lobsters exposed to 19.1 µg/l concentration of copper revealed less number of protein fractions and thicker bands. However, the effect was found to be severe with many interfering bands between high and low molecular weight peptides. Six protein fractions were observed and among them, five had high molecular weights and one, low molecular weight. The high molecular weight protein ranged from 168 kD to 62 kD. The low molecular weight protein was of 28 kD. Among the high molecular weight fractions the maximum was observed at 142 kD

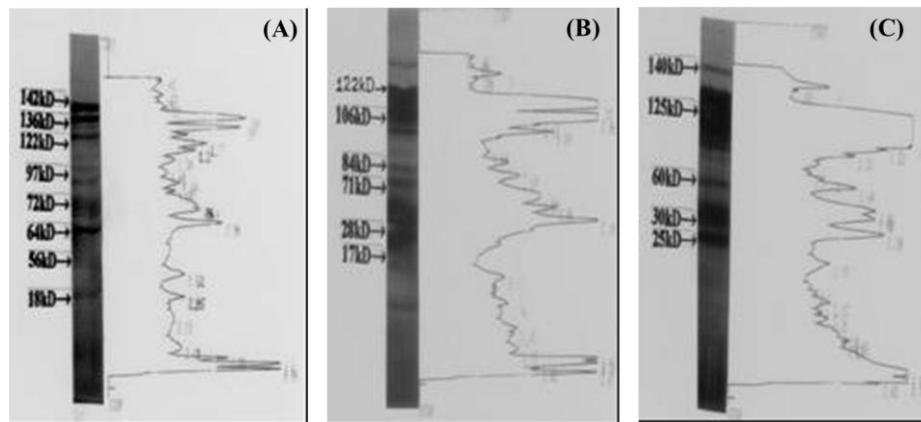


Figure 4. Electrophorogram and densitometric scan image of total proteins in the hepatopancreas of *P. homarus homarus* after 7 days of the experiment (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

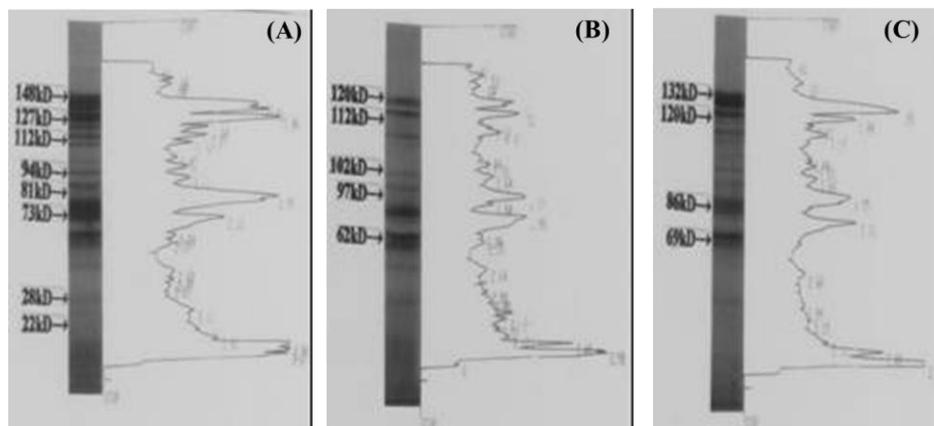


Figure 5. Electrophorogram and densitometric scan image of total proteins in the hepatopancreas of *P. homarus homarus* after 28 days of the experiment (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

(30.077 mg/g protein) at an area of 18.362% and 1.22 seconds retention time. The low molecular weight protein at 28 kD contained 22.331 mg/g protein at an area of 13.633% and 3.43 seconds retention time (Fig. 3C).

**Protein pattern in hepatopancreas:** The protein profile of hepatopancreas of control group after 7 days revealed eight distinct protein fractions seven with high molecular weights and one with low molecular weight. The high molecular weight proteins ranged from 142 kD to 56 kD and the low molecular weight fraction was of 18 kD. Among the high molecular weight fractions, maximum was observed at 64 kD (20.521 mg/g protein) at an area of 12.675% and 2.32 seconds retention time. The low molecular weight fractions at 18 kD had 15.170 mg/g protein at an area of 9.370% and 2.95 seconds retention time (Fig. 4A). After 7 days of exposure to 9.55 µg/l concentration of copper, the zymogram

pattern revealed six protein fractions, four with high molecular weights and two with low molecular weights. The high molecular weight proteins ranged from 122 kD to 71 kD. The low molecular weight proteins were found to be between 28 kD and 17 kD. Maximum high molecular protein (17.378 mg/g) was found at 125 kD at an area of 11.9275 and 0.93 seconds retention time. The low molecular weight fraction at 17 kD contained 16.092 mg/g protein at 2.14 seconds retention time and extending to an area of 11.045% (Fig. 4B). Similarly, after 7 days of exposure to 19.1 µg/l concentration of copper, the hepatopancreas revealed five fractions, three with high molecular weights and two with low molecular weights. The high molecular weight proteins ranged from 140 kD to 60 kD and the low molecular weight protein range was upto 25 kD (Fig. 4C). The high molecular protein having maximum quantity was found at 125 kD (9.808 mg/g) at 0.86 seconds

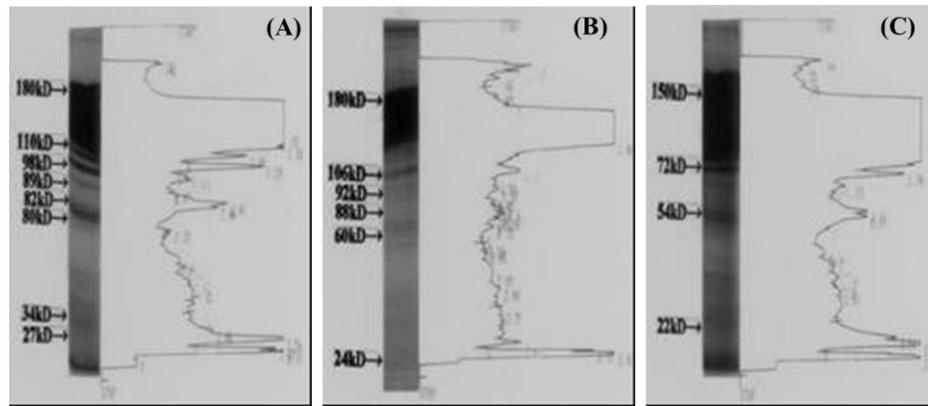


Figure 6. Electrophorogram and densitometric scan image of total proteins in the gills of *P. homarus homarus* after 7 days of the exposure (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

retention time having an area of 6.636%. Low molecular protein was maximum 12.140 mg/g at 25 kD at 2.10 seconds retention time, in 8.214% of area. Besides, several minor insignificant fractions were also exhibited.

The zymogram pattern of hepatopancreas in control lobster at the end of the 28 days revealed eight distinct protein fractions. Six distinct bands with high molecular weight proteins ranged from 148 kD to 73 kD (Fig. 5A). The 81 kD protein had the maximum quantity of 21.890 mg/g (1.95 seconds retention time and an area of 13.571%). Similarly, two low molecular weight proteins ranged from 28 kD to 22 kD. Maximum protein of low molecular weight was observed at 22 kD (12.280 mg/g) at an area of 7.613% and 3.50 seconds retention time. The effect of copper on hepatopancreas was found to be severe with indistinct protein profiles after 28 days of exposure to 9.55 µg/l concentration of copper. Five protein fractions were observed all with high molecular weights ranging from 120 kD to 62 kD (Fig. 5B). The 62 kD protein had the maximum quantity of 15.059 mg/g (2.08 seconds retention time and an area of 13.291%). After 28 days of exposure to 19.1 µg/l concentration of copper, the characterization of protein revealed only four distinct fractions of high molecular weight ranging from 132 kD to 69 kD. Few minor fractions of protein were also recorded. Among the high molecular weight fractions, maximum was recorded at 132 kD (16.221 mg/g) at an area of 13.395% and 2.08 seconds retention time (Fig. 5C).

**Protein pattern in gills:** The zymogram pattern of gills in control lobster after 7 days revealed eight distinct bands, six with high molecular weight proteins ranging from 180 kD to 80 kD and two with low molecular weights ranging from 34 kD to 27 kD (Fig. 6A). The 180 kD protein had maximum quantity 14.953 mg/g (0.85 seconds retention time and an area of 36.741%). Maximum low molecular weight protein was observed at 34 kD (3.647 mg/g) at an area of 3.458% and 3.72 second retention time. After 7 days of exposure to 9.55 µg/l concentration of copper, the gills revealed six distinct bands, five with high molecular weights ranging from 180 kD to 60 kD and one low molecular fraction at 28 kD (Fig. 6B). The 180 kD protein had the maximum quantity of 16.691 mg/g (1.06 seconds retention time and an area of 60.258%). The low molecular weight fraction at 28 kD constituted 2.018 mg/g protein with 7.287% coverage of area and 3.82 seconds retention time. After 7 days of exposure to 19.1 µg/l concentration of copper, four distinct protein fractions three with high molecular weights and one low molecular weight were observed. The high molecular weight proteins ranged from 150 kD to 54 kD. The 150 kD protein had the maximum quantity of 13.821 mg/g (0.87 seconds retention time and an area of 36.372%). The low molecular weight protein at 22 kD had 2.597 mg/g protein covering an area of 6.834% with a retention time of 3.76 seconds (Fig. 6C).

The zymogram pattern of gills in control lobster after 28 days revealed eight fractions, seven with high

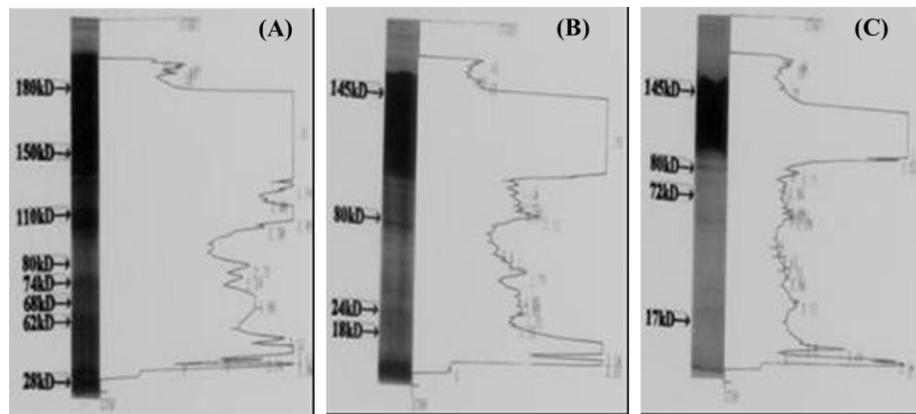


Figure 7. Electrophorogram and densitometric scan image of total proteins in the gills of *P. homarus homarus* after 28 days of the exposure (A) Control, (B) exposed to 9.55 µg/l and (C) exposed to 19.1 µg/l concentration of copper.

molecular weights and one with low molecular weight. The high molecular weight proteins ranged from 180 kD to 62 kD (Fig. 7A). The 180 kD protein had the maximum quantity of 15.128 mg/g (0.81 seconds retention time and an area of 45.431%). Similarly, the low molecular weight protein at 28 kD had 1.147 mg/g protein with a coverage of 3.446% and 3.80 seconds retention time. The zymogram pattern of gills in lobster exposed to 9.55 µg/l concentration of copper for 28 days revealed four fractions two with high molecular weights and two with low molecular weights. The high molecular weight proteins ranged from 145 kD to 80 kD with the 145 kD fraction having maximum quantity of 16.850 mg/g (0.78 seconds retention time and an area of 43.204%). The low molecular weight protein ranged from 18 kD to 24 kD and the 24 kD protein had the maximum quantity of 7.323 mg/g (3.50 seconds retention time and an area of 18.777%) (Fig. 7B). After 28 days of exposure to 19.1 µg/l concentration of copper, the gills revealed four distinct fractions, three with 145 kD to 72 kD and one with low molecular weight (17 kD). The 145 kD protein had maximum quantity of 20.731 mg/g (1.00 seconds retention time and an area of 47.877%). The low molecular weight protein at 17 kD had 2.939 mg/g covering an area of 6.787% and a retention time 3.79 seconds (Fig. 7C).

## Discussion

The electrophoretic technique remains a promising tool for identifying the protein profile in response to

stressful and sublethal level of heavy metals (Dutta et al., 1983; Costa et al., 2002). Heavy metal binding proteins are associated with copper and the lower molecular weight protein in particular is found to have a significant percentage of copper contained in the muscle, hepatopancreas and gills. The lower molecular weight protein probably plays a significant role in the metabolism of the copper. The hepatopancreas of decapod crustaceans has been implicated as being important in the metabolism of heavy metal, for it is the site of absorption of products derived from digested food and also it appears to act as a store for metal taken up from solution over body surface such as the gills (Bryan, 1968; Jennings and Rainbow, 1979; Jacqueline et al., 2010).

The disappearance of bands in the muscle, hepatopancreas and gills of *P. homarus homarus* on exposure to copper may be due to the interference of copper in the protein synthesis process and the reduction in the number of banding pattern as reported in freshwater prawn, *Macrobrachium lamerrei lamerrei* (Krishnamoorthy and Subramanian, 1997). In the present study, 12 distinct bands are accounted in the muscle tissue of *P. homarus homarus*. Extensive disruption in the number of banding is well-documented at 19.1 µg/l of concentration with seven and six polypeptide fractions during 7 and 28 days of copper exposed lobster. Low molecular myofibrillar proteins (troponin) hydrolyzed by specific proteinase are of interest, because these proteins are hampered by the

fragments produced from bigger molecule proteins such as myosin heavy chain (Pangkey et al., 2000). Lim and Lee (1970) working on prawn muscle myogen reported species specificity and used the electrophoretic technique to trace the toxicity levels between species. In addition, the muscle myogen are found to vary considerably between different concentrations and tissues. The present investigation confirms the above findings on copper toxicity. The muscle myogens are generally more in number than in the gills and hepatopancreas indicating the number of glycoproteins is more in muscle than in other tissues in *P. homarus homarus*.

In hepatopancreas, the number of protein bands reduced from 8 (control) to 6 and 5 at 9.55 µg/l concentration during 7 and 28 days, respectively. Similarly, at 19.1 µg/l of concentration, the total number of bands reduced from 8 (control) to 4 at either days of exposure (7 and 28). It is also evident that exposure to copper disturbed the banding pattern under stress conditions. Krishnamoorthy and Subramanian (1997) reported that the intensities of the major polypeptide bands in the gills of prawns when treated with heavy metal were less than that of the control. The result is in accordance with the current observation in *P. homarus homarus* in which the 8 distinct bands (control) decreased to 6 and 4 at 9.55 µg/l during 7 and 28 days, respectively. Similarly, the bands reduced to four at 28 days of exposure to 19.1 µg/l concentration of copper. Similar reduction in the numbers of protein fractions was found in *Scylla serrata* when treated with copper (Ramanibai 1986).

*Panulirus homarus homarus* has minimum protein residue in muscle, hepatopancreas and gills due to copper toxicity which interfered with the banding pattern of proteins as reported in other aquatic invertebrates (Wright, 1978). It is also evident that exposure to copper disturbed the banding pattern of protein under stress condition in *P. homarus homarus*. Similar alterations in the banding pattern of protein due to trace metal interaction has been reported in fishes *Salmo gairdnerii* due to arsenic (Kothary and Candido, 1982) and in *Oncorhynchus*

*tshawtsche* due to zinc and cadmium (Heikkila et al., 1982).

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