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# Haemolymph proteins of the Indian white shrimp, *Penaeus indicus* (H. Milne Edwards)

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## ABSTRACT

Haemolymph proteins of the Indian white shrimp, *Penaeus indicus* were characterised by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The most important fractions were the cuproproteins-hemocyanins, characterized by two fractions. The two fractions are also lipo- and glyco-proteic in nature. Males and females possess similar protein pattern and the maximal number of fractions separated was 16.

## Introduction

Electrophoresis in stabilizing media, has been widely used for accurate and rapid characterization of crustacean haemolymph proteins. However, these results, obtained by different electrophoretic techniques are not easily comparable and often lead to confusing results. Although data concerning the blood protein composition lack uniformity, they clearly demonstrate the occurrence of an important intraspecific variation of the haemolymph constituents. This may be partly attributed to differences in physiological factors such as sex, season, diet (Uglow, 1969a,b), moult cycle (Busselin, 1970; Chaix *et al*., 1981) and reproductive cycle (Feilder *et al*., 1971; Picaud, 1971, 1976; Munuswamy and Subramoniam, 1987; Rankin *et al*.,1989). In spite of these variations, the specific haemolymph pattern could be discerned through electrophoretic analysis (Manwell and Baker, 1963).

In *P. indicus*, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to characterize the haemolymph protein pattern. In this study, variations in the haemolymph protein pattern with respect to size and sex have been characterized. The two major fractions have been identified and their molecular weights determined.

## Materials and Methods

*P. indicus* used for the present study was collected from the brackish water growout farms of the Central Institute of Brackish water Aquaculture, Narakkal, as well as from the off-shore waters and transported to the laboratory at Central Marine Fisheries Research Institute, Cochin and maintained in 23-25‰ sea water in 1 ton fibre glass tanks provided with continuous aeration for acclimatization upto 16-20 hours. The haemolymph was extracted by piercing a 22-guage needle attached to 1 ml syringe, directly into the heart, just below the cephalothorax using 3% sodium citrate as anticoagulant. The samples

were stored at 4ºC until further use.

Haemolymph proteins of *P. indicus* was analysed electrophoretically to separate the species specific proteins. The haemolymph protein patterns of both, male and female of different size groups ( 60-80 mm, 80-100 mm, 100-120 mm and 120-140 mm) were compared with normal human serum. The polyacrylamide gel disc-electrophoresis (PAGE) method (Davis, 1964) was followed with necessary modifications. Separating gel concentrations of 5% Acrylamide and 2% Bisacrylamide and buffer Tris boric acid $Na_2$ EDTA (0.5M, pH 8.0) combined with 1% SDS and 2 - mercaptoethanol gave best separation and resolution. Spacer gel in the concentration of 1:2:1:4 (large pore monomer, large pore buffer, riboflavin and 40% sucrose) was used to obtain clear separation of fractions. 10 $\mu$l haemolymph sample was used for separation. For larger specimens of *P. indicus*, wherein the protein concentration is high, the haemolymph sample was diluted with double distilled water and 40 $\mu$l was used for separation.

### *Staining*

*Protein fractions:* 0.25% Coomassie Brilliant Blue in methanol, glacial acetic acid and water (5:5:1) was used for staining the protein fractions.

*Copper protein fractions:* To detect the presence of copper protein fractions, the gels were treated for 48 hours at room temperature in a saturated solution of Rubeanic acid in methanol, acetic acid and water (5:2:5). The appearance of greenish brown colour indicated the presence of copper (Horn and Kerr, 1969).

*Glycoprotein fractions:* Periodic acid-Schiff (PAS) was used to localise the polysaccharides associated with proteins.

*Lipoprotein fractions:* Oil Red 'O' in 50% methanol and 10% TCA was used to detect neutral *lipids.*

*Calcium binding fractions:* The calcium binding protein fractions were detected by Alizarin red S staining method . (Pearse ,1968).

*Determination of molecular weights of hemocyanins:* The method of Weber and Osborn (1969) and Davis and Stark (1970) was followed. The molecular weight markers of MW SDS-280 kit of Sigma Co. were used as standards.

## Results

Figure 1 represents the composite haemolymph protein pattern specific to *Penaus indicus* obtained after SDS-PAGE of haemolymph of males and females in the size range 60- 140 mm. Beginning with the components of highest mobility, the fractions are serially numbered in the ascending order. Generally 10-16 protein fractions were observed in the haemolymph, the major fractions being the copper bearing proteins i.e., the fast and slow hemocyanins.

*Fast hemocyanin (fraction 7) :* The broad, intensely staining somewhat dif-

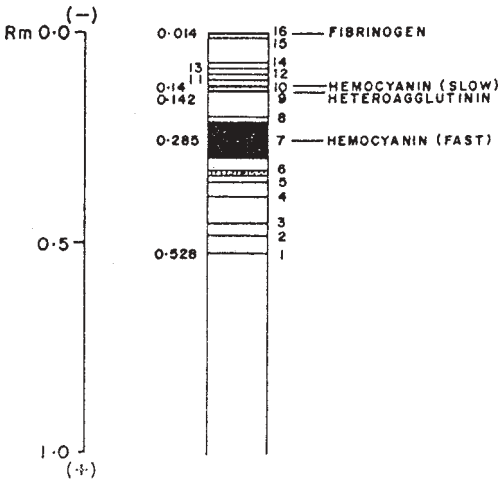


Fig. 1. Composite pattern of haemolymph protein fractions of *Penaeus indicus*

fuse but major component, represents hemocyanin. This component constituted nearly 80% of the total protein in the haemolymph along with the slow fraction. The Rf value of this fraction ranged between 0.285 to 0.30 and it varied quantitatively with size of the species. This fraction stained for copper, calcium and was also glycolipoproteic in nature.

*Slow hemocyanin (fraction 10)* : This copper bearing protein, a narrow zone of high intensity of stain is termed as the slow hemocyanin. This slow hemocyanin fraction was invariably present in all samples of haemolymph and stained positively for copper and calcium and was also a glyco-lipo-protein complex like the fast hemocyanin. The relative mobility (Rf) was invariably 0.14.

*Heteroagglutinin (Fraction 9)*: A streaky band closely associated with the slow hemocyanin fraction is considered to be the heteroagglutinin, a component associated with clotting of the haemolymph. (Terminology adopted from *Ghidalia et.al. (1970) and Baron, 1975*). This fraction is non-copper bearing but stained for calcium and rarely for PAS and Oil red O. The exact nature of this protein fraction is still unknown. The relative mobility in all cases was found to be 0.142.

*Clotting protein (Fraction 16):* This is a streaky band, with almost no mobility, observed near the cathodal end, intensely stained. This protein fraction is believed to represent the "Fibrinogen" (Manwell and Backer, 1963). This fraction was also a complex protein, staining for PAS and Oil red O, occasionally for calcium but not for copper. The relative mobility (Rf) in all cases was found to be 0.014.

The slow and fast hemocyanin fractions were present in all samples of haemolymph and no polymorphism was noticed in the population. Apart from the above mentioned specific major protein fractions, several other protein fractions of varying intensity was observed in the haemolymph. These vary greatly with respect to size, sex, moult stage, reproductive cycle and nutritional state. The fast moving fractions 1, 2, 3, 4 and 5 are

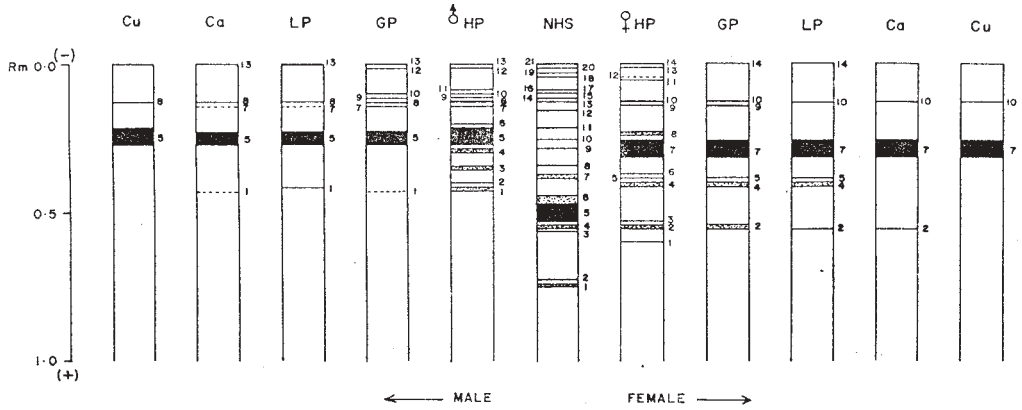


Fig. 2. Comparative electropherograms of haemolymph proteins of *Penaeus indicus* male and female, size group I (60-80 mm), intermoult stage, along with normal human serum proteins.

HP-Haemolymph proteins, NHS-Normal human serum, GP-Glycoproteins,
LP-Lipoproteins, Ca-Calcium binding proteins, Cu-Copper binding proteins.

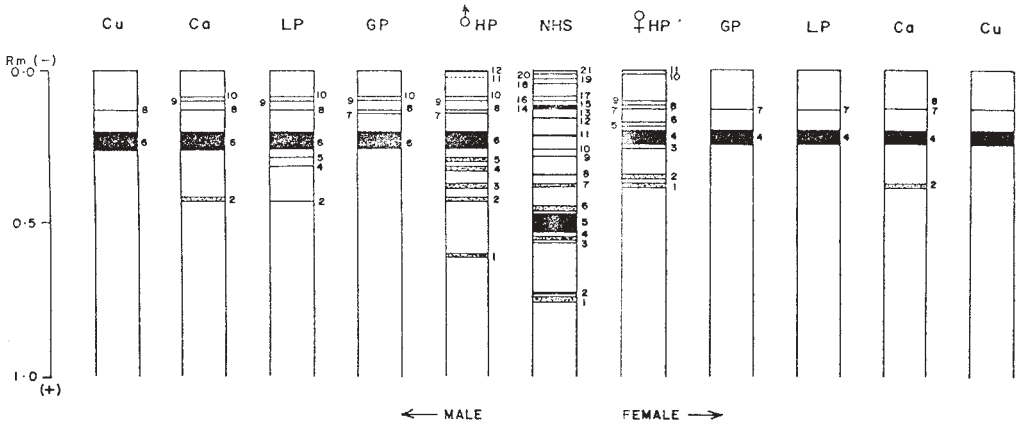


Fig. 3. Comparative electropherograms of haemolymph proteins of *Penaeus indicus* male and female, size group II (80-100 mm), intermoult stage, along with normal human serum proteins.
HP-Haemolymph proteins, NHS-Normal human serum, GP-Glycoproteins, LP-Lipoproteins, Ca-Calcium binding proteins, Cu-Copper binding proteins.

apparently simple proteins since they do not stain for PAS, Oil red O or Alizarin red S. The slow moving fractions apart from those mentioned above 6, 8, 11, 12, 13, 14 and 15 are complex proteins staining for either calcium, glycogen or lipid.

*Variations with respect to size and sex:* Haemolymph protein pattern of four size groups (60-80 mm, 80-100 mm, 100-120 mm, and 120-140 mm) are represented in figures 2, 3, 4, 5 and 6. In size group 1 (60-80 mm), the number of protein fractions varied between 12-14 . While the basic protein pattern does not vary, the quantitative difference in the protein content was represented by the reduced thickness of the copper staining fraction - the fast hemocyanin.

In the male haemolymph, 13 fractions were separated (Fig.2). While fractions 5 and 8 are copper bearing proteins staining positive with Rubeanic acid, fractions 1, 5, 7, 8 and 13 stained for calcium lipid and glycogen, indicating a complex protein nature. Fractions 7, 10 and 12 also stain for PAS and indicate that most of the fractions separated are glycoproteins. The copper bearing proteins (fractions 5 and 8) stain for Oil Red O, PAS and Alizarin Red S thus reflecting the complex glycolipo-protein nature of the hemocyanin molecule. The role of calcium in binding with hemocyanin molecule is reflected by Alizarin Red positivity.

In the female haemoloymph, 14 fractions were observed (Fig.2). Fractions 7 & 10 stained positive for copper, PAS, Oil red O and Alizarin red. Fractions 2, 4, 5, 7, 9, 10, 14 for PAS, fractions 2, 4, 5, 7, 10 and 14 for Oil Red O, fractions 2, 7, 10 for calcium. The apparent difference in the fractions separated from male and female haemolymph is attributed to intra-individual variation within the size groups. No female-specific protein fractions was observed in this group. The basic components in the haemolymph, namely the hemocyanin fractions (Slow & fast fractions), fibrinogen and heteroagglutinin remained unaltered in all cases, while variations occurred in the remaining fractions within individuals of

the same size group.

In size group II (80-100 mm), the number of protein fractions varied between 11-12. In this size group the quantitative variation in the protein content in the haemolymph was represented by the copper-bearing protein-the fast hemocyanin (Fig.3). In this size group, while fractions 8, 9 & 10 in the males stained for PAS fraction, fractions 4 and 7 stained only for PAS in females. Fractions 2, 4, 5, 6, 8, 9 and 10 stained for Oil Red O in the case of males and while in females only fractions 4 and 7 stained for lipid. Fractions 2, 6, 8, 9 and 10 stained for calcium in males and 2,4,7 and 8 in the case of females. Again qualitative difference in the fractions of males and females does not indicate sex specificity but intraindividual variation within the size group. In size group III (100-120 mm ), 11-12 fractions occurred in the haemolymph of males and females (Fig.4). The overall relative mobility of fractions appeared to be reduced compared to the smaller size groups. In this case, the increased protein content in the haemolymph was represented by the thicker fast hemocyanin fraction . The glycoproteins were more in females than males, fractions 2, 3, 5, 6, 8 & 9 stained for PAS, while fractions 2, 3, 8, 9 were glycolipo proteins. Calcium binding fractions were 3, 5, 6 and 9 in females, 4 and 8 in males. Slow and fast hemocyanin fractions were also present. There was an obvious increase in the glycoprotein in females from this 80 mm size onwards. No sex-specific proteins were present.

In size group IV (120-140 mm), 13-16 protein fractions were separated from the haemolymph. (Fig.5). The hemocyanin fractions were more thick and densely stained reflecting the increase in the protein content in the haemolymph. In the males, 13 fractions were observed. Fractions 4, 5, 6, 7, 8, 9, 10 and 13 stained for PAS, while fractions 4, 5, 6, 8, 9, 10, 12 and 13 were calcium binding fractions and fractions 5 and 8 hemocyanins.

In the female haemolymph, 16 protein fractions were observed among which most of them are glycoproteins

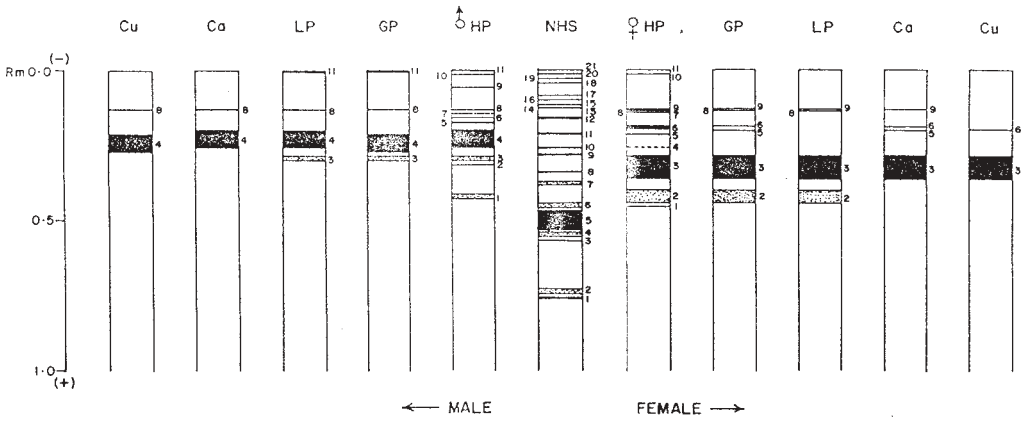


Fig. 4. Comparative electropherograms of haemolymph proteins of *Penaeus indicus* male and female, size group III (100-120 mm), intermoult stage, along with normal human serum proteins.

HP-Haemolymph proteins, NHS-Normal human serum, GP-Glycoproteins, LP-Lipoproteins, Ca-Calcium binding proteins, Cu-Copper binding proteins.

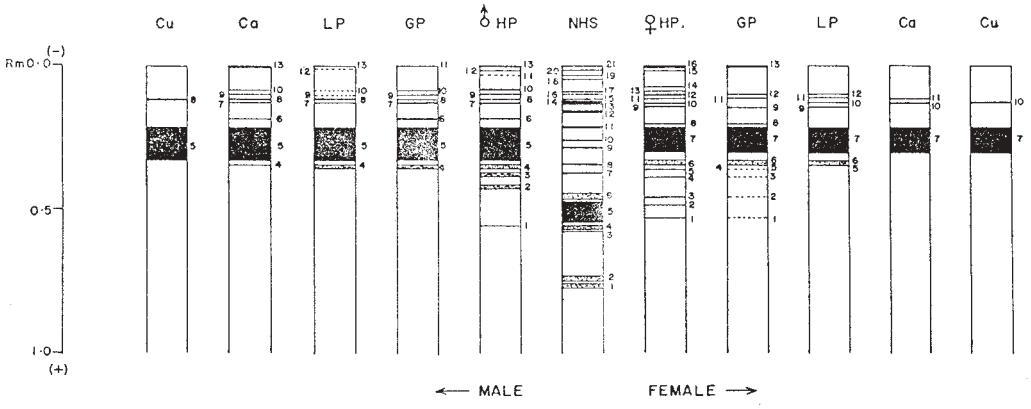


Fig. 5. Comparative electropherograms of haemolymph proteins of *Penaeus indicus* male and female of size group IV (120-140 mm), intermoult stage, along with normal human serum proteins.

HP-Haemolymph proteins, NHS-Normal human serum, GP-Glycoproteins, LP-Lipoproteins, Ca-Calcium binding proteins, Cu-Copper binding proteins.

(fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 16 ). Fractions 5, 6, 7, 9, 10, 11, and 12 were glycolipoproteins; 7, 10, and 11 are calcium binding proteins. There was an obvious increase in the glycoproteins in the haemolymph in the females. Fractions 7 and 10 were hemocyanin fractions. In this size group, the obvious difference between the male and female reflected the individual variations within the size group as well as sex. Comparison of the haemolymph protein pattern of the four size groups showed that the basic species specific pattern prevails among all the size group. Fig.6 represents the comparative electropherograms of haemolymph protein pattern of females in intermoult stage from the four size groups, compared with normal human serum protein pattern.

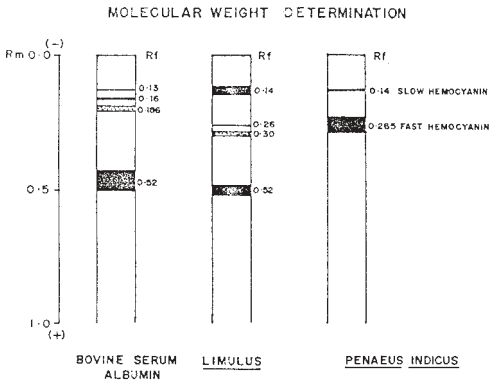


Fig. 6. Electropherograms of bovine serum albumin, *Limulus* hemocyanin and copper binding proteins (Hemocyanin) of *Penaeus indicus* for determination of molecular weight of *P. indicus* hemocyanin

### *Molecular weights of hemocyanins*

*P. indicus* haemolymph, when subjected to SDS-PAGE , more than 80% of the protein was found in two bands of slightly different mobility. The two bands stain for copper with Rubeanic acid and hence identified as hemocyanin protein fractions. The molecular weights of these two hemocyanin subunits were determined by calibration with proteins of known molecular weights namely Limulus hemocyanin and bovine serum albumin. This calibration method yielded values of 2,80,000 daltons for the slow fraction and 1,37,183.9 daltons for the fast fraction as molecular weights of the two hemocyanin units (Table 1, Fig. 6).

TABLE 1. *Determination of molecular weight of hemocyanin fractions in* P. indicus

| Relative mobility (Rf) | Approximate molecular weight in Daltons |
|---|---|
| Bovine serum albumin | |
| 0.13 | 2,64,000 |
| 0.16 | 1,98,000 |
| 0.186 | 1,32,000 |
| 0.52 | 66,000 |
| *Limulus* hemocyanin | |
| 0.14 | 2,80,000 |
| 0.26 | 2,10,000 |
| 0.30 | 1,40,000 |
| 0.52 | 70,000 |
| *Penaeus indicus* (Hemocyanin fractions) | |
| Slow Hc fraction 0.14 | 2,80,000 |
| Fast Hc fraction 0.285 | 1,37,183.9 |

## Discussion

In *P. indicus,* an unifrom specific protein banding pattern was observed, in spite of variations attributable to physiological factors such as size, moult cycle, season, nutritional state etc. Four main components - the slow and fast hemocyanin, heteroagglutinin and fibrinogen were invariably present, in both males and females, irrespective of size, along with other simple protein fractions. The species specificity is indicated by the characteristic relative mobility of the specific slow and fast hemocyanin fractions. The maximal number of protein fractions recorded is 16, while the minimum was 10. The basic pattern remained the same in all size groups studied. The quantitative variation in the haemolymph protein is indicated by the thickness and intensity of the fast hemocyanin fraction. In general, the least number of protein fractions were observed in the haemolymph of younger prawns (60-80 mm) which increased with size. This change is correlated with factors such as size, weight and nutritional state and reflects the progressive alteration of the nature of proteins in the haemolymph to suit the functional requirements of the species as size increases and probably corresponds with the change in size and properties of the various molecules. The increase in protein content with increase in size of the species is reflected by the broader and dense fast hemocyanin fraction and progressive occurrence of other simple protein fractions. Absolutely no change occurs to the slow hemocyanin, heteroagglutinin and fibrinogen in either of the sexes. Apparently, the respirartory role of the hemocyanin and role of heteroagglutinin and fibrinogen is significant in *P. indicus*, since these remain steady throughout the life cycle of the species.

Qualitative changes in haemolymph protein pattern associated with sex and age of the animal has been recorded by few authors. In *Ligia italica*, *P. laevis*, *A. vulgare* and *Armadillo officianalis*, 13, 14, 10 fractions were indentified respectively by PAGE (Sevilla and Lagarrigue, 1973 ). Only one major fraction is reported in all these species. The almost identical relative mobility (Rf value) and protein content in the four species reflected their systematic correlation as well as the functional significance of hemocyanin (Sevilla and Lagarrigue, 1973). Picaud (1971, 1976) by PAGE described 14 fractions in *L. italica* and re-

ported that the proteinogrammes were identical in both sexes. The hemocyanin fraction was not identified and probably represented by the intense staining of 9th and 10th fractions. In *L. oceanica*, 15 fractions with one hemocyanin fraction and 20 fractions in *A. vulgare* were separated. Picaud (1976) also noted characteristic fractions in the intersexual forms of both species.

Alikhan and Lysenko (1973) by means of PAGE separated the haemolymph proteins as *Porcellio laevis*, into seventeen stainable fractions, identifying three hemocyanin fractions, a lipoprotein, a glycoprotein and an esterase. The haemolymph protein pattern of *P. laevis* belonging to different growth instars clearly indicated a direct relationship between blood protein concentration and age/size/weight of the isopod. Alikhan and Akhtar (1980) compared the haemolymph protein pattern by PAGE in *P. laevis*, *A. vulgare* and decapod *Orconectes propinquus*. Typical patterns of adults revealed three bands for *P. laevis*, five in *A. vulgare* and eight in *Orconectes*. In *P. laevis*, a fast moving hemocyanin, a glycoprotein and a lipoprotein; in *A. vulgare*, a fast and slow moving liporotein were identified. Jazdewski *et al.* (1975) determined by acetyl cellulose electrophoresis, the protein composition of *Gammarus fossarum*, *G. lacustris*, *G. roesli* (amphipods), *Asellus acquaticus* and *Oniscus asellus* (Isopods) and described five major protein fractions-apohemocyanin, hemocyanin, heteroaggultinin, fibrinogen and glycoproteins. The haemolymph proteins pattern of *G. lacustris* was similar to that of *G. roesli* but not with that of *G. fossarum* which seems due to ecological affinity but not of systematic position. In *Niphargus virei* (amphipod), Gibert (1972), revealed 15-16 fractions by PAGE and reported wide intra-individual variations but the species specific pattern revealed three hemocyanin fractions exhibiting perodixase activity. Glycoproteins were many, while lipoproteins were few. Kaim-Malka *et al.*, (1983), separated 25 fractions in males and 22 in females of *Idotea balthica*. Two hemocyanin fractions were identified. In *Cirolana borealis*, 24 fractions were separated in both males and females and 4 hemocyanin fractions exhibiting peroxidase activity were identified (Kaim-Malka, 1993). Durliat and Vranckx (1976, 1982) separated the haemolymph proteins of *Astacus leptodactylus* by various preparative methods and revealed the presence of clottable protein in plasma, which is cathodic in position, analogous to the fibrinogen like factor. The fibrinogen- like factor indentified in *P. indicus* by SDS-PAGE, may be similar in properties and further immunological methods of identifications will provide the clue to the true nature of this protein factor. In the lobster *Homarus americanus* haemolymph the plasma pattern revealed six components whereas the serum pattern revealed only five viz. hemocyanin, fibrinogen and others. The hemocyanin fraction was a single homogenous component with similar mobility in serum and plasma. Fluid agar film electrophoresis has been used to resolve the haemolymph of *Carcinus maenas* into two distal fractions-the fast and slow haemocyanin exhibiting peroxidase reaction with O. dianisidine/peroxidase staining system; intermediate fraction-apohemocyanin (confirmed by treatment with KCN); proximal fraction comprising glycoprotein, lipoprotein and fibrinogen (Uglow, 1969). While the fast hemocyanin fraction did not exhibit significant monthly variations, all others varied greatly. Only the apohemocyanin showed significant differences between the sexes. In *P. indicus* also, the fast and slow

hemocyanin fractions (as confirmed by Rubeanic acid) exhibited glyco and lipoproteic nature. Unlike in *C. sapidus*, these fractions do not appear to vary with respect to seasonal factors or between sexes. Horn and Kerr (1969) used starch gel and paper curtain electrophoresis to study haemolymph protein in *C. sapidus*. They revealed the presence of two hemocyanins, fast and slow, interconvertible clotting protein in the serum and less in plasma and apohemocyanin which had different electrophoretic mobilities to hemocyanin when dialysed. In *P. indicus,* the four fractions namely the fast and slow hemocyanin, the heteroagglutinin and the fibrinogen apparently form the characteristic pattern of the species, all these present in all samples of haemolymph irrespective of size, sex and moult. Ceccaldi (1971) revealed by PAGE in *Scyllarus arctus* , 14 fractions, of which fractions 7, 9 and 10 were important quantitatively and fraction 7 was identified as hemocyanin. Ghidalia (1972) observed two cuproprotein fractions in *Macropipus puber* and heterogenecity between the cuproproteins is indicated by the absorption peaks. Such heterogenecity probably exists in *P. indicus* hemocyanin also, which can be proved only by further detailed studies.

Fielder *et al.* (1971) found 4 components in the light patch variants compared to the 3 hemocyanin components in the dark patch variant in *Uca pugilator*. Maguire and Fielder (1975 a, b) revealed species specific pattern in 12 species of portunid crabs., identified as hemocyanin. The hemocyanin fractions varied between 3 and 5, thus species specific. Fractions in the upper and middle regions reflected high individual variation. Hemocyanin was not affected by the physiological state of the crabs. In *P. indicus* while sex, and storage of haemolymph did not affect the basic pattern of protein fractions, preliminary standardisations revealed reduced mobility of the protein fraction with decrease in length of gel. The relative mobility of the two hemocyanin fractions in *P. indicus* were high and hence found confined to the upper region.

Cuzon and Ceccaldi (1972) by PAGE revealed the presence of 17 protein fractions in *Penaeus kerathurus*; only one hemocyanin fraction was identified and its role as reserve during starvation seems to be significant. As in *P. indicus* wide individual variation was recorded. In *P. monodon* also 17 protein fractions were identified in the adult; the number of fractions increased with increasing size and no marked difference in the male and female haemolymph protein was observed. The hemocyanin fractions were not identified (Pratibha, 1984). In adult *P. indicus*, 16 fractions were revealed by SDS- PAGE, also the number of fractions increase with size as in the case of *P. monodon*. In *Squilla mantis*, Ferrero *et al.* (1983) by SDS-PAGE, revealed 16 major proteins and quantitative variations of individual fractions. The hemocyanin molecule was reported to be homogenous represented by one fraction. In *P. indicus*, 2 hemocyanin fractions exist in the native state, in *P. monodon*, possibly 3 exist (Ellerton and Anderson, 1981) and in *P. kerathurus* only one fraction was revealed (Cuzon and Ceccaldi, 1972). These point to the possible polymeric existence of the hemocyanin fraction.

### *Molecular weight determination*

In *Penaeus indicus,* two hemocyanin components of slightly different mobilities, which differ in molecular weight by about 50%, has been detected by SDS-PAGE . Calibration of gels with proteins on the basis of their molecular

weight yeilded values of approximately 2,80,000 daltons and 1,37,184 daltons for molecular weights of the slow and fast hemocyanin components respectively. The native state of *P. indicus* hemocyanin occurs in the form of two components of differing mobilities in SDS-PAGE at pH 8.0 when almost all hemocyanin molecules remain in the native aggregation state (Markl, 1986). The two components were present in all individuals irrespective of sex, size (60-140 mm) or physiological condition except during the period of vitellogenesis wherein the slow hemocyanin component disappeared and reappeared after spawning. The functional significance of this phenomenon is inexplicable at the moment.

The molecular weight of the aggregate form of hemocyanin has been determined in several species; 950,000 daltons in *Cancer magister* (Ellerton *et al.*, 1970); 610,000 daltons for *Cancer pagurus* (Boone *et al.* (1983); 471,000 daltons by ultracentrifugation in *Penaeus monodon* (Ellerton and Anderson, 1981). By PAGE they determined three monomers of 82,000, 91,000 and 107,000 daltons and the main monomer 176,000 daltons in *P. monodon*. The subunits of hemocyanin in decapods have molecular weights of 70,000-80,000 daltons in *Cancer magister* (Ellerton *et al.*, 1970); 75,000 -84,000 for two other estimates for the same species (Carpenter and Van Holde,1973; Loer and Mason ,1973); 76,000 -78,000 for *Homarus americanus* (Waseman, 1975); 78,000 for *Neptunus validus*; 80,000 for *Macropipus puber*; 82,000 for *Panulirus regius* (Lambin *et al.*, 1976);60,000 in *Idotea balthica* (Kaim-Malka *et al.*, 1983); 400,000, 150,000, 76,000 and 24,000 daltons in *Cirolana borealis* (Kaim-Malka, 1993); 76,000 for *Panulirus regius* (Rochu *et al.*,1978), 75,000, in *P. interruptus* (Gaykema *et al.*, 1984), 70,000, 77,000 and 85,000 in *P. monodon* (Ellerton and Anderson, 1981). It is highly probable that the subunits of *P. indicus* hemocyanin are also of similar composition and magnitude as in other decapods. The subunits may be heterogenous as reported in *P. monodon*, a closely related species.

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