ISOLATION AND CHARACTERIZATION OF AGGLUTININ IN THE HEMOLYMPH OF *PENAEUS INDICUS* H. MILNE EDWARDS

Thesis submitted
under the Faculty of Science
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in partial fulfilment of the requirements
for the Degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

by

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April 1999

DECLARATION

I hereby declare that the thesis entitled **"Isolation and characterization of Agglutinin in the hemolymph of *Penaeus indicus* H. Milne Edwards"** is a bonafide record of research work done by me during the course of research under the guidance of Dr. G. S. Selvam, Reader, Department of Biotechnology. No part of this thesis has been included in any other thesis submitted previously for the award of any other degree.

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CERTIFICATE

This is to certify that the thesis entitled **"Isolation and characterization of Agglutinin in the hemolymph of *Penaeus indicus* H. Milne Edwards"** is a record of independant and bonafide research work carried out by Mrs. S. Jayasree under my guidance in the Department of Biotechnology, Cochin University of Science & Technology. No part of the thesis has been submitted for any other degree.

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To my family

&

my friends

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PREFACE

The role of aquaculture in the improvement of nutrition and socio-economic conditions of the fish farming communities in the rural and coastal regions need not be overemphasized. Aquaculture has been in vogue for thousands of years, especially in Asia, and is an important source of food for a widely dispersed population. Shrimp is a protein rich food with universal taste. Aquaculture contributes nearly 22% of the 72 million tonnes of global supply of food fish. China has been the traditional world leader in aquaculture and contributes nearly 50% of the world's production, while India with 10% is the second largest aquaculture producer. The crude / destructive method of fishing with open access to the seas and other water bodies has led to overexploitation, especially in the coastal waters and estuaries. To recoup the over -exploited species like shrimps, the governments and aquaculture scientists in many countries have started sea ranching with hatchery - reared young ones. Thus, without aquaculture, fish workers will not be able to earn their daily bread in the future and the demand and supply gap will widen to astronomical levels, pushing the price of fish beyond the reach of the majority of the population.

India is endowed with rich fishery resources for the development of aquaculture. It's strategic location in the Indian ocean with two monsoons a year, tropical climate, numerous inland water bodies, coastal estuaries and

lagoons are a unique gift of nature and with aquaculture it is possible to increase the aquatic food production from the present 5 million tonnes to at least 15 million tonnes. The modern shrimp aquaculture farms that have come up in recent years are widely classified as extensive, semiintensive and intensive with the stocking density ranging from 20,000 shrimp seed per hectare to 35,000 per hectare across the range. Increase in stocking density bring with it the heightened risk of disease and hence the use of drugs and chemicals to combat the problems. Aqua industry should go hand in hand with the environment. Compared to poultry and dairying the technology for culturing aquatic animals is still in its infancy, especially in prevention and treatment of diseases. Unlike higher terrestrial animals, aquatic animals such as shrimp do not have antibodies in their blood to fight pathogens. The success of invertebrate animals (shrimps) in every ecological niche must be due to the presence of an effective immune system capable of combating infection.

Growth of an organism is governed not only by its genetic potential but also by its immediate environmental conditions. Certain environmental factors such as water temperature and food supply augment growth rate and food conversion efficiency of an organism. Physico- chemical disturbances and social dominance of one species by another can act as powerful environmental stresses, interferes the growth process, results in altered transcription and translation. These changes are reflected in the hemolymph and evoke immune mechanism of the organism. Immunity in incertebrates has been proved to involve both humoral and cellular defense mechanisms. Cellular defense

mechanisms are based on activities of hemocytes includes phagocytosis, hemocytosis, coagulation and encapsulation. Humoral defense mechanism depends on cellular secretions or cell disruptions include agglutinins, lysins, precipitins and bactericidins and acts synergistically with cellular defense mechanism. Investigations into the defense mechanism of invertebrates are significant for several reasons.

- Helps in the understanding of the interaction between pathogens and invertebrate immune system would be valuable in assessing the value of microbes in the biological control of invertebrate pests.
- Detailed knowledge of invertebrate immunity may assist in preventing the outcome of disease in molluscs and crustaceans, which are commercially farmed often in crowded and stressful conditions.
- It helps to monitor environmental pollution by carefully assessing changes in invertebrate immune function.
- Isolated and purified invertebrate immunoreactive molecules may provide novel chemotherapeutic agents for use in man.
- Isolated and characterised immunoreactive molecules will help to synthesise analogues such as antibacterial, antiviral and antifungal against potential pathogens.

The main objectives of the present study are:

(i) to study the relationship between growth rate, RNA, DNA, RNA/ DNA and body size with reference to Nervous system, Eyestalk and Muscle tissue in prawn, *Penaeus indicus* H. Milne Edwards;

(ii) to study the biochemical constituents of hemolymph during developmental stages and to study the impact of environment on the biochemical constituents;

(iii) to study the hemocytes, hemolymph clotting, isolation, purification and partial characterization of agglutinin;

(iv) immunochemical characterization of purified agglutinin through developing ELISA and

(v) to determine the amino acid composition of agglutinin - Amino acid analysis.

Each of these objectives are met with in 5 Chapters. Each chapter has an introduction to the particular aspects of study which includes a review of literature, methodology adopted, the results obtained and discussion on the subject. A summary of the thesis and literature cited in the text are listed at the end.

ABBREVIATIONS

%G/d	Percentage growth rate /day
AAS	Atomic absorption spectrophotometer
Ag	Agglutinin
AMP	Adenosine mono phosphate
BSA	Bovine serum albumin
cAMP	cyclic AMP
DEAE- cellulose	Diethylaminoethyl – cellulose
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
E	Eyestalk
EDTA	Ethylene diamino tetraacetic acid
EEP	Emission flame photometer
ELISA	Enzyme- linked immunosorbent assay
G I – G VIII	Group I – Group VIII
GSH	Growth stimulating hormone
HPLC	High – performance liquid chromatography
HRP	Horseradish peroxidase
LB	Luria broth
LBA	Luria broth agar
M	Muscle tissue
MF	Methyl fernesoate

MIH	Molt inhibiting hormone
N. S	Nervous system
NSCS	Neurosecretory cells
O. D	Optical density
ODD	Ouchterlony double immunodiffusion
OPA	Orthophthalaldehyde
OPD	O – phenylene diamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RBC	Red blood corpuscles
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS – PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
SGR	Specific growth rate /day
TCA	Trichloro acetic acid
TE	Tris – Ethylene diamine tetraacetic acid
TFAA	Total free amino acid

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Chapter I

Relationship between growth rate, nucleic acid, protein and body length with reference to Nervous system, Eyestalk and Muscle tissue in *Penaeus indicus* H. Mine Edwards

1.1. INTRODUCTION

Shrimps, especially *Penaeus indicus* H. Milne Edwards is an aquatic organism inhabiting the sea, estuaries and backwaters, remain the backbone of Indian marine product export. The Vembanad Lake and connected backwaters of Kerala comprise a system of connected lagoons, bays and swamps penetrating the mainland. The paddy fields adjoining the backwaters of Kerala with its "Chemmeenkettu" constitute a lucrative prawn fishery sector with high productivity indices. Shrimp farms provide excellent employment opportunities and generate income, particularly to India's coastal villages. Prawn is a protein rich food with universal taste. Growth monitoring studies increasingly become essential to assess the growth rate and conditions hampering the normal growth process of shrimps. Certain environmental factors such as water temperature and food supply have an obvious and major influence on growth rate and food conversion efficiency of an organism (Pickering, 1993).

Growth in crustacean, with an inextensible exoskeleton becomes essentially a discontinuous process due to the succession of molts separated by intermolts (Hartnoll, 1982). Growth can be expressed as increase in time of length, volume or wet weight (Hartnoll, 1982). In order to increase in size, crustaceans must replace their confining exoskeleton with a larger one and subsequently grow to fill it. This periodic shedding of the old exoskeleton is accomplished by molting, which is the external manifestation of a

discontinuous growth process (Lachaise *et al.*, 1993). Several physiological functions of crustaceans such as reproduction and growth are known to be under the control of neuroendocrine hormones (Adiyodi and Adiyodi, 1970). Histomorphology and secretory activity of neurosecretory systems of *P. indicus* was studied by Mohamed *et al.* (1993). Eyestalk in decapods is known to contain a number of hormones which governs molting, growth, metabolism, water balance, pigment depression and sexual maturity (Fingerman and Rosenberg, 1988; Sarojini *et al.*, 1995). During growth, protein synthesized is efficiently converted into muscle protein. Prawn growth as defined by increase in prawn flesh, is mainly accomplished through synthesis of protein and growth can be measured through taking the measure of the organizer (RNA) of protein synthesis. DNA content per nucleus is constant in somatic cells of a given species (Brachet, 1955) and variations in the number of cells in an organism may be estimated by variation in DNA content of that organism. RNA content directly related to protein synthesis (Brachet, 1961; Kennel and Magasanik, 1962) and considered as an indicator of cellular activity. Protein synthesis is the integrator of all the various external stimuli such as food supply, stress and pollution etc. (Cooper, 1981).

Relative growths through length weight analysis of crustaceans have been worked out by Hartnoll (1978). The concentrations of DNA, RNA and protein of *Artemia salina* (L) were measured to investigate the usefulness of RNA - growth rate relationship in estimating growth or productivity (Dagg and Littlepage, 1972). Regnault and Luquet (1974) studied the growth of

shrimp, *Crangon vulgaris* by taking RNA as indicator. Growth rate and RNA concentrations were determined on a laboratory population of the brine shrimp, *Artemia salina* (Sutcliffe, 1965). The measurement of RNA concentration might permit a prediction of growth rate of mixed populations such as amphipod, *Orchestia platensis* (Sutcliffe, 1965). Sutcliffe (1969) studied the relationship between growth rate and ribonucleic acid concentration in growing invertebrates such as cockroach, *Blatella germanica.* Since the amount of DNA per cell is constant within a species, the ratio of RNA to DNA (RNA per unit DNA) is indicative of the amount of RNA per cell (Hotchkiss, 1955). This ratio is considered as more accurate index of metabolic activity than RNA concentration alone and the ratio is not affected by difference in cell numbers. Growth of the shrimp *Crangon vulgaris* was studied by following the evolution of nucleic acid concentration ($RNA/_{DNA}$) and protein content (Regnault and Luquet, 1974). Relationship between $RNA/_{DNA}$ ratio and growth rate in Atlantic cod, *Gadus morhua* was studied by Buckley (1979). Bulow (1970) reported that $RNA/_{DNA}$ ratios were sensitive to changes in feeding levels and could be used as an indicator of recent growth rates in adult golden shiners (*Notemigonus crysoleucas*). Buckley (1982) found that the RNA - DNA ratio was useful for the diagnosis of the starving condition in winter flounders (*Pseudopleuronectes americanus*).

Growth in terms of length weight analysis have been established of few decapods inhabiting the western Indian ocean (Ivanov and Krylov, 1980). A study of length weight relationships to estimate growth of *P. indicus* of

Singapore area was made by Hall (1962). Growth estimation of *P. indicus* of Indian waters was carried out by Brusher (1972). Subramanyan and Mitra (1974) analysed the relation between the total weight and the weight of abdomen in *P. indicus* from Indian waters. Crosnier (1965) estimated the growth of prawns collected from Madagascar waters based on carpace length. Growth pattern of *Penaeus* sp. such as *P. indicus* of Mozambique waters and *P. marginatus* (Kenya coast) were analysed by Ivanov and Krylov (1980). Morphometric studies, including length weight relationship, muscle production and proximate composition of the fresh water crayfish, *Austropotomobius pallipes* was studied (Rhodes and Holdich, 1984). Morphometric studies including length weight analysis during reproductive stages were reported in *Palaemon xiphias* by Guerao *et al.* (1994). Detailed morphometric studies in *P. indicus* from Veli Lake, Trivandrum, Kerala was studied by Jayachandran *et al.* (1996). Morphometric studies will help in establishing sexual dimorphisms. Genetic information coded into deoxyribonucleic acid (DNA) molecules are translated into the structure of proteins (Ochoa, 1963) and the application of separation and structural studies of proteins to taxonomic studies is the simplest indirect approach for obtaining phylogenetic information proteins (Crick, 1963). Electrophoretic myogen patterns are reported to be helpful in species identification (Thompson, 1960).

Electrophoretic separation of tissue proteins of fish and prawns has been generally acclaimed as a promising technique for the detection of population units which also been used to study systematics and population

genetics of penaeid prawns (Thomas, 1981). Electrophoretic studies will be helpful in estimating genetic variation in a stock. Growth of prawn is dependent upon the quantity of protein stored in muscle (Zeitoun *et al.*, 1977). George and Diwan (1990) made a preliminary attempt to study the growth rate of *P. indicus* with changes in nucleic acids and protein in relation to body size.

From the above available literature it was found that growth study of *P. indicus* with the help of RNA/ DNA is rather limited. The present study is an attempt to find out the relation between $RNA/_{DNA}$ ratio, protein, percentage growth rate and specific growth rate of prawn, *P. indicus* with respect to Nervous system, Eyestalk and Muscle tissues during ontogenesis.

1.2. MATERIALS AND METHODS

1.2. 1. Experimental animals

Indian white prawn, *Penaeus indicus* H. Milne Edwards (Plate 1.1) was collected from Vallarpadam area of Cochin backwaters, Kerala, in the southern west coast of India (Fig. 1.1). They were divided into eight homogenous size groups - (post larvae to spent stage) according to body length. The groups are G I (10 mm), G II (30 mm), G III (50 mm), G IV (70 mm), G V (90 mm), G VI (110 mm), G VII (130 mm) and G VIII (150 mm). The average size of each group varies by 20 mm from the preceding and subsequent groups. Whole animal was used for the analysis of G I and G II

Plate 1.1. The study material, *Penaeus indicus*

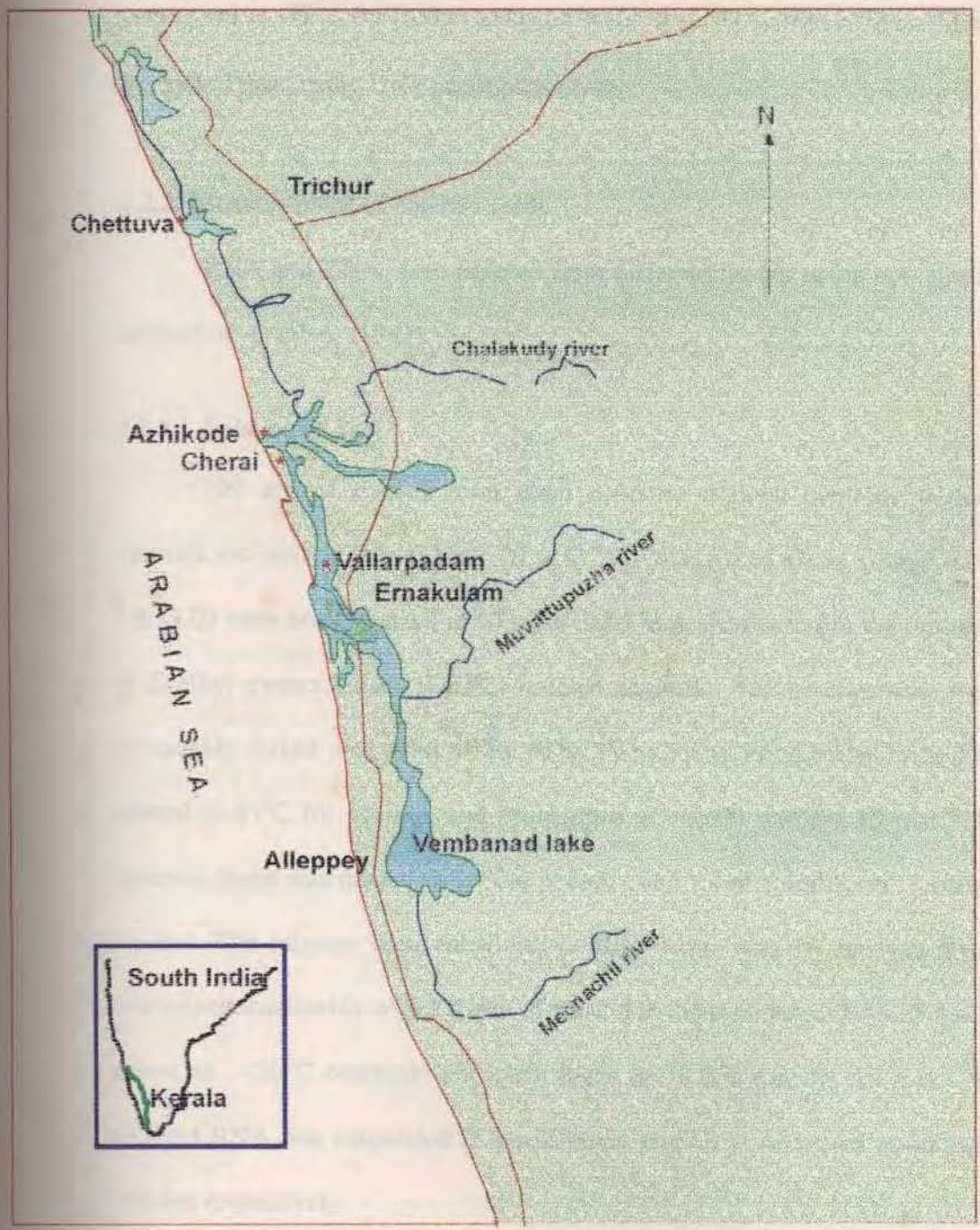


Fig. I.1. Map showing the sample collection centres for the present study along the west-coast of Kerala

after removing the hard integument and nervous system (N. S), eyestalk (E) and muscle tissue (M) (abdomen) were dissected out from the body for G III to G VIII, washed with cold crustacean saline (Cooke *et al.,* 1977), weighed and stored at -70^{0}C for further analysis. RNA and DNA were estimated from the hydrolysate using UV Spectrophotometer.

1.2.2. Measurement of Nucleic acids

RNA and DNA were isolated from different tissues using hot- phenol method of Ansubel (1987).

1.2.2.1. Isolation of RNA

100 mg of tissues from three different regions (nervous system, eyestalk and muscle tissue) for G III to G VIII (100 mg of whole animal for G I & G II) were homogenized in 10 ml of 0.05 M acetate buffer in the presence of Diethyl pyrocarbonate (DEPC) (pooled analysis). The homogenates were thoroughly mixed with 1 ml of 2% SDS. The aliquots equilibrated with 80% phenol at 65^{0}C for 15 min. and centrifuged at 10,000 rpm for 15 min. The aqueous phase was treated with 80% phenol and 1 ml of chloroform : isoamyl alcohol. The mixture was centrifuged at 10,000 rpm and the aqueous phase was mixed thoroughly with 2.5 vol. of ethanol in the presence of 0.17 M NaCl, stored at -20^{0}C overnight and centrifuged at 10,000 rpm for 10 min. The pelleted RNA was suspended in sterile water and O. D measured at 260 and 280 nm respectively.

1 O. D at 260 nm = 40 ng of nucleic acid.

1.2.2.2. Isolation of DNA

Tissues (100 mg) from three different regions (N. S, E & M) for G III to G VIII and whole tissues for G I and G II were homogenized in saline EDTA. The homogenates were separately treated with 1 ml of 0.2% SDS and 1 ml of 80% phenol, centrifuged at 10,000 rpm for 15 min. The aqueous phase was collected, treated with 2 vol. of phenol : chloroform : isoamyl alcohol and centrifuged at 10,000 rpm for 10 min. The separated aqueous phase was treated with 2 vol. of chloroform : isoamyl alcohol. The precipitate was removed after centrifugation for 15 min. at 10,000 rpm. The supernatant was precipitated using 2 vol. of ethanol in the presence of 1 ml 0.47 M NaCl. DNA pellet was suspended in TE buffer after centrifugation and O. D measured at 260 and 280 nm respectively.

1.2.3. Estimation of Protein

Protein contents of the tissues were estimated by the method of Lowry *et al.* (1951).

Alkaline Copper Reagent

A. 0.2% solution of Na_2CO_3 in 0.1N NaOH

B. 0.5% solution of $CuSO_4.5H_2O$ in 1% solution of potassium sodium tartarate

C. 50 ml of reagent A was mixed with 1 ml of reagent B.

D. Commercially available Folin - ciocaltcau reagent (SRL) diluted twice was used.

Standard

100 mg of Bovine albumin serum (BSA) was dissolved in distilled water and made upto 100 ml. This gave a standard of 1 mg/ml.

Procedure

100 mg of tissue from three different regions (N. S., E., M) for G III to G VIII and whole tissue for G I to G II were homogenized in sterile distilled water. 0.1 ml of the tissue homogenate was taken and made upto 1 ml with water. To this 5 ml of alkaline copper reagent was mixed and allowed to stand at room temperature for 10 min. Later, 0.5 ml of folin - phenol regent was added, shaken well and kept for 45 min. with intermittent shaking. The blue complex formed was measured at 660 nm against blank.

1.2.4. Percentage growth rate (% Growth/day)

Growth, as percent per day (% G/d) with respect to body length and body weight was calculated using the formula adopted by Sutcliffe (1969).

$$\% \text{ G/d} = \left[\exp\left(\frac{\text{In Wt} - \text{In Wo}}{t - to} \right) - 1 \right] X100$$

Wt = dry weight at time 't', Wo = initial dry weight.

1.2.5. Specific growth rate (SGR)

The protein growth coefficient (GP) expressed as the percentage change in protein content per day (SGR_p) was calculated from the equation (Buckley, 1979).

$$SGR = 10^{\left[\left(\frac{\log Wn+t \; - \; \log Wn}{t}\right)-1\right]X100}$$

Wn = protein content at time t 'o'

Wn + t = protein content after 't' interval.

SGR_{RNA}, SGR_{DNA}, $SGR_{RNA/DNA}$ can be calculated using the same formulae.

1.2.6. Electrophoretic studies

Discontinuous polyacrylamide gel electrophoresis (PAGE) was performed under non - dissociating conditions (Hames, 1990) to study the protein profile of nervous system, eyestalk and muscle tissues.

Reagents

1. Acrylamide stock - 44%

   Acrylamide - 44 g and

   Bisacrylamide - 1.15 g

   dissolved in 50 ml and made upto 100 ml using double distilled water.

2. Separating gel buffer (1.5M Tris, pH 8.8)

   18.16 g Tris dissolved in 50 ml of distilled water and adjusted to pH 8.8.

3. Stacking gel buffer (0.5M Tris, pH 6.8)

   6.056 g Tris dissolved in 25 ml of distilled water adjusted to pH 6.8.

4. Running gel buffer - pH 8.3

Tris - glycine buffer, pH 8.3.

30 g Tris and 14.4 g glycine dissolved in 500 ml and made upto 1,000 ml with distilled water.

5. Staining solution

250 mg of Coomassie brilliant blue dissolved in 500 ml of Methanol : Acetic acid : H_2O mixture in the ratio 145:10:45.

6. Destaining solution

Methanol : Acetic acid : Water in the ratio 145:10:45.

Procedure

Electrophoresis was carried out in 20 cm slab gel of 0.7 mm thickness. The separating gel concentration was 20% for nervous system, 12% for eyestalk, 7.5% for muscle protein and 2.5% for stacking gel. Separating gel solution was prepared and poured between the glass plates that were sealed on three sides by 2% agar. Water saturated n-butanol was used to overlay the gel surface. After 1 hr, polymerised gel was washed with water followed by running buffer. After 1 hr, placing the comb in between the plates and stacking gel, 4% solution was poured. The comb was removed after 60 min. and the slot was washed with water and filled with buffer. Running buffer was poured into the buffer tanks. Protein samples were loaded in each well and electrophoresed at 70 V until the tracking dye entered the resolving gel, then

the voltage was raised to 120 V. Electrophoresis was continued until the tracking dye reached the bottom of the gel. The gel was stained in Coomassie brilliant blue R-250 for 30 min. after fixing it in TCA and destained for 60 min.

1.2.7. Statistical analysis

One- way analysis of variance (ANOVA) was used to determine the level of significance between age groups with respect to body length and regions (N. S., E., M) and in relation to all the biochemical parameters studied (Ross, 1987).

1.3. RESULTS

Percentage growth rate (% G/d) of *P. indicus* decreased significantly as a linear function of increase in body size from G I to G VIII with the highest growth rate in G II prawns *($P < 0.05$)* (Fig. 1.2 and Table 1.1). The quantitative results of RNA, DNA and protein levels of nervous system, eyestalk and muscle tissues were summarized in Figs. 1.3a, b & c; Table 1.2. RNA, $RNA/_{DNA}$, protein, SGR_{RNA}, $SGR_{RNA/DNA}$, SGR_{P} concentration varies significantly among the eight age groups *($P<0.05$)*. Variation in DNA concentrations were not affected *($P>0.05$)*. No significant variation was observed between nervous system, eyestalk and muscle tissue *($P>0.05$)* (Table 1.4a & b). RNA concentrations of nervous system, eyestalk, muscle

tissue and protein concentration of nervous system were found to increase from G I to G II and declined from G II to G VIII, which reflects a negative correlation with increase of body size. The highest DNA content was recorded for G II prawns and declined thereafter, but remained steady for N. S, E and M. Protein concentration showed an increasing trend form G I to G VIII in muscle tissue, a positive correlation with increase of body size (Fig. 1.3c). Protein content of eyestalk and $RNA/_{DNA}$ ratio of all the regions exhibited a cyclic pattern in the order G II > G VII > G V. Interestingly, group II prawns exhibited highest level of RNA, DNA, $RNA/_{DNA}$ ratio. $RNA/_{DNA}$ ratio (Fig. 1.4) and specific growth rates (Figs. 1.5a, b & c and Table 1.3) of three regions showed cyclic fluctuation in the order G II > G VII > G V ($P<0.05$). The quantitative data of nucleic acid and protein reveals that growth being maximum in G II prawns and % G/d decline from G I to G VIII.

Electropherogram of protein bands of three regions followed the same pattern with increase of band numbers from G I to G VIII. The intensity of protein bands of nervous system proteins decreased and thinned while the intensity and density of protein bands of eyestalk and muscle tissue increased from G I to G VIII (Plate 1.2a, b & c).

Table 1.1. Percentage growth rate of *P. indicus* with reference to body length.

Body length (mm)	Weight (g)	t-to	% growth rate/day *
10	0.39	20	43.49 ± 0.63
30	3.74	20	43.21 ± 0.09
50	6.96	20	41.01 ± 1.0
70	9.23	20	39.89 ± 0.062
90	10.75	20	38.61 ± 0.85
110	11.72	20	38.57 ± 0.08
130	12.67	20	37.90 ± 0.12
150	13.27		

$$* \ \% \ G/d = \left[\exp\left(\frac{\text{In Wt} - \text{In Wo}}{t - to} \right) - 1 \right] X100$$

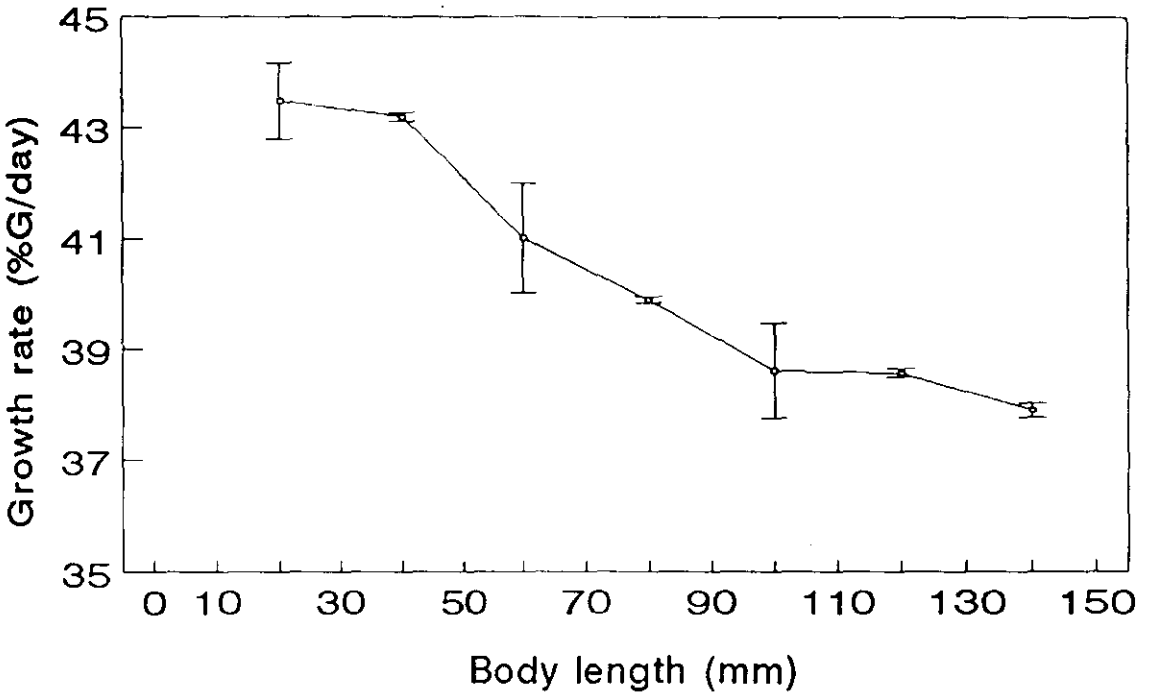
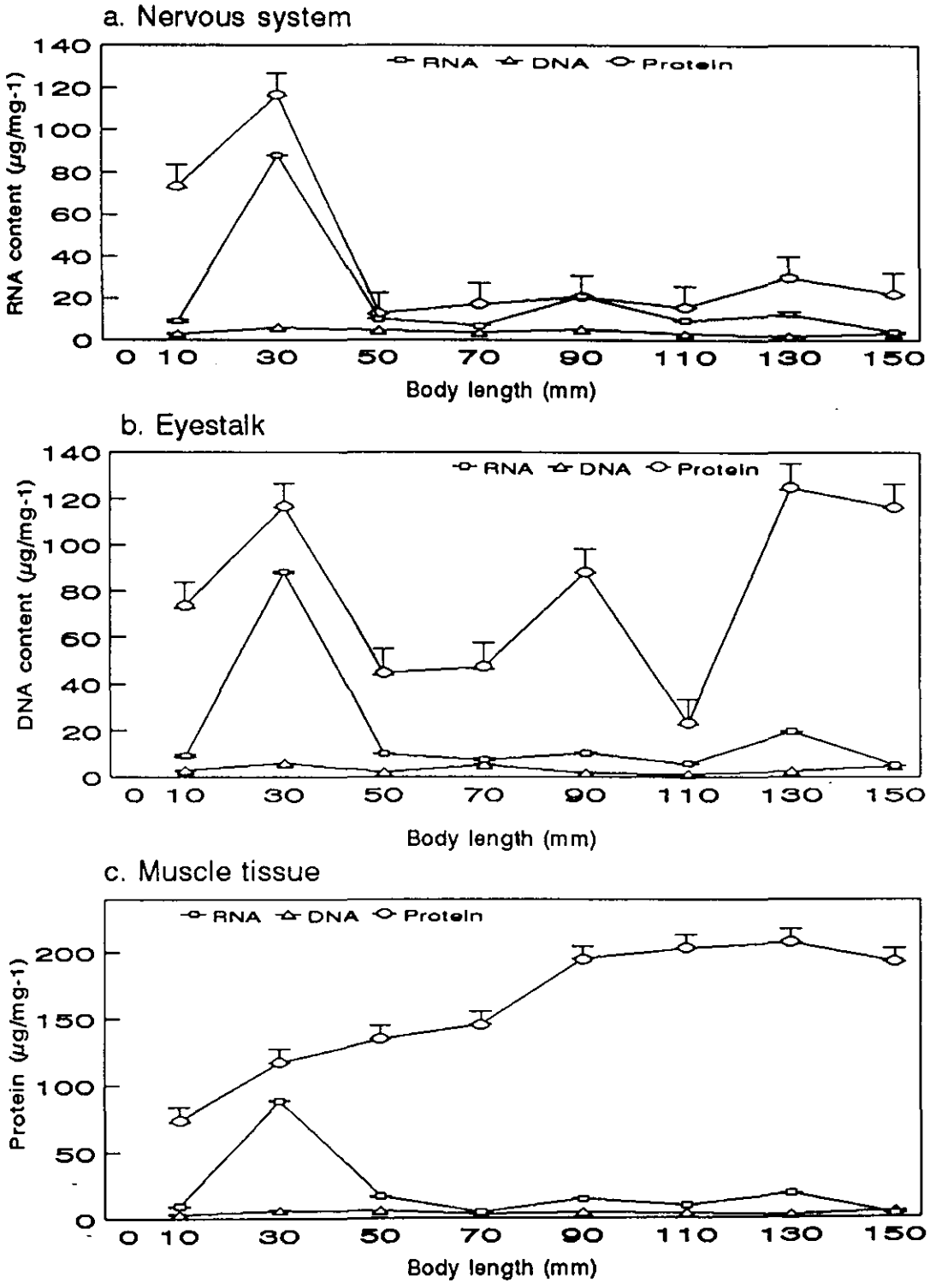


Fig. 1.2. Percentage growth rate of *P. indicus* with reference to length weight analysis. Error bars are SEM. N = 10

Table 1.2. Relationship between body length, RNA, DNA, $RNA/_{DNA}$ and Protein levels as an index of growth rate in *P. indicus vs.* Nervous system (N. S), Eyestalk (E) and Muscle tissue (M).

Parameters (µg/100 mg)	Regions	Body length (mm)							
		10 ‡	30 ‡	50	70	90	110	130	150
RNA*	N. S	9.18 ± 0.54	88.13 ± 0.35	12.18 ± 0.15	6.91 ± 0.02	20.89 ± 0.56	9.49 ± 0.11	12.77 ± 0.99	4.15 ± .09
	E	9.18 ± 0.54	88.13 ± 0.35	10.1 ± 0.12	7.51 ± 0.58	10.22 ± 0.36	5.75 ± 0.053	19.63 ± 0.07	5.27 ± 0.083
	M	9.18 ± 0.54	88.13 ± 0.35	16.64 ± 0.37	4.33 ± 0.15	14.17 ± 0.03	9.36 ± 0.03	18.01 ± 0.14	2.86 ± 0.13
DNA [na]	N. S	2.98 ± 0.166	6.06 ± 0.2	4.73 ± 0.17	3.73 ± 0.08	5.15 ± 0.12	3.27 ± 0.12	2.17 ± 0.19	3.36 ± 0.11
	E	2.98 ± 0.2	6.06 ± 0.16	2.25 ± 0.2	5.32 ± 0.24	1.85 ± 0.12	1.21 ± 0.017	2.54 ± 0.073	4.72 ± 0.13
	M	2.98 ± 0.166	6.06 ± 0.2	5.84 ± 0.16	2.82 ± 0.2	4.22 ± 0.08	3.77 ± 0.01	1.83 ± 0.23	4.78 ± 0.01
$RNA/_{DNA}$*	N. S	3.07 ± 0.054	14.54 ± 0.33	2.14 ± 0.1	1.64 ± 0.46	4.04 ± 0.49	2.9 ± 0.13	5.9 ± 0.69	1.21 ± 0.3
	E	3.07 ± 0.07	14.54 ± 0.054	4.42 ± 0.33	1.406 ± 0.27	5.58 ± 0.29	4.67 ± 0.37	7.73 ± 0.16	1.114 ± 0.24
	M	3.07 ± 0.4 7	14.54 ± 0.33	2.84 ± 0.05	1.51± 0.021	3.35 ± 0.07	2.48 ± 0.07	9.85 ± 0.04	0.598 ± 0.08
Protein*	N. S	73.51 ± 0.9	116.8 ± 0.41	12.87 ± 0.126	17.35 ± 0.04	20.87 ± 0.12	15.81 ± 0.06	30.03 ± 0.85	22.11± 0.15
	E	73.51 ± 0.9	116.8 ± 0.41	44.82 ± 0.91	47.32 ± 0.74	88.15 ± 0.12	23.38 ± 0.7	125.06 ± 0.85	116.42 ± 0.51
	M	73.51 ± 0.9	116.8 ± 0.41	134.48 ± 0.065	145.18 ± 0.01	194.58 ± 0.05	203.18 ± 0.21	207.3 ± 0.01	192.62 ± 0.074

* $P < 0.05$; [na] - not significant. (N=10); ‡ triplicate pooled analysis of entire tissue for three regions.



Figs. 1.3a, b & c. Changes in Nucleic acids and Protein in different body lengths of *P. indicus* (a. Nervous system, b. Eyestalk & c. Muscle tissue). Error bars are SEM. N = 10

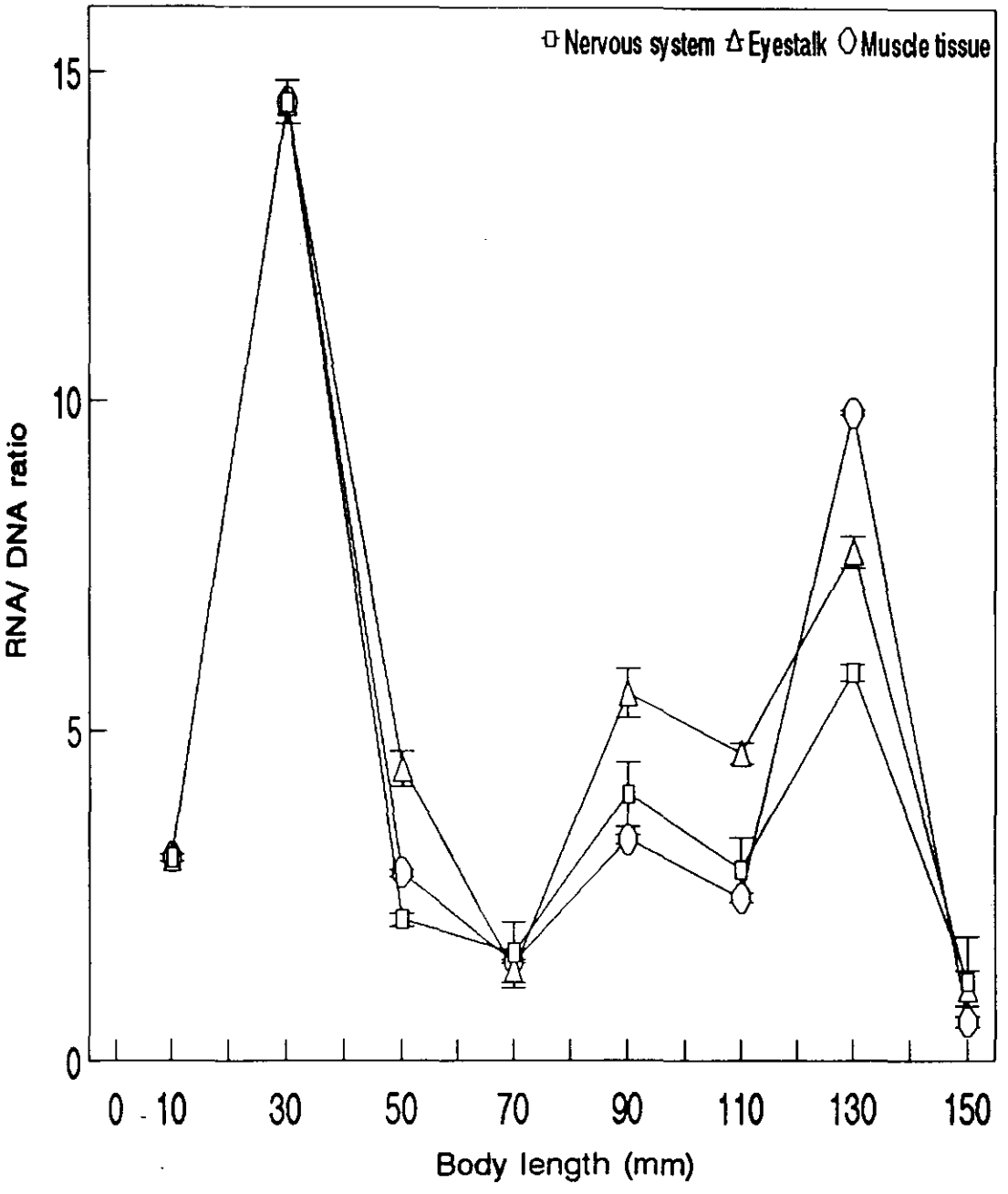
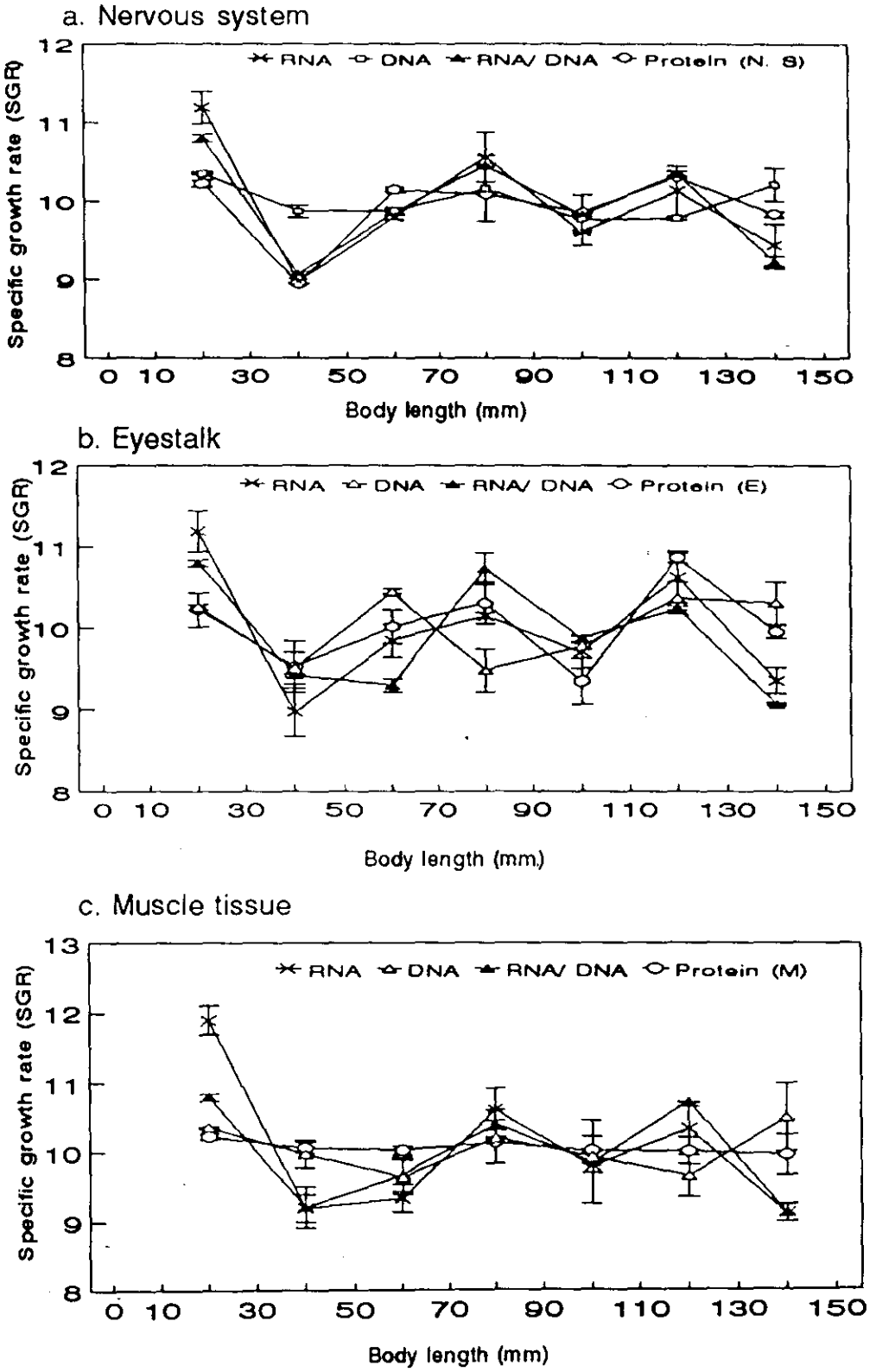


Fig. 1.4. Variations in $RNA/_{DNA}$ ratio in different body lengths of *P. indicus* (Nervous system, Eyestalk & Muscle tissue). Error bars are SEM. N = 10

Table 1.3. Relationship between body length and specific growth rates (SGR) *vs.*Nervous system, Eyestalk and Muscle tissue of *P. indicus*.

Regions	Parameter ‡	Body length (mm)						
		10-30	30-50	50-70	70-90	90-110	110-130	130-150
Nervous system	SGR_{RNA}*	11.19 ± 0.2	8.97 ± 0.03	9.8 ± 0.03	10.56 ± 0.32	9.61 ± 0.02	10.14 ±0.32	9.45 ± 0.26
	SGR_{DNA} [na]	10.36 ± 0.02	9.87 ± 0.08	9.88 ± 0.04	10.16 ± 0.42	9.77 ± 0.32	9.79 ± 0.02	10.22 ± 0.21
	$SGR_{RNA/DNA}$*	10.8 ± 0.05	9.08 ± 0.02	9.86 ± 0.04	10.09 ± 0.02	9.86 ± 0.03	10.32 ± .03	9.84 ± 0.08
	SGR_{P}*	10.23 ± 0.04	8.95 ± 0.03	10.15 ± .034	10.46 ± 0.02	9.83 ± 0.02	10.36 ± .01	9.23 ± 0.04
Eyestalk	SGR_{RNA}*	11.9 ± 0.25	8.97 ± 0.3	9.85 ± 0.2	10.09 ± 0.04	9.86 ± 0.2	10.32 ± 0.32	9.84 ± 0.16
	SGR_{DNA} [na]	10.26 ± 0.03	9.51± 0.2	10.45 ± 0.03	9.48 ± 0.27	9.78 ± 0.04	10.37 ± 0.2	10.31 ± 0.26
	$SGR_{RNA/DNA}$*	10.8 ± 0.04	9.42 ± 0.02	9.30 ± 0.08	10.73 ± 0.2	9.88 ± 0.04	10.25 ± 0.02	9.07 ± 0.08
	SGR_{P}*	10.23 ± 0.21	9.53 ± 0.31	10.02 ± 0.2	10.31 ± 0.26	9.35 ± 0.28	10.87 ± .06	9.96 ± 0.02
Muscle tissue	SGR_{RNA}*	11.9 ± 0.2	9.2 ± 0.2	9.34 ± 0.2	10.61 ± 0.3	9.79 ± 0.1	10.33 ± 0.31	9.12 ± 0.12
	SGR_{DNA} [na]	10.36 ± 0.02	9.98 ± 0.2	9.64 ± 0.25	10.2 ± 0.02	9.93 ± 0.08	9.65 ± 0.3	10.49 ± 0 .49
	$SGR_{RNA/DNA}$*	10.8 ± 0.05	9.21 ± 0.3	9.68 ± 0.24	10.4 ± 0.2	9.85 ± 0.6	10.71 ± 0.01	9.13 ± 0 13
	SGR_{P}*	10.23 ± 0.04	10.07 ± .08	10.03 ± 0.06	10.14 ± 0.3	10.02 ± 0.2	10.01 ± 0.19	9.96 ± 0.29

* $P < 0.05$; [na] - not significant; [‡] $SGR = 10^{\left[\left(\frac{\log Wn+t \;-\; \log Wn}{t}\right)-1\right]}X100$



Figs. 1.5a, b & c. Specific graowth rate (SGR) of *P. indicus* with reference to RNA, DNA, $RNA/_{DNA}$ and Protein (**a.** Nervous system, **b.** Eyestalk & **c.** Muscle tissue). Error bars are SEM. N = 10

Table 1.4a & b. ANOVA table showing level of significance between age groups and regions with reference to DNA, RNA, $RNA/_{DNA}$, proteins and specific growth rates of *P. indicus*.

a.

Source of variation	DNA		RNA		$RNA/_{DNA}$		Protein	
	F	P	F	P	F	P	F	P
Between age groups	2.601>0.05^{ns}		258.24< 0.05*		62.92< 0.05*		30.84<0.05*	
Between regions (N. S, E, M)	0.393>0.05^{ns}		0.896 > 0.05^{ns}		1.71 > 0.05^{ns}		17.49<0.05*	

b.

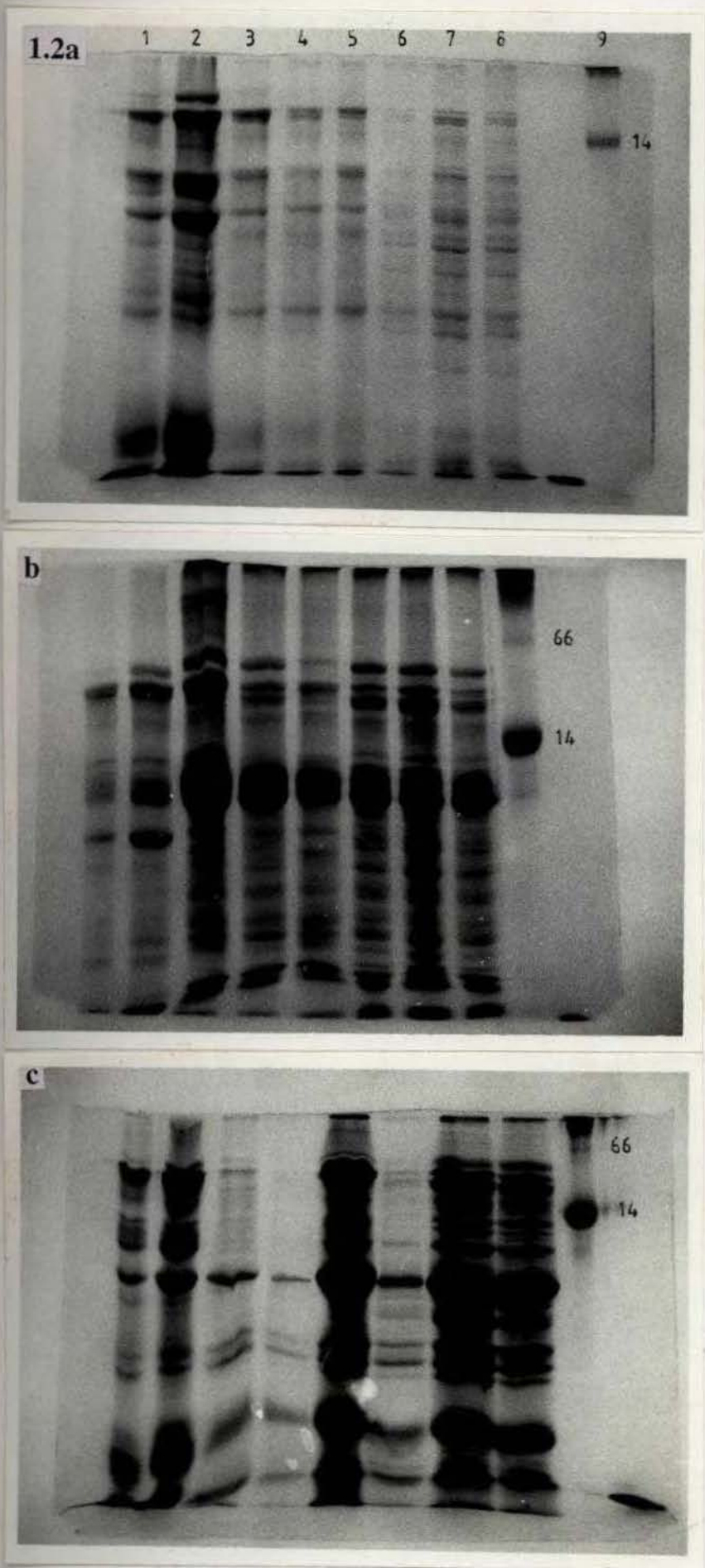
Source of variation	SGR_{RNA}		SGR_{DNA}		$SGR_{RNA/DNA}$		SGR_{P}	
	F	P	F	P	F	P	F	P
Between age groups	9.19 < 0.05*		1.45 > 0.05^{ns}		14.2 < 0.05*		37.12< 0.05*	
Between regions (N. S, E, M)	0.658>0.05^{ns}		0.514 >0.05^{ns}		0.514>0.05^{ns}		1.45 > 0.05^{ns}	

* - significant at 5 % level; ^{ns} - not significant

Plate1.2a. Electropherogram (20%) showing Eyestalk protein bands of *P.inidcus* among 8 size groups. Lane 1-8 corresponds to 8 groups; Lane 9 - MW marker 14 kDa.

b. Electropherogram (12%) showing Eyestalk protein bands of *P. indicus* among 8 size groups. Lane 1-8 corresponds to 8 groups; Lane 9 - MW marker 66 kDa & 14 kDa.

c. Electropherogram (7.5%) showing Muscle protein bands of *P. indicus* among 8 size groups. Lane 1-8 corresponds to 8 groups; Lane 9 - MW marker 66 kDa & 14 kDa.


1.2a
1 2 3 4 5 6 7 6 9
14
b
66
14
c
66
14

1.4. DISCUSSION

In crustaceans, growth is defined as increase in wet weight of body, which generally occurs in the periods between molts when the absorbed water is replaced by proteins. Higher level of RNA, DNA and $RNA/_{DNA}$ ratios with increase in protein levels reveals highest growth rate in group II (30 mm) prawns. Dagg and Littlepage (1972) reported that growth is faster in the early phase of life cycle of animals. Thus the juveniles with high nucleic acid titres and $RNA/_{DNA}$ ratio leads to faster growth. Buckley (1979) observed a direct linear relation between $RNA/_{DNA}$ ratio and growth rate in cod larvae. Direct linear relation between percentage change in protein content per day (SGR_P) and $RNA/_{DNA}$ ratio was found to be significant ($P < 0.05$) in the case of nervous system, eyestalk and muscle tissue. The RNA content of a wide variety of organisms has been related to growth rate (Sutcliffe, 1969). Prawn with a high $RNA/_{DNA}$ ratio is expected to grow (accumulate protein) at a faster rate than prawn with a low $RNA/_{DNA}$ ratio. RNA serves as both a template and organizer for protein synthesis, the loss of RNA results in a decrease in the potential maximum rate of protein synthesis and may consequently affect growth rate. Where as rate of protein synthesis is regulated by the rate of messenger RNA (m- RNA) synthesis and breakdown. $RNA/_{DNA}$ ratio can be considered as a measure of the amount of protein synthetic machinery per cell, since DNA content of an organism is an indicator of cell numbers. Hence the direct positive relationship between $RNA/_{DNA}$ ratio and growth rate observed for adult golden shiners (Bulow, 1970) support our studies.

The declining trend of protein concentration observed in nervous system may be due to the transport of biogenic amines into various target organs through hemolymph during development (Fingerman *et al.*, 1994). The neurosecretory material contained in the neurosecretory cells (NSCS) of *P. indicus* was predominantly composed of a protein rich in Cystine (- S - S) and Cystine (- SH group). In *P. indicus*, two morphologically diverse secretory cycles were observed, these have been correlated with the process of molting (Vijayan, 1988) and reproduction (Mohamed and Diwan, 1991). Increased production of eyestalk proteins during development may be due to the augmented secretion of eyestalk hormones as a process of molting and sexual maturity (Mattson and Spaziani, 1985). Neurohormones also regulate gonadal maturation (Sarojini *et al.*, 1995) and results in accumulation of muscle protein. Increased muscle protein concentration observed in G I to G VIII of our study coincide with report of Sarojini *et al.* (1995). During developmental process growth results due to anabolic drive in which dietary protein is efficiently converted to muscle protein and consequent tissue growth (Millward, 1989). In mature individuals weight increases faster than the body length due to muscle growth attributed through accumulation of protein as flesh. In our results the declining trend of growth rate and increased protein concentration from G I to G VIII were observed. Conversely in juveniles (G I to G V) weight increased far less than the body length. Lack of substantial gonad development or cuticular thickening and muscle growth in juveniles may be the causal factors (Rhodes and Holdich, 1984).

The RNA synthesis occurs at different times in different parts of the body and varies with molting process as observed in crustacean, *Crangon vulgaris* (Regnault and Luquest 1974). Cyclic fluctuations of $RNA/_{DNA}$ ratio and specific growth rate in the order G II > G VII > G V exemplifies the differential transcription and translation process taking place during ontogenesis due to molting process. Hence $RNA/_{DNA}$ ratio can be used as a predictive tool to study gene action. Growth of all crustaceans can be represented as a stepwise growth pattern. Thus at each molt, a sudden increase take place as a result of absorption of water and remain unchanged until the next molt (Kurata, 1962). The investment of energy in reproduction inhibits growth in larger individuals and extends the intermolt period (Hartnoll, 1982) explains the decreasing growth rate during sexual maturity. Growth proceeds in a series of phases, each composed of from one to several instars. Within each phase there is simple allometric growth. But between both the levels of allometry, the size of the variables often changes at the transition phase. These transition change may be minimal determined through changes in nucleic acid or prominent with molting due to differential gene action (Hartnoll, 1978).

The differential gene action observed as G II > G VII > G V, the manifestation of specific growth rate and $RNA/_{DNA}$ ratio was comparable to the above findings. The $RNA/_{DNA}$ ratio thus expresses cellular metabolic activity and translates cell potentiality to synthesize proteins. High $RNA/_{DNA}$ ratio could indicate either healthy rapidly growing fish, while low ratios for

stress conditions (Buckley, 1984). The high $RNA/_{DNA}$ profile and protein concentrations in G VII results from the high turnover rate of protein during sexual maturity, as a function of gene action in gonadal cells during reproductive phase (Guraya, 1985). Percentage growth rate shows that growth in crustaceans is a continuous process as in all other animals (Highman and Hill, 1979). In decapoda, gonadal and somatic growth occurs simultaneously (Adiyodi and Subramanian, 1983; Charniaux -Cotton, 1987). Higher $RNA/_{DNA}$ ratio in nervous system of G VII is expressed from the increased secretion of GSH in brain and thoracic ganglia (Fingerman *et al.*, 1994).

The banding pattern of protein in electropherogram of nervous system, eyestalk and muscle tissue reveals the increasing trend of protein synthesis during ontogenesis. The decrease of intensity of nervous system protein bands from G I to G VIII explains the process of transport of various synthesized metabolic products from nervous system to the hemolymph. The increasing trend of intensity and density of protein bands from G I to G VIII in the electropherogram of eyestalk and muscle tissue reveals the growth through accumulation of protein. Electropherogram of muscle myogen pattern of our report was comparable to the preliminary study of Thomas (1981) in *P. indicus*. The high protein content and low levels of lipids in comparison with other tissues (George and Asokan, 1983) characterize the muscle of the prawn. Protein synthesis in living organisms is controlled by fundamental genetic systems and their expression lead to structural differentiation. The products of which is a measure of genetic differences that helps to study systematics (Lim

and Lee, 1970). According to this hypothesis the electropherogram predicts the differential gene action with changes in banding pattern due to stress, growth and environmental conditions etc.

Present study conclude statistically significant ($P < 0.05$) correlation with $RNA/_{DNA}$ ratio and growth rate with respect to body size reveals that group II prawns exhibited fastest growth, thus the relationship is valid. Growth rate decreases with increase of body size in prawns, a general process of ageing and maturity (Miquel, 1971). Differential gene action during ontogenesis is reflected as significant fluctuations ($P < 0.05$) in $RNA/_{DNA}$ ratio and specific growth rates (SGR_{P}, SGR_{RNA}, SGR_{DNA}, $SGR_{RNA/DNA}$) in the order G II > G VII > G V. Discontinuous growth due to molting process explains differential gene action with highest growth rate in group II prawns. Increasing trend of band numbers from G I to G VIII in the electropherogram of three regions explains the growth resulted with the accumulation of protein in flesh, a manifestation of growth. The electropherogram of N. S region reflects the transport of protein to the target organs eyestalk and muscle tissue which exemplifies the synthesis and accumulation of different protein during ontogenesis. These results indicate that $RNA/_{DNA}$ ratio, protein and growth relationship might be useful in estimating the growth of mixed population of animals.

C h a p t e r I I

Biochemical analysis of hemolymph

2.1. INTRODUCTION

The successful establishment of a species in a given habitat depends on the ability of each of its developing stages to adapt to the environment. Members of the genus *Penaeus* are distributed over a wide range of salinities and cultured under a variety of conditions in many tropical and subtropical areas of the world (Cawthorne *et al.*, 1983). Many fundamental features of the class Crustacea are reflected in the nature of the internal medium, the hemolymph. Thus, the exoskeleton and consequent periodic molting, aquatic habitat and ammonotelic nitrogen metabolism, all have important correlation in crustacean blood chemistry (Florkin, 1960). The hemolymph composition of organisms reflects the metabolism of the tissues and organs bathed by this fluid, which is a carrier of substrates, end products of catabolism and hormones. The hemolymph was chosen as a biological agent susceptible and modified by the pollutants. Changes in the protein content of the hemolymph may reflect specialization and adaptation in the organisms based on subtle metabolic alternations, and may possibly be used as taxonomic tool (Marynard, 1960). Euryhaline shrimps adapt physiologically to the alteration in the surrounding medium. These changes are most obviously manifested in the composition of the hemolymph (Pequeux, 1995). Solute concentration in the hemolymph, however is a function of solute concentration in the medium as well as molt stages (Mantel and Farmer, 1983). Exposure to polluted waters

can also affect the hemolymph in various ways. Thus studies on hemolymph composition of shrimp will provide indication about the physiological modifications associated with molting process, developmental stages, defense mechanism and pollution etc.

The level of biochemical constituents in the hemolymph like total protein, agglutinin, total free amino acids (TFAA), proline and ions such as Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} etc. serve as useful indicators of physiological and pathological conditions of the particular species to the surrounding environment. Among the crustacean, hemolymph characterization has been done in several decapods and few isopods. *Brachyura* and *Macrura* have received much attention compared to the *Nantantians*. Osmoregulatory studies of penaedian shrimp has been made in *Metapenaeus dobsoni* (Panikkar, 1951), *M. monoceros* (Panikkar and Viswanathan, 1948) and in *Penaeus carinatus* (Panikkar, 1951). The osmoregulatory capabilities of penaedian shrimp can be correlated with their salinity distributions. Thus stenohaline penaedians are osmotic conformers and euryhaline penaedians osmotically regulate at low and high salinities (Mc Farland and Lee, 1963). The occurrence of osmoregulation is based on efficient ionic regulation (mainly Na^{+} and Cl^{-}) and increased levels of Na^{+} - K^{+} ATPase activity (Guy Charmantier, 1998).

The protein, which is synthesized in various tissues during metabolism, is transported to the hemolymph (Fingerman *et al.*, 1994) to accomplish growth. Total protein in the sera has been recorded in six decapods -

Callinectus sapidus, Cancer magister, C. irroratus, Libinia emergina and in *Homarus americanus*. Bursey and Lane (1971) reported changes in ionic and protein concentration during molt cycle of *Penaeus duorarum*. Lynch and Webb (1973) studied the variation in total protein of the blue crab *Callinectus sapidus*. Changes in blood serum protein levels during molting cycle of the *Homarus gamarus* have been done by Hepper (1978). Pugach and Crawford (1978) studied seasonal changes in hemolymph amino acids, proteins and inorganic ions of *Orthoporus ornatus*. Gilles (1970) studied about the effect of osmotic stresses on the protein concentration of *Eriochier sinensis* blood. Hemolymph is a repertoire of different types of proteins of which hemocyanin protein of *P. indicus* was isolated and characterized by Laxmilatha (1991). The physiological and nutritional status of the crustaceans influence the composition of the hemolymph (Dall, 1974).

The natural agglutinins in the hemolymph of spiny lobster, *Panulirus interruptus* have been isolated and characterized by Tyler and Scheer (1945) and 17 species of Japanese coastal crustaceans by Ueda *et al.* (1991). Pauley in 1974 isolated the natural agglutinin in the hemolymph of *Callinectus sapidus*. Tyler and Metz (1945) have reported species specific haemagglutinins in spiny lobster, *Panulirus interruptus* serum. This was the first report of agglutinin activity against bacteria in lobster serum and established its importance as an integral part of the lobster's defense mechanism.

The free amino acid content constitutes one of the biochemical parameters characterizing body fluids. Numerous studies have dealt with the role of free amino acids in different vertebrates and invertebrate species. The absorption of nutrients such as free amino acids, simple sugars and protein from the digestive tract influence the concentration of these nutrients in blood, which in turn governs nutrient uptake of tissues and modulates the growth of cells. Clark (1968) made a survey of the effect of osmotic dilution on free amino acids of various polychaetes. The role of amino acids during osmotic adjustments on aquatic invertebrates have been studied by Chaplin *et al.* (1967). The effect of low salinity on amino acid metabolism in the tissues of the common mud crab, *Panopeus herbstii* have been studied (Boone and Claybrooth, 1977). Composition of free amino acids in different organs of the crayfish have been studied by Van Marrenwijk and Ravestein (1974). Cowey and Forster (1971) attempted to study the essential amino acid requirements of the prawn, *Palaemon serratus*. A comparative study of the free amino acids in the hemolymph of a millipede *Spirosteptus* was made by Sundara Rajulu (1970). Rogala *et al.* (1978) done a comparative study of free amino acids of 3 crayfish species, *Astacus astacus, A. leptedactylus* and *Orconettes limosus.* Pugach and Crawford (1978) studied about the seasonal changes in hemolymph amino acids, proteins and inorganic ions of a desert millipede, *Orthoporus ornatus.* Bounias *et al.* (1989) studied the functional relationship between free amino acids in the hemolymph of *Aedes aegyptii.* Faug *et al.* (1992) estimated the concentration of proline in the hemolymph of *Penaeus*

monodon. Proline content among free amino acid pool in *Palaemon serratus* was studied by Cowey and Forster (1971).

Marine animals that regulate the osmolality of body fluids encounter the dual problems of internal dilution at low salinities and concentration of the body fluids at high salinities. The capability to regulate solute concentration can be determined by comparing the concentration of the hemolymph to that of the ambient seawater over a range of salinities. Within a certain molt stage or period after eedysis, the organism can maintain permeability characteristics in body surface available for water or ion exchange with the medium, specific to this particular stage or time period. As a consequence, the organism response to media of different osmotic and ionic concentration is unique to the molt stages. At the same time, different molt stages respond differently upon exposure to media of similar osmotic and ionic concentrations. Thus despite considerable data on osmoregulatory ability of representative species, very few work has been done in the field of ionic concentrations in penaeids.

Carbonates of calcium and magnesium comprise a major portion of the exoskeleton of crustaceans and large amount of these materials are reabsorbed during the premolt period (Greenaway, 1985). These salts pass through the epidermis into the hemolymph and transported to a storage or excretion site (Greenaway and Farelly, 1991). Related changes in the hemolymph electrolytes of intermolt crayfish, *Procambarus clarkii* have been reported by Wheatly *et al.* (1996). Comparison of the hemolymph content of Ca, Mg, Na

and K levels in three species of crayfish have been studied by Gondko *et al.* (1981). Castille and Lawrence (1981) studied the effect of salinity on the osmotic, sodium and chloride concentrations in the hemolymph of *Penaeus aztacus, P. duorarum, P. setiferus* and *P. vannamei. Panulirus longiceps* hemolymph concentration was studied by Dall (1974). The effect of salinity on calcium concentration in the hemolymph of *Penaeus monodon* was reported by Ferraris *et al.* (1986). Calcium and magnesium contents were measured in the terrestrial crabs, *Cardisoma hertipes, Gecarcoidea natalis, Geograpsus grayi* and *Ocypode cordimana* in relation to the intermolt cycle by Greenaway (1993). Sodium balance and adaptation to fresh water in the amphibious crab, *Cardisoma hirtipes* was studied by Greenaway (1989). Sodium and potassium concentration and their transport across membranes have been reported in crustaceans by Pequeux (1995). Hemolymph levels of manganese, chloride, copper and iron levels in the hemolymph of *Penaeus indicus* have been reported by Laxmilatha (1991).

From the available literature it is clear that hemolymph studies in *Penaeus indicus* is rather limited. The present study involves the evaluation of certain organic and inorganic constituents in the hemolymph of *P. indicus* with respect to developmental stages (body length) and also a comparative study between three localities along the west - coast of Kerala. Biochemical constituents such as total protein, agglutinin, total free amino acid, proline and cations Na, K, Ca, Mg were studied.

One of the localities, Chettuva selected for comparison was a temporary mud bank formed during July 1997. Mud bank is a calm, undisturbed semicircular area of sea water boarded by disturbing waves, formed during the wake of south- west monsoon along the Kerala coast. This is a unique phenomenon elsewhere noticed in the world. Mud bank is rich in organic load, clay, macrofauna and microfauna that form the food of crustaceans, especially prawns. Mud bank area is rich in prawn fishery (Kurian, 1966; Damodaran, 1973; Kurian and Sebastian, 1986; Nair, 1983).

2.2. MATERIALS AND METHODS

2.2.1. Experimental animals

P. indicus were collected from Vallarpadam, Chettuva (mud bank), Azhikode and Cherai during the south - west monsoon period (July - August 1997) (Fig. 1.1). *P. indicus* collected from Vallarpadam area were divided into 8 homogenous size groups, based on the body length G I to G VIII (see section 1.2.1) and were used for the study of biochemical constituents in the hemolymph during developmental process. *P. indicus* of body length 130 mm collected from Chettuva (mud bank), Azhikode and Cherai during the south - west monsoon period of July - August 1997 was used to study the influence of environment on biochemical constituents in the hemolymph.

Hemolymph was withdrawn from the pericardial sinus using No. 24 hypodermic needle after swabbing the body surface with 70% ethanol and transferred to a sterile vial with anticoagulant 3% ($^{W}/_{V}$) sodium citrate (9:1). The hemolymph was allowed to clot at 4°C for 12 hrs. The supernatant was collected after centrifugation at 5,000 rpm for 15 min. and used for biochemical analysis except cations. Citrate free hemolymph was used for cation (Na, K, Ca & Mg) studies.

The biochemical constituents such as total protein, agglutinin, total free amino acid, proline, cations - Na, K, Ca & Mg in the hemolymph were estimated with standard procedures. Hemolymph samples from specimens of intermolt stages and same sex were pooled together for all estimations of the smaller size groups such as G I, G II, G III & G IV.

2.2.2. Estimation of total protein

The total serum protein was estimated by the method of Lowry *et al.* (1951) as in section 1.2.3.

2.2.3. Isolation and estimation of agglutinin

Isolation of agglutinin was done according to the extraction procedures of Miller *et al.* (1972). The *P. indicus* serum was separated into several fractions using Sephadex G - 200 column chromatography. The column (1.6 X 15 cm i.d.) was prepared by applying the slurry of Sephadex G - 200 prepared in distilled water. The column was equilibrated for 24 hrs with 0.02M

phosphate buffered saline (PBS), pH 8.0. 3 ml of *P. indicus* serum was applied to the column and eluted using 0.02M PBS buffer at a constant flow rate of 1 ml/min. Each fraction was monitored for the presence of protein at 280 nm and used for agglutination assay. The fractions of +ve response for agglutination assay were pooled and precipitated out at 45% saturation of ammonium sulphate at 4°C for 1-2 hrs. The precipitate was separated after centrifugation at 5,000 rpm for 20 min. at 4°C and dissolved in 0.85% NaCl. This agglutinin solution was dialysed in distilled water and used for biochemical studies. The concentration of agglutinin solution was determined following the method of Lowry *et al.* (1951) as in section 1.2.3.

2.2.4. Estimation of total free amino acid (TFAA)

Total free amino acid concentration was calculated by Ninhydrin method of Yemm and Cocking (1955) and Sadasivam and Manikam (1992).

Reagents

1. 0.2M citrate buffer, pH 5.0

   A. 0.2M solution of citric acid (42.028 g/l)

   B. 0.2M solution of sodium citrate (58.829 g/l)

   20.5 ml of solution A was mixed with 29.5 ml of solution B, diluted and made upto 100 ml with water.

2. Ninhydrin solution

A. 0.8 g stannous chloride ($SnCl_2$ $2H_2O$, SRL) was dissolved in 500 ml of 0.2M citrate buffer, pH 5.0.

B. 20 g ninhydrin dissolved in 500 ml of methyl cellosolve (2-methoxyethanol).

100 ml of solution A was mixed with 100 ml of solution B to get 200 ml of ninhydrin solution.

3. 80% & 60% ethanol

4. Standard

solution of Glycine and glutamic acid was prepared by dissolving 50 mg each in 100 ml of distilled water. 1 ml of this standard gives a concentration of 1mg/ml.

Procedure

50 µl of hemolymph was treated with 80% ethanol and centrifuged at 5,000 rpm for 5 min. The supernatant was then treated with 0.5 ml of citrate buffer (pH 5.0) and 1.2 ml of ninhydrin solution, heated for 15 min. and subsequently cooled in running water. 2.3 ml of 60% ethanol was added and the absorbance was measured at 550 nm against a reagent blank.

2.2.5. Estimation of Proline

Proline content in the serum was estimated using the method of Sadasivam and Manikkam (1992).

Principle

Sulphosalicyclic acid precipitates proteins as a complex. The extracted proline reacts with ninhydrin in acidic conditions (pH 1.0) to form coloured chromophore and read at 520 nm.

Reagents

1. 3% aqueous sulphosalicyclic acid.

   3 g sulphosalicyclic acid dissolved in 25 ml and made upto 100 ml.

2. Acid ninhydrin

   1.25 g ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6M phosphoric acid warmed with agitation until dissolved.

3. Glacial acetic acid

4. Toluene

5. Standard

   50 mg of Proline dissolved in 50 ml of distilled water. 1 ml of this standard gives a concentration of 1 mg/ml proline.

Procedure

The serum was deproteinized with 3% sulphosalicyclic acid and the filtrate was treated with 1 ml of acid ninhydrin reagent and glacial acetic acid. The mixture was incubated in boiling water bath for 1 hr. and reaction was terminated by placing the tubes in ice bath. 2 ml of toluene was added and shaken for 20 - 30 sec. Toluene layer was separated and warmed at room temperature. The red colour developed was measured in UV Spectrophotometer at 520 nm using toluene as blank.

2.2.6. Estimation of cations (Na, K, Ca & Mg)

Na, K, Ca and Mg content were measured using standard procedure. Na, K and Ca concentration was estimated by Emission Flame Photometer (EEP) (ELICO Ltd, INDIA) and Mg content by Atomic Absorption Spectrophotometer (AAS) (ELICO Ltd, INDIA).

Procedure

Standard stock solutions for Sodium, Potassium, Calcium and Magnesium were prepared in deionized water and made upto 1000 ml. 100 ppm solution of sodium was prepared by dissolving 0.0254 g NaCl/100 ml, potassium (0.01906 g KCl/100 ml), calcium (0.0367 $CaCl_2$/100 ml) and magnesium (0.0211 $MgCl_2$/ 100 ml) respectively. Propane - Oxygen flame and 589 mμ (sodium), 766.5 mμ (potassium) and 612 mμ (calcium) filters

were used in EEP. Oxygen - acetylene flame and 285.2 mμ filter was used in AAS. Test hemolymph was diluted with deionized water and sprayed into the respective flame.

2.2.7. Statistical analysis

One- way analysis of variance (ANOVA) was used to determine the level of significance between body length (age groups) and between locality (Ross, 1987).

2.3. RESULTS

Table 2.1a shows the data of four biochemical parameters; Total protein, agglutinin, Total free amino acid (TFAA) and proline in the hemolymph of *P. indicus* with reference to increase of body length. The total protein content in the hemolymph of 8 size groups ranged between 51.32 mg/ml to 105.17 mg/ml. The agglutinin concentration ranged between 0.07μg/ml to 2.52 μg/ml among 8 size groups. TFAA concentration ranged between 0.46 mg/ml to 5.09 mg/ml among 8 size groups. Proline concentration varies between 0.08 μmol/100 ml to 2.68 μmol/100 ml among these size groups. All the parameters exhibited an increasing trend from G I to G VIII

(Fig. 2.1a, b, c & d). Significant variation was observed *(P <0.01)* among 8 size groups (Table 2.1b).

Table 2.2a summarises the concentration of protein, agglutinin, total free amino acid and proline content of hemolymph of prawn collected from three different localities, Chettuva (mud bank), Azhikode and Cherai. The mean total protein recorded in the sample from three localities includes 119.06 mg/ml, 108.91 mg/ml and 102.91 mg/ml and total agglutinin concentration 3.47 μg/ml, 0.97 μg/ml and 0.25 μg/ml, respectively. The mean total FAA in the hemolymph of three locality include 9.31 mg/ml, 3.21 mg/ml and 2.37 mg/ml. and proline concentration in these three localities include 3.31μmol/100 ml, 1.55 μmol/100 ml and 0.25 μmol/100 ml, respectively. The hemolymph of prawn collected from mud bank (Chettuva) recorded maximum concentration of protein, agglutinin, TFAA and proline (Fig. 2.2a, b, c & d). Statistically significant variation was observed among the three locality samples *(P <0.001)* (Table 2.2b).

Four cations Na, K, Ca and Mg concentrations with respect to body length were shown in Table 2.3a. Na concentration ranges between 68.28 to 99.44 mmol/l among 8 size groups with increase in body length. K concentration varies between 18.28 - 33.27 mmol/l. Ca concentration

Table 2.1a. Concentrations of biochemical parameters (Protein, Agglutinin, Total free amino acid (TFAA) & Proline) *vs.* Age groups.

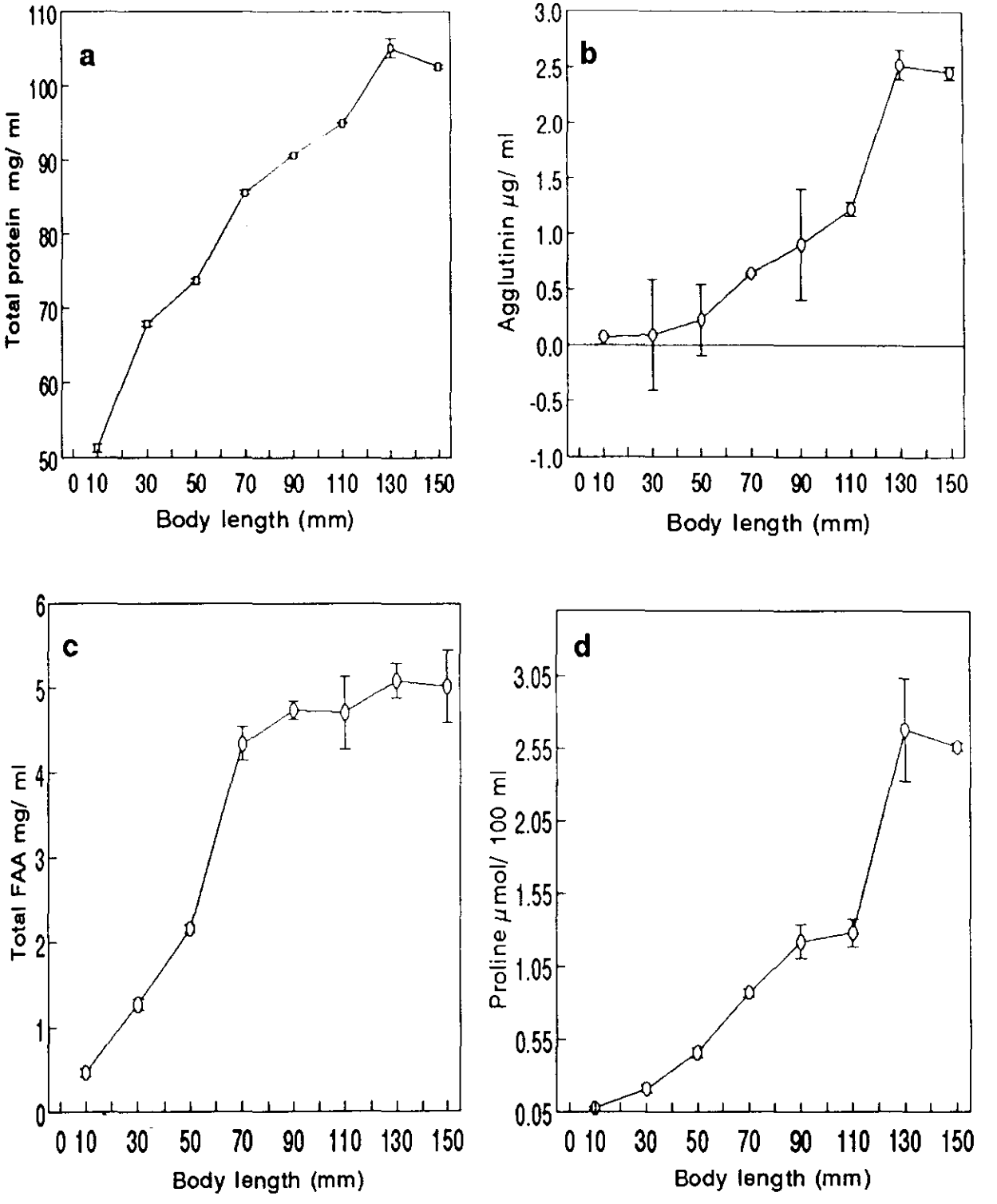
Groups	Biochemical parameters *				
	Body length (mm)	Total protein mg/ml	Agglutinin µg/ml	Total FAA mg/ml	Proline µmol/100 ml
I	10	51.32 ± 0.543	0.07 ± 0.018	0.46 ± 0.048	0.08 ± 0.01
II	30	67.94 ± 0.4	0.09 ± 1.35	1.27 ± 0.063	0.21 ± 0.026
III	50	73.75 ± 0.23	0.22 ± 1.2	2.16 ± 0.05	0.46 ± 0.037
IV	70	85.77 ± 0.293	0.64 ± 0.01	4.34 ± 0.20	0.87 ± 0.029
V	90	90.68 ± 0.20	0.91 ± 0.5	4.74 ± 0.11	1.22 ± 0.116
VI	110	95.02 ± 0.086	1.23 ± 0.06	4.71 ± 0.432	1.28 ± 0.097
VII	130	105.17 ± 1.28	2.52 ± 0.133	5.09 ± 0.2	2.68 ± 0.354
VIII	150	102.63 ± 0.312	2.45 ± 0.06	5.02 ± 0.432	2.56 ± 0.029

* mean of triplicate analysis.

Table 2.1b. ANOVA table showing levels of significance between Age groups with reference to Protein, Agglutinin, TFAA and Proline.

Source of variation	Protein		Agglutinin		TFAA		Proline	
	F	P	F	P	F	P	F	P
Between age groups*	4880.39	<0.001	40442.25	<0.001	417.402	<0.001	79.803	<0.001
Within age groups [ns]	1.369	ns	1.49	ns	2.26	ns	0.371	ns

* *P <0.01*; [ns] - not significant



Figs. 2.1a, b, c & d. Comparison of biochemical parameters (Protein, Agglutinin, Total free amino acid (TFAA) and Proline)in the hemolymph of *P. indicus vs.* body length.

Table 2.2a. Concentrations of biochemical parameters (Protein, Agglutinin, TFAA and Proline) *vs.* Locality.

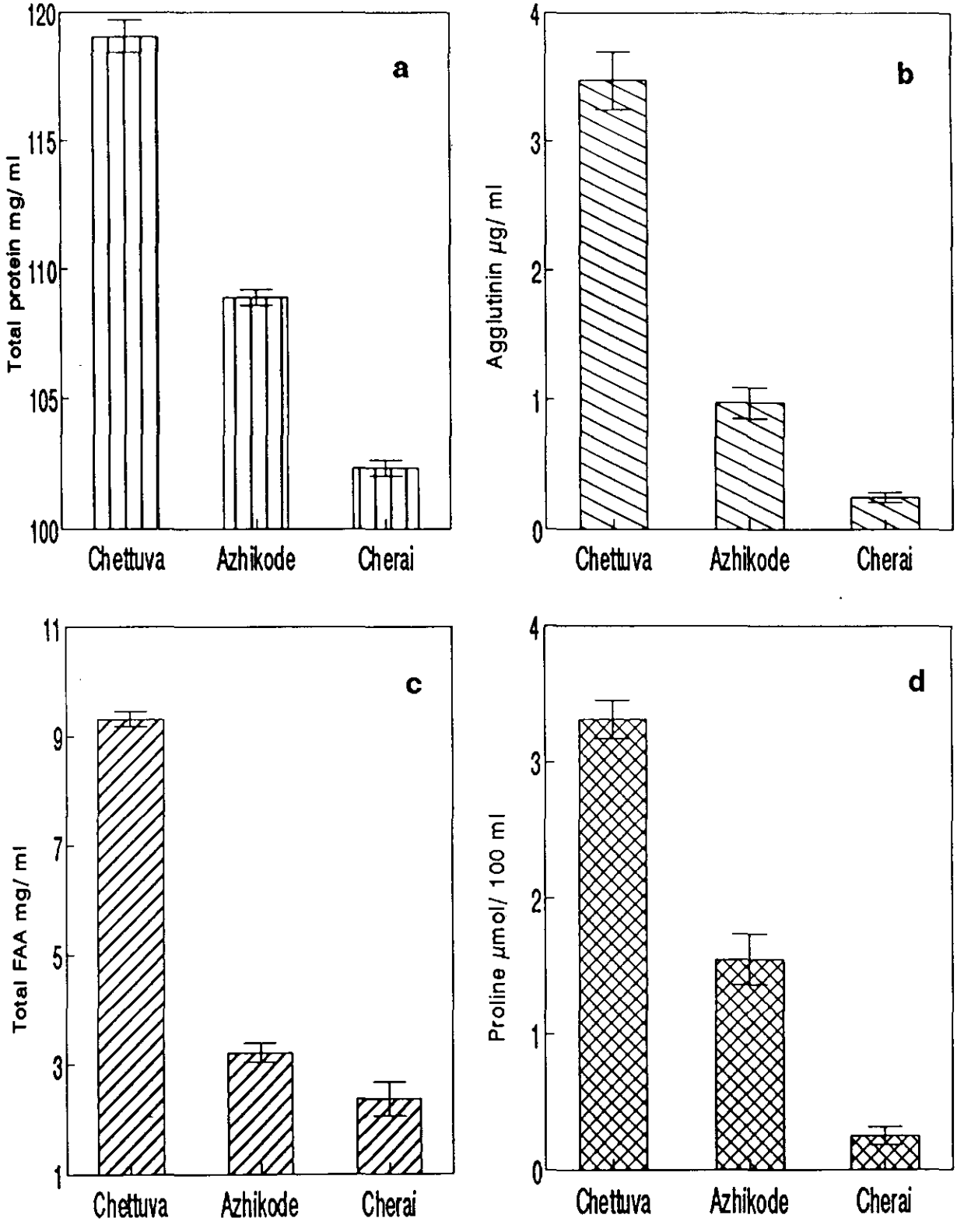
Parameter	Locality *		
	Chettuva	Azhikode	Cherai
Total protein mg/ml	119.06 ± 0.628	108. 91 ± 0.293	102.33 ± 0.31
Agglutinin µg/ml	3.47 ± 0.223	0.97 ± 0.117	0.25 ± 0.038
Total FAA mg/ml	9.31 ± 0.556	3.21 ± 0.18	2.37 ± 0.317
Proline µmol/100 ml	3.31 ± 0.138	1.55 ± 0.186	0.25 ± 0.067

* mean of triplicate analysis.

Table 2. 2b. ANOVA table showing levels of significance between Locality with respect to Protein, Agglutinin, TFAA and Proline.

Source of variation	Protein		Agglutinin		TFAA		Proline	
	F	P	F	P	F	P	F	P
Between Locality *	2238.5369	<0.001	560.56425	<0.001	546.801	<0.001	444.657	<0.001
Within Locality ^{ns}	1.017	ns	0.571	ns	1.006	ns	0.251	ns

* *P <0.001*; ^{ns} - not significant



Figs. 2.2a, b, c & d. Comparison of biochemical parameters (Protein, Agglutinin, Total free amino acid (TFAA) and Proline)in the hemolymph of *P. indicus vs.* Locality.

ranges between 0.09 - 2.54 mmol/l and Mg between 0.03 - 1.64 mmol/l among 8 size groups. All the parameters exhibited a linear increase from G I to G VIII (Fig. 2.3a, b, c & d). Significant variation were observed among all the age groups ($P < 0.01$) (Table 2.3b). The following sequence of cation concentration was observed.

$$Na^{+} > K^{+} > Ca^{2+} > Mg^{2+}$$

Table 2.4a shows the concentration of 4 cations (Na, K, Ca & Mg) in the hemolymph of *P. indicus* collected from three localities - Chettuva (mud bank), Azhikode and Cherai. Sodium concentration recorded from these three localities were 122.94 mmol/l, 97.08 mmol/l, 88.94 mmol/l and the K concentration was 49.0 mmol/l, 46.2 mmol/l and 36.92 mmol/l, respectively. Ca concentration among three localities was 6.36, 4.04, 2.14 mol/l and Mg concentration recorded were 1.23, 0.73 and 0.3 mmol/l. The sample collected from Chettuva (mud bank) recorded maximum levels of Na, K, Ca and mg concentration (Fig. 2.4a, b, c & d). Significant variations were observed in cation concentration between locality ($P < 0.05$) (Table 2.4a).

Table 2.3a. Cation concentration (Na, K, Ca & Mg) *vs.* body length

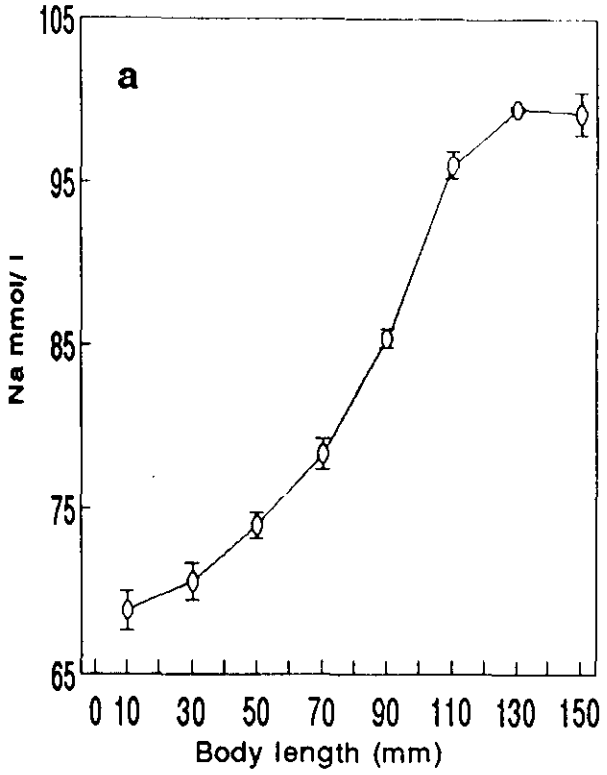
Groups	Biochemical parameters *				
	Body length (mm)	Na mmol/l	K mmol/l	Ca mmol/l	Mg mmol/l
I	10	68.28 ± 1.2	18.28 ± 0.66	0.09 ± 0.95	0.03 ± 0.72
II	30	70.63 ± 1.1	20.08 ± 0.8	0.37 ± 0.62	0.05 ± 0.58
III	50	74.03 ± 0.8	25.11 ± 0.8	0.43 ± 1.0	0.06 ± 0.01
IV	70	78.38 ± 0.92	28.24 ± 0.28	0.55 ± 0.6	0.69 ± 0.89
V	90	85.42 ± 0.6	30.20 ± 0.82	0.91 ± 1.3	0.82 ± 1.1
VI	110	96.02 ± 0.8	31.20 ± 0.9	1.12 ± 0.8	0.95 ± 0.82
VII	130	99.44 ± 0.12	33.14 ± 0.8	2.54 ± 0.6	1.64 ± 0.2
VIII	150	99.16 ± 1.3	33.27 ± 1.2	2.36 ± 2.3	1.38 ± 1.2

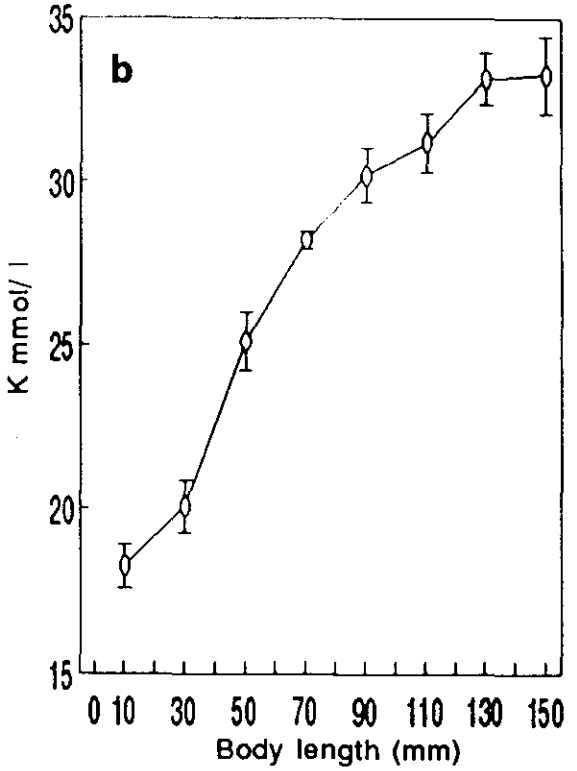
* mean of triplicate analysis.

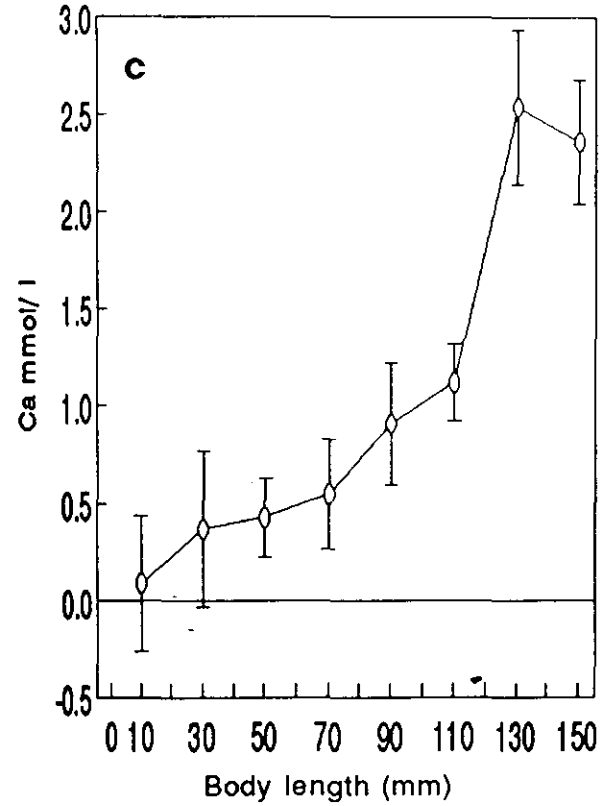
Table 2.3b. ANOVA table showing levels of significance between body length with reference to cations (Na, K, Ca & Mg).

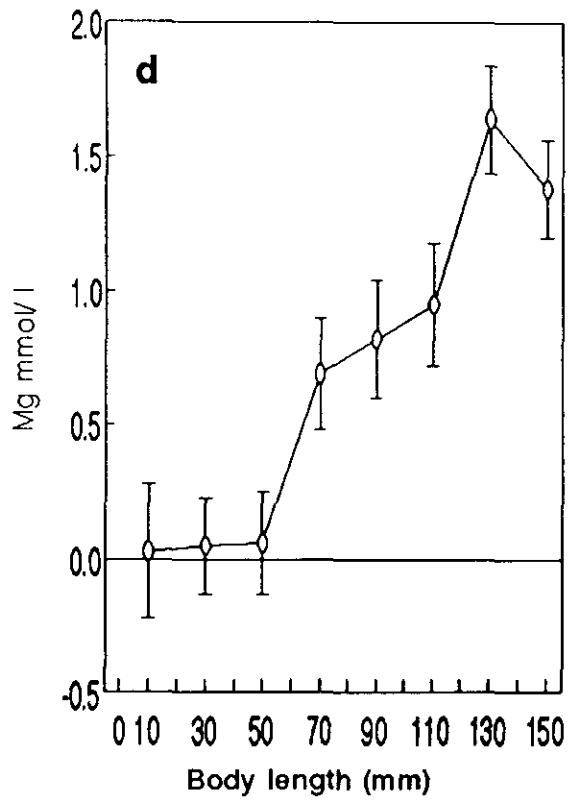
Source of variation	Na		K		Ca		Mg	
	F	P	F	P	F	P	F	P
Between age groups *	5096.17	<0.05	18.959	<0.05	111.253	<0.05	319.544	<0.05
Within age groups ns	0.84	ns	1.07	ns	1.804	ns	0.371	ns

* $P < 0.01$; ns - not significant









Figs. 2.3a, b, c & d. Comparison of Cations (Na, K, Ca & Mg) in the hemolymph of *P. indicus vs.* body length.

Table 2.4a. Cation concentration (Na, K, Ca & Mg) *vs.* Locality.

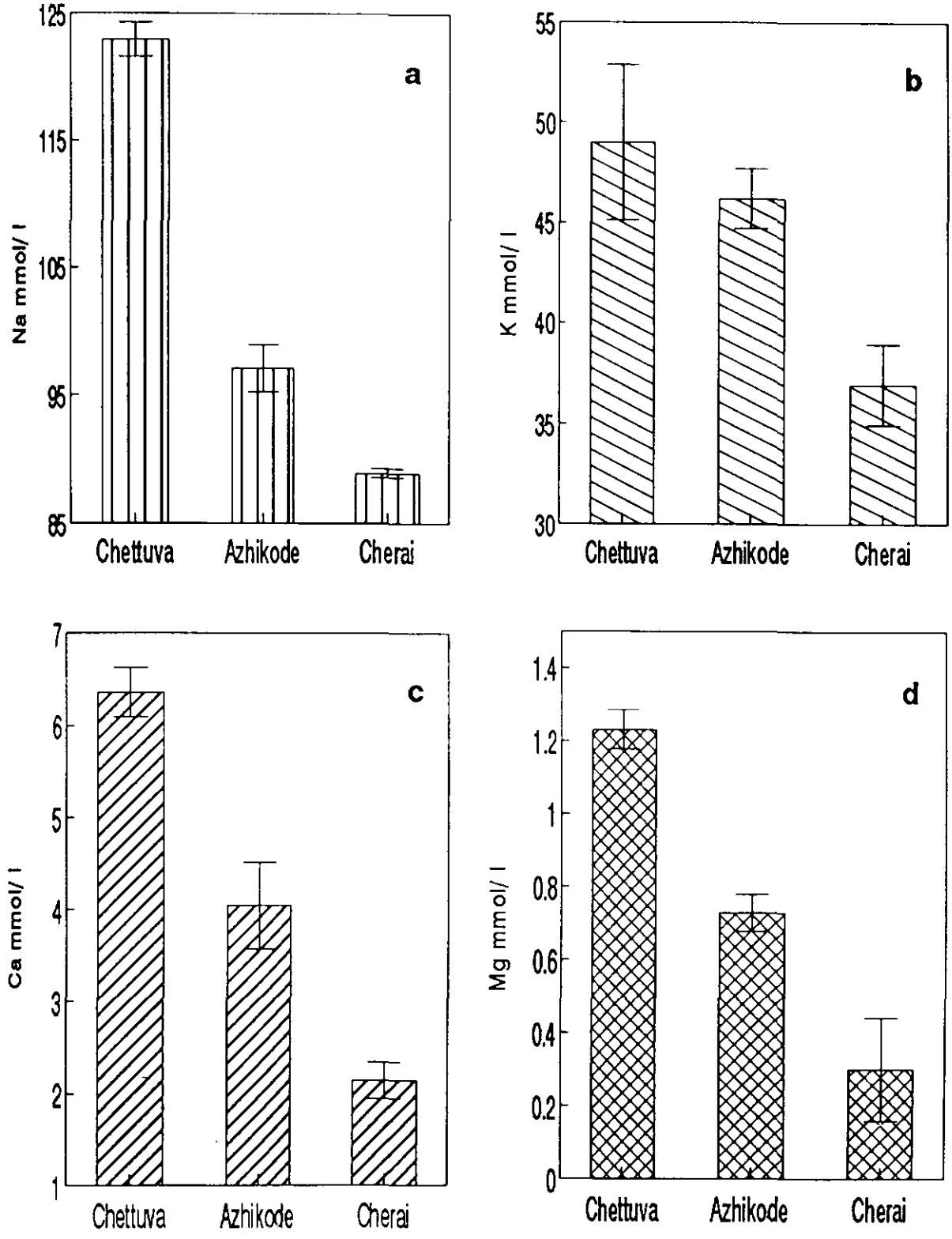
Parameter	Locality *		
	Chettuva	Azhikode	Cherai
Na	122.94 ± 1.35	97.08 ± 1.84	88.94 ± 0.338
K	49.0 ± 3.88	46.20 ± 1.48	36.92 ± 1.99
Ca	6.36 ± 0.27	4.04 ± 0.475	2.14 ± 0.195
Mg	1.23 ± 0.054	0.73 ± 0.05	0.30 ± 0.142

* mean of triplicate analysis.

Table 2.4b. ANOVA table showing levels of significance between Locality with reference to Cations (Na, K, Ca & Mg).

Source of variation	Na		K		Ca		Mg	
	F	P	F	P	F	P	F	P
Between Locality *	647.336	<0.05	31.518	<0.05	113.659	<0.05	130.544	<0.05
Within Locality ns	0.056	ns	2.199	ns	0.158	ns	1.937	ns

* $P < 0.05$; ns - not significant



Figs. 2.4a, b, c & d. Comparison of Cations (Na, K, Ca & Mg) in the hemolymph of *P. indicus vs.* Locality.

2.4. DISCUSSION

The chemical composition of the hemolymph in crustacea depends more or less directly on the nature of the environment and varies during development. Despite the ontogenic and phenotypic variation, taxonomic interference can often be based on the hemolymph composition. Since hemolymph is the carrier of every kind of biochemical constituents from one point of the body to the other, hemolymph chemistry is concerned with the number of specific role of the substances in transit. The chemical composition varies with respect to the environment (Florkin and Scheer, 1971).

Biochemical parameters such as total protein, agglutinin, total free amino acid, proline and cation (Na, K, Ca & Mg) concentrations were studied during the developmental stages and influence of environment on hemolymph concentration was also analysed by comparing the samples from three different localities - Chettuva (mud bank), Azhikode and Cherai.

The total protein content is the most important biochemical constituent in the hemolymph. In crustaceans, growth is defined as increase in wet weight of the body, which generally occurs in the periods between molts when the absorbed water is replaced by proteins. During growth process proteins synthesized at various levels are transported to the hemolymph. Significant increase in total protein concentration was noticed from G I to G VIII *($P < 0.05$)*. The protein concentration exhibited a wide range of 51.32 mg/ml to

105.17 mg/ml among 8 size groups. The result was comparable to the finding of Gondko *et al.* (1981) and Leone (1953) in *Callinectsu sapidus, C. magister* and *Homarus americanus.* Fingerman *et al.* (1994) opined that wide range of values presented might be due to the differential translation as a result of growth process. Leone (1953) reported that wide range of values presented may be expected to occur in a natural population at any given time. *P. indicus* samples were assembled from a single population (stock) and hence the wide variation occurred in the natural population. Significant variation was observed in different parameters in the hemolymph samples from three different locality despite the salinity change. Increase in protein concentration observed from Chettuva sample (Fig. 2.1a) may be due to the intake of protein rich food items from the mud bank area. All the biochemical parameters were increased in the sample collected from Chettuva (mud bank) (Fig. 2.2a, b, c & d). The increase in biochemical constituents were noticed in the hemolymph of shrimps, *P. aztacus* and *P. setiferus* during developmental stages (Castille and Lawrence, 1989). Protein concentration in the hemolymph was also greatly affected by nutritional state as well as dietary source (Dall, 1974; Hepper, 1978). Salinity might not be an important factor determining protein concentration in the hemolymph except in unusually high or low salinities when osmotic forces would prevail (thereby dehydrating or hydrating the hemolymph respectively) or in conditions where excess amino acid would be utilised as osmotically active compounds to increase or decrease hemolymph osmolality (Ferraris *et al.*, 1986).

The agglutinin concentration in the hemolymph also exhibited a range between 0.070 to 2.52 µg/ml from G I to G VIII. Significant variation was observed in agglutinin concentration between the sample collected from different locality *(P<0.001)*. The hemolymph from Chettuva sample exhibited higher concentration of agglutinin (3.47 µg/ml). The agglutinin was considered as one among the hemolymph natural defense protein or antimicrobial protein. Agglutinin synthesis can be stimulated by changes in salinity, environmental stresses such as temperature, pathogens or pollution (Nair, 1983; Kurian and Sebastian, 1986). The samples collected from Chettuva (mud bank) were expected to perform high titers of agglutinin. The stress conditions generated in the mud bank augments the agglutinin synthesis from the hemocytes in crustaceans. This may be the reason for the variation of agglutinin concentration in the hemolymph collected from three localities. Increase in metallo - protease secretion was noticed in *Cryptobia salmositca* living in the medium with pathogens (Xzuo and Woo, 1998).

The concentration of FAA was found to be low in hemolymph compared to muscle. Dalla (1986) studied the effect of FAA composition in the hemolymph of *Penaeus japonicus*. In *P. japonicus*, glycine, proline and alanine functions as osmo - effectors after hypo - osmotic shock and increases after hyper - osmotic shock (Dalla, 1986). In the present study, total free amino acid (TFAA) concentration and proline concentration exhibits an increasing trend from G I to G VIII *(P <0.01)*. Wide range of free amino acid (0.46 - 5.09

mg/ml) and proline (0.08 - 2.68 μmol/100 ml) was noticed during the growth process. The free amino acid concentration in the hemolymph was considered as the product of metabolism in *Aedes aegypti* (Bounias, 1989). The TFAA and proline concentration exhibited variation between the samples collected from three localities (Table 2.2a). The increase of free amino acids may be due to low salinity of mud bank area consequent to the inflow of fresh water during heavy monsoon. The stress mediated while mud bank formation may be the reason for the increase of proline concentration in sample. Proline is a basic amino acid that can be used as an indicator of external environmental changes. Methyl fernesoate (MF) in the hemolymph, like proline was found to increase in the hemolymph followed by physiological stress such as increased temperature, anoxia and decreased salinity in the green crab, *Carcinus maenas* (Lovett, 1997).

Moreover, free amino acid content in the hemolymph also depends on the composition of food and the physiological state of the animal (Dean and Scott, 1965). Increased titers of FAA in the hemolymph of prawns collected from mud bank (Chettuva) may be due to the abundance of food materials (Damodaran, 1973). Gilbert (1959) found that the level of free amino acids in crustacean hemolymph depended on body size and sex of the animals and also observed higher concentrations of free amino acids in males than in females.

Our observation of FAA and proline content is in agreement with the findings of Faug *et al.* (1992) in *Penaeus monodon*. During growth process the

normal flux direction of FAA is from hemolymph to cell, not from cell to hemolymph. This may be due to the low level of FAA in the hemolymph. On the other hand, the low level of total FAA implied that more organic molecules could be used for building blocks of cells instead of just an osmolarity-regulating reservoir in the hemolymph. At higher salinities urea is produced that functions as an osmolite, which is more energy efficient than amino acid. Due to thermal stress, in the intercellular pool of amino acids in *Eriochier sinensis* living in fresh water show a marked drop of free proline concentration (Duchätean and Florkin, 1955a). An increased FAA concentration can be observed in euryhaline crustacea transferred from seawater to brackish water in crabs, *Carcinus maenas* and *Eriochier sinensis* (Duchätean and Florkin, 1955b). In isopods, *Porcellio laevis*, proline represents the highest level of TFAA (2.18 µmol/100 ml) followed by serine and glutamic acid. In eutrophied water dwelling animals of mud bank area reports higher levels of proline (Nair, 1983).

The growth and success of an organism is governed not only by its genetic potential but also by its immediate environmental conditions. Certain environmental factors like poor water quality, physical disturbances and social dominance of one species by another can act as powerful environmental stresses, thereby reducing the performance of the organism. The aquatic organisms with the high levels of natural agglutinin, higher concentration of proline and reduced cortisol response to common aquacultural stresses would

have benefits for disease resistance, reproductive performance and growth rate (Pickering, 1993).

Table 2.3a summarises the content of 4 cations in the serum of *P. indicus* with reference to developmental stages. The following sequence of cation concentration was observed.

$$Na^{+} > K^{+} > Ca^{2+} > Mg^{2+}$$

The serum of *P. indicus* is high in sodium and low in magnesium level. The cation concentration also increases significantly with size groups from G I to G VIII *($P < 0.05$*. All the parameters were found to be higher in G VII and slightly declines in G VIII due to spent gonads. The movements of ions are the likely mechanism of pH maintenance in aquatic crustaceans (Cameron, 1978; Wolcolt, 1991). Morris and Butler (1996) reported that ionic status may provide a method of maintaining acid - base balance in purple crab, *Leptograpsus variegatus*. In the present study, high sodium index (99.44 mmol/l and low magnesium index (1.64 mmol/l) was recorded. This feature is a characteristic property of all marine crustacea (Florkin, 1962). The existence of a relatively efficient ionic regulation correlated with molting has allowed numerous adaptations to life in brackish water, fresh water and for euryhaline forms in a considerable range of salinities.

Table 2.4a summarises the 4 cation concentration in the hemolymph of samples collected from three localities. Sodium and potassium concentration

was found to be higher and unchanged. The sample from the mud bank (Chettuva) recorded higher concentration of all the 4 cations studied. Significant increase of cation concentration in the serum of sample from the mud bank (Chettuva) may be due to the various types of stress conditions (osmotic, thermal, pollution, stocking density etc.). Infection of *Carcinus maenas* by Sacculina has a number of metabolic changes leading to increase of protein, Ca^{2+} and Mg^{2+} concentration in the hemolymph, where as its Na^{+} and K^{+} contents remain unchanged (Drilhon, 1935). The nature and range of animal's possible colonising ability depend on the biochemical capability. The restoration of the normal composition of blood plasma at the end of the post-molt period require an effective system of ionic regulation present in marine crustaceans and are responsible for the observed differences of blood inorganic ions compared to sea water. This type of regulation result in a high sodium index and low magnesium index in marine euryhaline crustaceans. The higher concentration of Ca^{2+} and Mg^{2+} found in the hemolymph of Chettuva sample may be due to low salinity. A change in the salinity of seawater normally results in an equivalent change in its calcium concentration. In species which osmoregulate, there is some evidence for active regulation of calcium in the hemolymph in the dilute media. In *Carcinus maenas* and *Callinectes sapidus* calcium concentration in the hemolymph increased in dilute medium (Greenaway, 1976). The sodium and calcium concentration of few crustaceans studied were *Holothuisana transversa* (Sparkes and Greenaway, 1984)

Gammarus pulex (Wright, 1980), *Macrobrachium rosenbergii* (Castille and Lawrence, 1981) and *Carcinus maenas* (Zenders, 1980).

The hemolymph total calcium remains fairly constant throughout the molting cycle in most aquatic crustaceans, despite significant translocation between tissues, hemolymph and external water (Greenaway, 1985). Any change in total Ca during the molting cycle have been attributed to the protein bound fraction (Neufeld and Cameron, 1993), free calcium which is the more relevant variable, remains unaltered. *In vitro* experiments have shown that extracellular Ca can block molt inhibiting hormone (MIH) suppression of ecdysteroidogenesis (Watson *et al.* (1989). The mechanism of this involves stimulation of cAMP - phosphodiesterase activity by a Ca calmodulin complex resulting in the lowering of cellular cAMP (Mattson and Spaziani, 1986). The calcium requirements of crustaceans are indicated by the total calcium content of the body. In animals at hard intermolt stage, the bulk of this calcium is in the skeleton and its proportion in the soft tissues and hemolymph will be very small. The total content or calcium is best expressed as the regression of Ca content of body weight and also linearly related to body size (Greenaway, 1985). The above result of our studies were comparable to the observation of Bursey and Lane (1971) in *Penaeus duorarum*, in *Macrobrachium rosenbergii*, (Fieber and Lutz, 1982) and in *Cancer maenas* (Greenaway, 1976). Intracellular level of K^+ and Na^+ ions control the Na^+ / K^+ pump which in turn control the movements of these ions between hemolymph and intracellular fluid. Na^+/K^- pump is rheogenic, extruding 3 Na^+ against 2 K^+

ions (Pequeux, 1995). This may be the reason for higher levels of Na^+ and K^+ in the hemolymph.

Biochemical constituents such as total protein, agglutinin, TFAA, proline and cations are well regulated during growth process through differential gene action and exhibits an increasing trend from G I to G VIII. Biochemical constituents are secreted at their target sites, transported to the hemolymph and exchange of materials takeplace between hemolymph, intercellular and extracellular fluid. Hormones released during growth process regulate the mineral balance. The comparative studies of biochemical constituents from different localities establishes the organic and inorganic regulatory capability of *P. indicus* hemolymph to cope with the surrounding environment.

Chapter III

Isolation and partial characterization of agglutinin in the hemolymph

3.1. INTRODUCTION

Crustaceans have an open circulatory system, hence the blood is termed the hemolymph. Hemolymph is a fluid tissue whose characteristics vary markedly during molt cycle and in response to environmental changes. It usually includes two components, the plasma and the corpuscles named as hemocytes. Hemolymph carries different kinds of hemocytes and a few dissolved plasma proteins. Plasma proteins such as hemocyanin, fibrinogen and heteroagglutinins have been traced by few authors (Tyler and Metz, 1945; Tyler and Scheer, 1945; Fuller and Doolittle, 1971a, 1971b). These proteins function as a pivot for the operation of humoral and cellular defense mechanism. The success of invertebrate animals and their ability to colonise every ecological niche must be due to the presence of an effective immune system operating through hemocytes and dissolved plasma proteins capable of combating infection.

Aquaculture in most instances is an intensive animal husbandry in which large numbers of animals are maintained in relatively limited spaces. Regardless of the quality of the husbandry practised, infectious disease outbreaks are always possible. Bacterial, viral, fungal or parasitic pathogens may originate from feral prawn or fish and be transmitted in the facility's water supply, may be transported to the farm with purchased eggs, juveniles or from farm to farm by humans (Takahashi *et al.*, 1985; Lightner, 1993). The

penaeid prawns are susceptible to attack by fungi, bacteria and protozoa. In Japan, the fungus, *Fusarium solanum* caused black gill diseases in *Penaeus japonicus*. Pathogenic bacteria of *Vibrio* sp. have been isolated from the blood and liver of dead *P. japonicus*. At Central Marine Fisheries Institute, Cochin, three bacterial diseases were found on adult prawns, the frequent one being caused by *Vibrio anguillarum*, due to which muscles got emaciated and softened and cuticle got thinner (Kurian and Sebastian, 1986). Dwivedi *et al.* (1983) have mentioned the protozoan like *Ephelota* sp. parasitises on penaeidean prawns. Aquatic species of bacteria, including commensals of the crustacean gut, are most prominent as external stress and as internal pathogens. Gomez-Gil *et al.* (1998) isolated several species of bacteria from hepatopancreas, hemolymph and digestive tract of *P. vannamei*. During diseased condition one or two species predominate (Song *et al.*, 1993; dele Peña *et al.*, 1993). It has been suggested that the presence of bacteria in the hemolymph is indicative of septicaemia and a common seqûelae to stress (Lightner, 1988). Monitoring of the health condition of populations under rearing is not very easy because techniques to make this control are not available. The study of hemocytes and hemogram is one possibility to perfect such techniques; it has been shown that infectious diseases reflect back on the blood of crustaceans (De Backer, 1961; Bang, 1971).

Classification of hemocytes provide a means of reference to further studies concerning their function, changes due to normal physiological processes under abnormal conditions. Very few work has been published about

the hemogram of crab, *Eriocheir sinensis* by Bauchau and Plaquet (1973). Tsing *et al.* (1989) classified the hemocytes of *P. japonicus, P. monodon, Macrobrachium rosenbergii* and *Palaemon adsperrus* into three cell types. Classification of hemocytes of Ridgeback prawn, *Sicyonia ingentis* has been based on subtle morphological features (Hose and Martin, 1989). Ratcliffe and Rowley (1979) classified insect blood cells according to their structure and function. Nakayama *et al.* (1997) studied the hemocytes of giant clam *Tridacna derasa.* Using the Ridgeback prawn, *Sicyonia ingentis* as a model for decapod crustaceans, Hose and Martin (1989) developed a hemocyte classification. Based on morphological (Martin and Graves, 1985; Martin *et al.*, 1987) and cytochemical (Hose *et al.*, 1987) criteria, two major categories of hemocytes have been identified such as hyaline cells and granulocytes. The purpose of hemocyte classification is to describe the involvement of hemocytes in phagocytosis, encapsulation, coagulation, nodule formation, cytotoxic reactions, transplant rejections and agglutination of foreign material.

Clottable factors are found either inside the amoebocytes (Horseshoe crabs) or in both the hemocytes and cell - free plasma of Crustacea Decapoda (Ravindranath, 1980). In horseshoe crab, coagulation processes have been analysed by Shishikura and Sekiguchi (1978). Clotting processes in Crustacea, Decapoda have been studied in detail by Durliat (1985). In Crustacea, three types of clotting processes have been defined (Tait and Gunn, 1918). In type A, agglutination of the blood cells takesplace without subsequent gelation of plasma; in type B, agglutination occurs with subsequent gelation of plasma.

This is indicated by the occurrence of degenerated cells which probably have released the content of their granules into the plasma, initiating coagulation and resulting in gellification. In *P. indicus*, type B coagulation takesplace. In type C, gelation of the plasma is in the shape of islands of coagulation around Hardy's explosive corpuscles. Clotting is initiated by the rupture of specific fragile hemocytes releasing enzymes. The clotting protein is naturally dissolved in the plasma. The clottable factor permitting the gelation of plasma is a high molecular weight plasma protein which can be converted to covalently cross linked gel by the action of Ca^{2+} dependent cellular transglutaminase (Bruner *et al.*, 1966; Lorand, 1972). The plasma coagulation appears in the form of heterogeneous polymers on polyacrylamide gradient gels (Durliat *et al.*, 1975) but shows a single precipitate arc on crossed immunoelectrophoresis performed with the blood of *Astacus leptodactylus* (Durliat and Vranckx, 1976). In Decapoda, clotting factors are found in both cell - free plasma and hemocyte compartments (Durliat, 1985). This type of clotting mechanism have been studied in *Homarus* (Stewart *et al.*, 1966), in *Panulirus interruptus* (Doolittle and Fuller, 1972) and in *Astacus leptodactylus* (Durliat and Vranckx, 1976). Endotoxins from diverse bacteria can activate clotting process in Decapoda. Endotoxins from gram -negative bacteria (*E. coli*) activated both protease and prophenoloxidase in hemolyte lysates from *Astacus astacus* (Söderhall and Häll, 1984) and elicited a clotting reaction. *Vibrio* initiated clotting in *Saculina* (Levin, 1967); *Gaffkya homari* in lobster, *Homarus* sp. (Durliat and Vranckx, 1983a); *Callinectes sapidus* in which

Paramoeba pernicosa initiated clotting process (Johnson, 1976); Shigellatoxin in *Homarus americanus* (Levin, 1967); *Vibrio* sp. in *Cancer irroratus* (Newman and Feng, 1982). The blood clotting factors of crayfish exhibit bactericidal properties against the endotoxins through the initiation of clotting process (Durliat and Vranckx, 1976; McKay and Jenkin, 1969). Analysis of clotting defects in diseased lobsters have been attempted by Durliat and Vranckx (1983b). The clotting mechanism of *P. indicus* using different bacterial species have been attempted. Natural heteroagglutinin present in the hemolymph helps to study clotting mechanism, agglutination and lysis of endotoxins and foreign bacteria.

Crustacea possess both humoral and cellular defense mechanisms like vertebrates and other invertebrates (Sindermann, 1971; Stewart and Zwicker, 1972). The free hemocytes in the hemolymph invoke cellular responses and also secrete humoral factors such as agglutinins. Invertebrates possess a variety of humoral substances which is involved in immunity such as lysins, agglutinins, antimicrobial factors that differ considerably from classical vertebrate antibodies (Miller and Ratcliffe, 1994). Agglutinins or receptor specific proteins are widely distributed in nature and provide a new research tool for the study of cell biology, immunology and cancer research. Invertebrate agglutinins have usually been detected in the hemolymph by their ability to agglutinate particles such as vertebrate erythrocytes, bacteria, protozoa or fungal cells. Agglutinins function as recognition molecules in defense reactions (Pendaland *et al.*, 1988), help in the lysis of foreign cells

(Komano and Natori, 1985) and functions as activator of hemocyte coagulation (Minnick *et al.*, 1986). Agglutinin occur throughout invertebrate phyla and have specific carbohydrate binding sites (Olafsen, 1988).

Cornick and Stewart (1973) attempted the isolation and partial characterization of a natural agglutinin in the hemolymph of *Homarus americanus*. Natural heteroagglutinins in the serum of spiny lobster, *Panulirus interruptus* have been studied by Tyler and Scheer (1945). Horseshoe crab, *Limulus polyphemus* agglutinin was studied by Cohen *et al.* (1965) and Finstad *et al.* (1974). Agglutinin of crayfish, *Parachaeraps bicarinatus* (McKay *et al.*, 1969) and *Procambarus clarkii* (Miller *et al.*, 1972) was characterized. Tripp (1992) studied the role of agglutinins in the hemolymph of *Mercenaria mercenaria*. Fuke and Sugai (1972) and Coombe *et al.* (1984) carried out the preliminary characterization of Ascidian agglutinins. Agglutinin in the serum of Acorn Barnacle, *Balanus roseus* was isolated and characterized by Kamiya and Ogata. (1982). A multimeric hemagglutinin from the coelomic fluid of sea urchin, *Anthocidaris crassispina* (Giga *et al.*, 1987) have been isolated and characterized to homogeneity. Fisher and Dinuzzo (1991) attempted to study the agglutination property of hemolymph of mollusc towards bacteria and erythrocytes. Roch *et al.* (1981) characterized agglutinin as haemolytic/ bacteriostatic factor naturally present in the coelomic fluid of earthworm. Among crustaceans, Brown *et al.* (1968) attempted to study the agglutinin of shore crabs like *Macropipus puber, Arabus pisonicalappa flammea, Callinectes danae, Dardanus venosus, Mithrax* sp. and spiny lobster,

Panulirus anceps. Ueda *et al.* (1991) isolated the agglutinin from 12 species of crab, 2 hermit crab, *Aniaus aniaus*, *Pugurus similis* and 3 species of lobsters. Antibacterial property of agglutinins have been studied in a few species. Bactericidal property of agglutinins have been examined in the hemolymph of *Homarus americanus* against *Gaffkya homari* (Cornick and Stewart, 1968). Chain and Anderson (1983) characterized the bactericidal molecule from the coelomic fluid of polychaete, *Glycera dibranchiata.* Lassègues *et al.* (1989) studied the bacteriostatic activity of *Eisenia fetida andrei* coelomic fluid. Antibacterial activity of crude whole body hemolymph of the hemichordate, *Saccoglossus ruber* was reported by Miller and Ratcliffe, (1987). Hirigoyenberry *et al.* (1992) characterized the antibacterial activity of *Eisenia fetida andrei* coelomic fluid. Antibacterial activity in the hemolymph of Myriapods (Arthropoda) have been studied by Xylander and Nevermann (1990).

From the above survey it is clearly indicated that very few work has been carried out in the hemolymph agglutinin of *P. indicus.* In this chapter, the present study involves the classification of hemocytes, its clotting mechanism, agglutination assays with bacteria and RBC, effect of sugars on agglutination property, isolation and physico- chemical characterization of agglutinin in the hemolymph and determination of its antibacterial property.

3.2. MATERIALS AND METHODS

3.2.1. Experimental animals.

Prawns, *Penaeus indicus* of both sex having body length 110 mm to 130 mm were collected from Vallarpadam area of Cochin backwaters, Kerala.

3.2.2. Preparation of Bacterial and RBC suspension

A total of 8 bacterial strains were isolated from prawn gut, sea water, marine sediments and estuarine water listed in Table 3.1 were used for agglutination test. Each bacterial strain was incubated aerobically in Luria broth (LB) (in g/1,000 ml of 50% aged sea water) at 37°C for 18 hrs. The cells were centrifuged at 4,000 rpm for 15 min. and washed 3 times with phosphate buffered saline (PBS) containing 0.12% Na_2HPO_4, 0.07% KH_2PO_4 and 0.68% NaCl (pH 7.5). The cells were then prepared at a concentration of 1 x 10^6 cells/ml in a 0.5% formalin - 0.85% NaCl solution and stored at 4°C until use (Ueda *et al.*, 1991). Vertebrate RBC (Human, Rat and Rabbit) were collected and centrifuged at 4,000 rpm for 5 min., washed with PBS, 0.85% NaCl (twice) and in 50% aged sea water (Fisher and Newell, 1986) and diluted with 0.85% NaCl to give a 2% RBC suspension.

3.2.3. Simple sugars used for inhibition studies

Glucose, Galactose, Lactose, Maltose, Cellobiose, Mannose, Xylose, Raffinose, Trehalose, L-Arabinose, D- glucosamine and $CaCl_2$ purchased from SRL (AR), Bombay were used for inhibition studies. 10% ($^{w}/_{v}$) sugar solution was prepared in sterile double distilled water.

3.2.4. Collection of hemolymph

3.2.4.1. Plasma

Hemolymph was withdrawn from the pericardial sinus using No.24 hypodermic needle after swabbing the body surface with 70% ethanol and transferred to a sterile vial with the anticoagulant, 3% sodium citrate($^{W}/_{V}$) [9 part hemolymph : 1 part anticoagulant]. The anticoagulant prevents clumping and breakdown of the cells. The plasma was collected as the supernatant after centrifugation at 5,000 rpm at 4°C for 20 min.

3.2.4.2. Serum

The serum was obtained by allowing the hemolymph to clot at 4°C overnight, and by centrifugation at 4,000 rpm for 15 min.

3.2.4.3. Plasmatic clottable protein (heteroagglutinin) extraction

The plasmatic clottable protein was extracted according to the method of Stewart *et al.* (1966). Hemolymph was allowed to precipitate using 45% ammonium sulphate saturation at 4°C for 1-2 hrs and centrifuged at 5,000 rpm for 20 min. The precipitate was dissolved in 0.85% NaCl to get clottable solution and dialysed against distilled for 12 hrs with intermittent changes. The clottable solution was used immediately for agglutination assays.

3.2.5. Smear preparation

A drop of freshly extracted hemolymph was placed on a clean glass slide. A thin uniform smear was drawn by using a rectangular cover slip at 45°. The smear was air dried for few minutes, fixed in methanol for 5 min. and

stained with Wright's stain for 5 - 10 min., washed with double distilled water and mounted in DPX. The hemocytes were identified and classified under Phase contrast microscope based on morphology and function.

3.2.6. Clotting mechanism

Hemolymph was withdrawn from the pericardial sinus using No. 24 hypodermic needle after swabbing the body surface with 70% ethanol was transferred to a sterile glass slides and allowed to clot without anticoagulant. Clotting time was measured by examination of clot formation with a glass rod. Clotting mechanism was studied under Phase contrast microscope.

3.2.7. Agglutination assay

Agglutination assays were performed using hemolymph plasma, serum and clottable solution against bacterial cells and RBC. Assay was conducted on glass slides and titer values were determined after serial dilutions in test tubes. A serial two fold dilution of the serum, plasma and clottable solution were made in a final volume of 25 µl using 0.85% NaCl as a diluvent. Test for the occurrence of agglutination were conducted by mixing 50 µl of serum, plasma or clottable solution with 50 µl of bacterial suspension or RBC suspension. Control wells contained 50 µl of the assay particles and 50 µl of sea water for bacteria and PBS saline for RBC. Bacterial assays were incubated for 24 hrs at 37°C and RBC assays for 2 - 3 hrs at 37°C. Agglutination activity was expressed in terms of titer values. Titers were designated as the reciprocal of the highest dilution showing positive

agglutination at the level with test particles (Fisher and Dinuzzo, 1991). Minimum concentration of plasma, serum and agglutinin required for agglutination assay was also carried out by the method of Lowry *et al.* (1951).

3.2.8. Testing for antibacterial properties

Inhibition zone test described by Xylander and Nevermann, (1990) was adopted for testing antibacterial property of agglutinin using *E. coli* and *Vibrio* sp. Test bacterial strains (*E. coli* and *Vibrio* sp.) were cultured in sterile nutrient broth and suspended in LBA. 10 µl of broth with bacterial suspension was dispersed into petridishes under a laminar flow to get standard agar plates with about 10^4 test bactria/ ml. Holes of about 3 mm in diameter were punched into the solid agar and filled with 10 µl of saline as control, 10 µl of plasma, serum or electroeluted agglutinin as test particle. The petriplates were incubated at 37°C for 24 hrs. The zone of clearance was observed using a white light transilluminator.

3.2.9. Isolation of agglutinin

Isolation of agglutinin was done according to the extraction procedures of Miller *et al.* (1972) and Mosconi *et al.* (1998).

3.2.9.1. Chromatography

The *P. indicus* serum was separated into several fractions in molecular sieve column chromatography using Sephadex G - 200 as column material. The column (1.6 X 15 cm i.d.) was prepared by applying the slurry of

Sephadex G - 200 prepared in distilled water. The column was equilibrated for 24 hrs with 0.02M PBS) phosphate buffered saline (pH 8.0). 3 ml of *P. indicus* serum was applied to the column and eluted using 0.02M PBS buffer at a constant flow rate of 1 ml/min. Each fraction was monitored for the presence of protein at 280 nm and used for agglutination assay. The fractions of +ve response for agglutination assay were pooled and precipitated out at 45% saturation of ammonium sulphate at 4°C for 1 - 2 hrs. The precipitate was separated after centrifugation at 5,000 rpm for 20 min. at 4°C and dissolved in 0.85% NaCl. The agglutinin solution was dialysed against distilled water. Polyacrylamide gel electrophoresis (Native and SDS - PAGE), agglutination assays, inhibition studies, antibacterial studies and physcio- chemical characterization was carried out using the dialysed agglutinin solution. The concentration of purified agglutinin was determined by the method of Lowry *et al.* (1951).

3.2.9.2. Electrophoresis - Native PAGE

Purity of agglutinin was determined by Native PAGE in 5% polyacrylamide gel according to the method of Hames (1990).

Reagents

1. Acrylamide stock solution - 30%

   Acrylamide and Bisacrylamide (30 : 0.08 g) dissolved in 50 ml and made upto 100 ml using double distilled water.

2. Separating gel buffer (1.5M, pH 8.8, Tris)

   18.16 g Tris dissolved in 50 ml of distilled water and adjusted to pH 8.8 with HCl.

3. Stacking gel buffer (Tris - Glycine buffer, pH 6.8)

   6.056 g Tris dissolved in 25 ml of distilled water, adjusted to pH 6.8 with HCl.

4. Running gel buffer (Tris - Glycine buffer, pH 8.3)

   30 g of Tris and 14.4 g Glycine dissolved in 500 ml and made upto 1,000 ml with distilled water.

5. Staining solution

   250 g of Coomassie brilliant blue R-250 was dissolved in 500 ml of Methanol : Acetic acid : H_2O (145 : 10 : 45) mixture.

6. Destaining solution

   Methanol : Acetic acid : H_2O in the ratio 145 : 10 : 45 was used as destainer.

Procedure

Electrophoresis was carried out in 20 cm slab gel of 0.7 mm thickness with 2.5 and 5% polyacrylamide for stacking and resolving gel, respectively. Agglutinin fraction (7th) collected from Sephadex G - 200 column was loaded in the gel and electrophoresed at constant current (30 mA) at 10°C for 5 - 6 hrs

in an electrophoresis buffer containing Tris and glycine, pH 8.3. Gels were fixed in 10% TCA for 30 min., stained in 0.25% Coomassie brilliant blue R-250 (30 min.) and destained. The bands were visualised using a white light transilluminator and stored in 7% acetic acid at 4°C.

3.2.9.3. Electroelution

Electroelution was performed in Electroeluter apparatus (Bio-Rad, USA) according to the method of Girardie *et al.* (1987). After electrophoresis (Native PAGE) Coomassie brilliant blue stained band corresponding to agglutinin protein was cut from the gel, soaked in distilled water, and placed on the cathodic side of the apparatus in 400mM $NH_4 HCO_3$, 2% SDS. Elution was performed at a constant voltage (70V) at room temperature for 9 hrs in elution buffer (50mM $NH_4 HCO_3$, pH 7.8, 0.1% SDS). Electroeluted agglutinin was concentrated in the anodic chamber and their concentration was determined according to the method of Lowry *et al.* (1951).

3.2.9.4. SDS – PAGE

The purity, polypeptide subunits and molecular weight of the purified agglutinin were determined by sodium dodecyl sulfate (SDS) - PAGE according to the method of Laemmli (1970).

Reagents

1. Acrylamide stock solution - 30%
   Acrylamide and Bisacrylamide (30 : 0.08 g) dissolved in 50 ml and made upto 100 ml using double distilled water.

2. Separating gel buffer (1.5M, pH 8.8, Tris)

18.16 g Tris dissolved in 50 ml of distilled water and adjusted to pH 8.8 with HCl.

3. Stacking gel buffer (Tris - Glycine buffer, pH 6.8)

6.056 g Tris dissolved in 25 ml of distilled water, adjusted to pH 6.8 with HCl.

4. Running gel buffer (pH 8.3)

Tris (15.1 g), glycine (72 g) and SDS (5 g) were dissolved in 50 ml distilled water and made upto 1000 ml.

5. Staining solution

250 g of Coomassie brilliant blue was dissolved in 500 ml of Methanol : Acetic acid : H_2O (145 : 10 : 45) mixture.

6. Destaining solution

Methanol : Acetic acid : H2O in the ratio 145 : 10 : 45 was used as destainer.

7. Sample solubilizing buffer

SDS	- 1 g
Stacking gel buffer (pH 6.8)	- 2.5 ml
β - mercaptoethanol blue	- 1 ml
Sucrose	- 3 g
Bromophenol blue	- 1 pinch

made upto 10 ml using distilled water and heated for 30 min. at 100°C.

Procedure

Electrophoresis was carried out in 20 cm slab gel of 0.7 mm thickness with 2.5% and 7.5% sodium dodecyl sulfate (SDS) - PAGE for stacking and resolving gel, respectively in the presence of 0.5% SDS dissolved in 1.5 M Tris - HCl, pH 8.8. Electroeluted agglutinin and molecular weight markers of wide range (6,500 - 205,000) were boiled at 100°C for 3 min. with sample solubilizing buffer. The denatured agglutinin and molecular weight standards were loaded in separate wells and electrophoresed at constant current (30 mA) at 30°C in an electrophoresis running buffer, pH 8.3. The gels were fixed in 10% TCA for 30 min., stained in 0.25% Coomassie brilliant blue (30 min.) and destained. Molecular mass of the protein bands was determined by the method of Weber and Osborn (1969). The presence of a disulfide bond between polypeptide subunit was also investigated based on the reduction with 2 - mercaptoethanol. The gels were stored in 7% acetic acid.

3.2.10. Physico - chemical characterization

Physico – chemical characterization was done according to the method of Miller *et al.* (1972).

3.2.10.1. Physical test for stability

a. Temperature stability

Effect of lowered temperature was studied by

(1) Storing purified agglutinin at -70°C and testing agglutinin activity after 1, 2, 3, 4, 5 and 6 months.

(2) Measuring agglutination activity of agglutinin after repeated freezing and thawing.

(3) Heat stability was studied by exposing agglutinin to temperatures ranging 10°C to 100°C for 20-30 min. and was rapidly cooled by plunging the tube into ice bath. Any formed precipitate were removed by centrifuging the fluid at 5,000 rpm for 20 min., the withdrawn supernatant was tested for agglutination assay.

b. pH stability

P. indicus purified agglutinin was pooled and placed into a number of test tubes and to various pH levels (pH 1-14). The samples were incubated at 37°C for 24 hrs, centrifuged at 5,000 rpm/ 20 min. and the supernatant was tested for agglutination assay.

3.2.10.2. Chemical test for stability

Precooled chloroform, and agglutinin were gently mixed (3:1) for 3 min. The chloroform phase containing precipitate was separated from the aqueous phase and the latter centrifuged at 5,000 rpm for 3 - 4 min. to remove excess precipitate. The serum phase was dialysed at 4°C for 24 hrs against PBS (pH 8.0). Toluene and Xylene extractions were carried out in the same manner (5:1). Trichloroacetic acid (TCA) extraction was performed by

incubating 0.5 ml serum in a test tube containing 0.5 ml of 20% TCA for 2 hrs at 4°C. The precipitate was removed by centrifuging at 3,000 rpm/ 20 min., and the supernatant was tested for agglutination activity.

3.2.10.3. Trypsin digestion

Trypsin 0.1 ml (5 mg/ml, Sigma) was incubated with purified agglutinin (0.3 ml) in 0.1 ml of 0.2M phosphate buffered saline (PBS), pH 8.0. The mixture was incubated at 37°C for 24 hrs. The reaction was terminated by boiling the mixture at 100°C for 10 min. The digestion product was centrifuged to remove the precipitate at 4,000 rpm for 15 min. and the supernatant was used for agglutination assay (Lee *et al.*, 1997).

3.2.10.4. Dialysis

The purified agglutinin was dialysed against different solutions like distilled water, 2% NaCl, PBS (pH 7.5), Tris - HCl (pH 7.5) etc. at 55°C. The fluid was replaced after the first 3 hours and dialysis continued for 24 hrs. The dialysed sample was used for agglutination assay.

3.2.10.5. Inhibition assay by simple sugars

Purified agglutinin was diluted with different sugar solutions containing 10% ($^{W}/_{V}$) sugar in 0.85% NaCl ($^{W}/_{V}$) and same volume of bacterial suspensions. The mixture was allowed to incubate at 37°C for 24 hrs and read the titer value.

3.2.11. Determination of Molecular weight and Biological activities of Purified agglutinin.

Molecular weight of purified agglutinin was determined using Sephadex G - 200 column chromatography and electrophoresis. Native PAGE (5%) and SDS - PAGE (7.5%) were employed to ascertain the molecular weight of the agglutinin using standard molecular weight markers of wide range (6,500 – 205,000), according to the method of Weber and Osborn (1969).

Purified agglutinin was subjected to bacterial assay against *Vibrio* v-5 strain and RBC assay against different RBC's to check the properties like agglutination and hemolysis (see section 3.2.7). Inhibition zone test was performed to determine antibacterial property (see section 3.2.8).

3.3. RESULTS

3.3.1. Identification of hemocytes

Three different types of hemocytes have been recognized by the size, shape and functional role during clotting mechanism. They are hyaline cells, dense granulocytes and semi- dense granulocytes (Plate 3.1a, b & c).

3.3.2. Clotting mechanism

The non- citrated hemolymph was allowed to form firm clot (Plate 3.2). A phenomenon occurred *in vitro* (observed under Phase contrast microscope) which resulted in the formation of extensive network of protoplasmic strands interconnecting with hemocytes. The process, seen *in vitro*, was first observed 7 min. to an hour after a preparation was made and proceeded slowly for few hours. Usually about an hour after, activity was first observed as the network become quite extensive, dense mass of closely interwoven plasma fibres and fibroblast like structures connecting hemocytes, shrivelled hyaline cells and agglutinated granulocyte with a central core of plasma. The first phase was the migration of granulocytes to the periphery to agglutinate. Concurrent event was anastomosis of the distal portion to form the network. Rupture of hyaline cells follows immediately before network formation. The protoplasmic stranding occurred in the immediate proximity of the granulocytes and even appeared to be attached to intact large protoplasmic strands. Anastomosis with other strands appeared to rejuvenate or increase the vigour of the strands and promote continued formation.

3.3.3. Assay of agglutinin against bacterial cells

As shown in Table 3.1, the plasma, sera and clottable solution (agglutinin) from *P. indicus* hemolymph were found to contain naturally occurring agglutinins against many bacterial strains. *Vibrio* v-5 strain isolated from diseased prawn gut were agglutinated strongly by *P. indicus* plasma (titer

64), serum (64) and clottable solution (agglutinin) (titer 128). Where as two strains of *Pseudomonas* pf MS - 47 and pf MW - 51 were weakly agglutinated (titer 16, 16 and 32). Among the different bacterial strains *Vibrio* v-5 strain was selected as the target cells for performing agglutination assay for the rest of the experiments (Plates 3.6, 3.7 & 3.8).

3.3.4. Assay of agglutinin against RBC

P. indicus plasma, serum and clottable solution (agglutinin) agglutinated with red blood cells from human, rabbit and rat blood with high titers (128, 128 and 256) (Table 3.1) (Plates 3.9, 3.10 & 3.11).

3.3.5. Minimum protein required for agglutination

Minimum protein required for agglutination using plasma, serum and clottable solution (agglutinin) against v-5 strain and RBC varies between 1.28-0.8 μg/ml and 1.20-0.6 μg/ml (Table 3.2). Minimum concentration of 0.8 and 0.6μg/ml is required for agglutination while using clottable solution, agglutinin.

Table 3.1. Agglutination assay of plasma, serum and clottable solution (agglutinin) of *P. indicus* (N = 10) against bacteria and RBC.

Test cells	Source	Agglutinin titer [a]		Clottable solution (agglutinin)
		Plasma	Serum	
Vibrio sp. (v-1)	Diseased prawn gut	64	64	128
" (v-2)	"	64	64	"
" (v-3)	"	64	64	"
" (v-4)	"	64	64	"
" (v-5)	"	64	128	256
" VEW - 27	Estuarine water	64	64	128
Pseudomonas fluorescence				
pf MS - 51	Marine sediments	16	16	32
pf MW - 47	Marine water	16	16	32
Sterile water	Sea	na	na	na
Red blood cells				
Human RBC	Human	128	128	256
Rabbit RBC	Rabbit	128	128	256
Rat RBC	Rat	128	128	256
Water		na	na	na

[a] Titer value 2^n (reciprocal of serum dilution); na - No agglutination

Table 3.2. Minimum protein content and agglutination titer values of *P. indicus* plasma, serum, clottable solution (agglutinin) for *Vibrio* (v-5) and Rat RBC.

Test cells	Titer values (2^n)			Minimum concentration of protein required for agglutination (μg/ml) *		
	Plasma	Serum	Clottable agglutinin	Plasma	Serum	Clottable agglutinin
Vibrio sp. (v-5)	64	128	256	1.28 ± 0.4	1.04 ± 0.7	0.8 ± 0.3
Rat RBC	64	128	256	1.20 ± 0.4	1.02 ± 0.8	0.6 ± 0.91

* triplicate analysis were performed.

3.3.6. Antibacterial activity

Plate 3.14 demonstrates the presence of antibacterial property of agglutinin against *Vibrio* v-5 strain. The zone of clearance (Z) in the bacterial lawn was appeared around the well containing *P. indicus* plasma, serum and agglutinin, but not around the control well (C) inoculated with saline alone. The bacterial strain in the lawn was isolated from diseased prawn gut.

3.3.7. Isolation of agglutinin and determination of biological properties

The crude hemolymph electropherogram showed 16 - 17 bands (Plate 3.3). Serum proteins were separated by Sephadex G - 200 column chromatography. Only the 7th fraction (Fig. 3.1) eluted from the column showed agglutinating, hemolytic and antibacterial properties against *Vibrio* v-5 strain and RBC (Plates 3.8, 3.11 & 3.14). The purity was tested in Native PAGE (Plate 3.4) and subunits resolved in SDS - PAGE (Plate 3.5).

3.3.8. Physical stability

The influence of temperature on the agglutinin activity against the *Vibrio* v-5 strain was shown in Fig. 3.2a. Agglutinin was stable at temperature ranging between 30 - 40°C but completely inactivated at 85°C.

3.3.9. pH

The activity of *P. indicus* agglutinin was stable at pH values ranging from 7 - 8, 10 - 11 and was reduced but never completely inactivated at extreme acid or alkaline values (Fig. 3.2b)

3.3.10. Chemical stability, Trypsin digestion and Dialysis

Results of experiments carried out to determine the stability of purified agglutinin of P. Indicus are listed in Table 3.3. Chloroform, TCA, Phenol and Trypsin digestion completely inactivated the agglutinin. Naturally occurring agglutinin of *P. indicus* serum was stable to dialysis, storage at low temperature, repeated freezing and thawing. The above result indicates proteinaceous nature of agglutinin in the hemolymph.

3.3.11. Inhibition assay with simple sugars

Table 3.4 shows the effect of sugars on agglutination activity against *Vibrio* v-5 strain. Glucose, Galactose, Lactose, Maltose, Cellobiose and Mannose stimulate agglutination with titer value 64 and 128. Xylose, Raffinose and Trehalose were inhibitory (titer 0) (Plate 3.12). L - Arabinose and D - glucosamine were found to be partially stimulate agglutination (titer 16). $CaCl_2$ solution that releases Ca2+ ions also found to stimulate

agglutination with 128 titer values (Plate 3.13). But it was found that Ca^{2+} supplementation was not necessary for agglutination.

3.3.12. Biological activities and Molecular weight of purified agglutinin

The plasma, serum, clottable protein and purified agglutinin (7th fraction from column) showed agglutinating, hemolytic and antibacterial properties (Plates 3.6 to 3.14). Based on these studies natural agglutinin in the plasma, serum and clottable solution is inferred to be a protein with antigenecity, agglutinating, hemolytic and antibacterial properties.

The stability of the agglutinin under dialysis indicated the presence of a macromolecule in the serum. Separation of agglutinin rich fraction from Sephadex G - 200 column and its band position in the native PAGE indicated that agglutinin's apparent molecular weight (MW) was ~ 181,000 Dalton (Plate 3.4) The SDS - PAGE polypeptide profile of agglutinin enriched fraction gave two bands of 97 kDa and 84 kDa (Plate 3.5) Based on these study, natural agglutinin of *P. indicus* is a high MW protein, dimer of approx. 181,000 dalton (97 kDa and 84 kDa) with antigenecity, agglutinating and antibacterial properties.

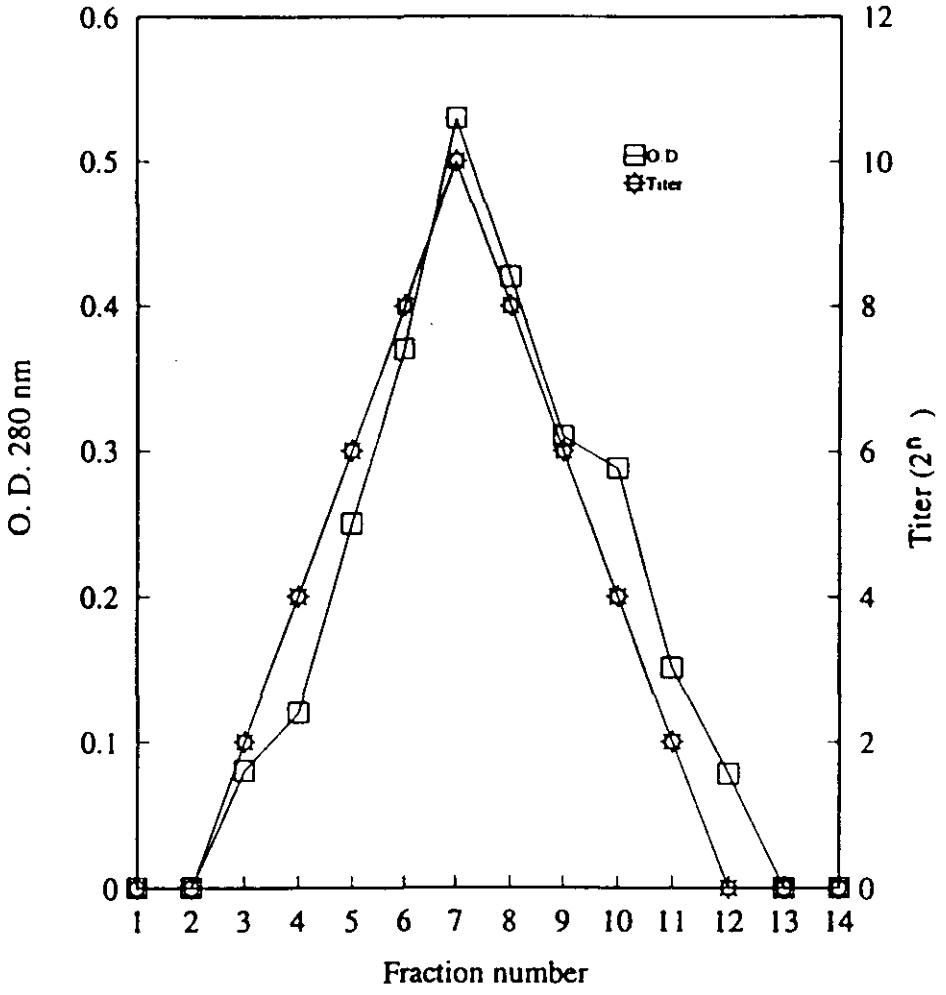
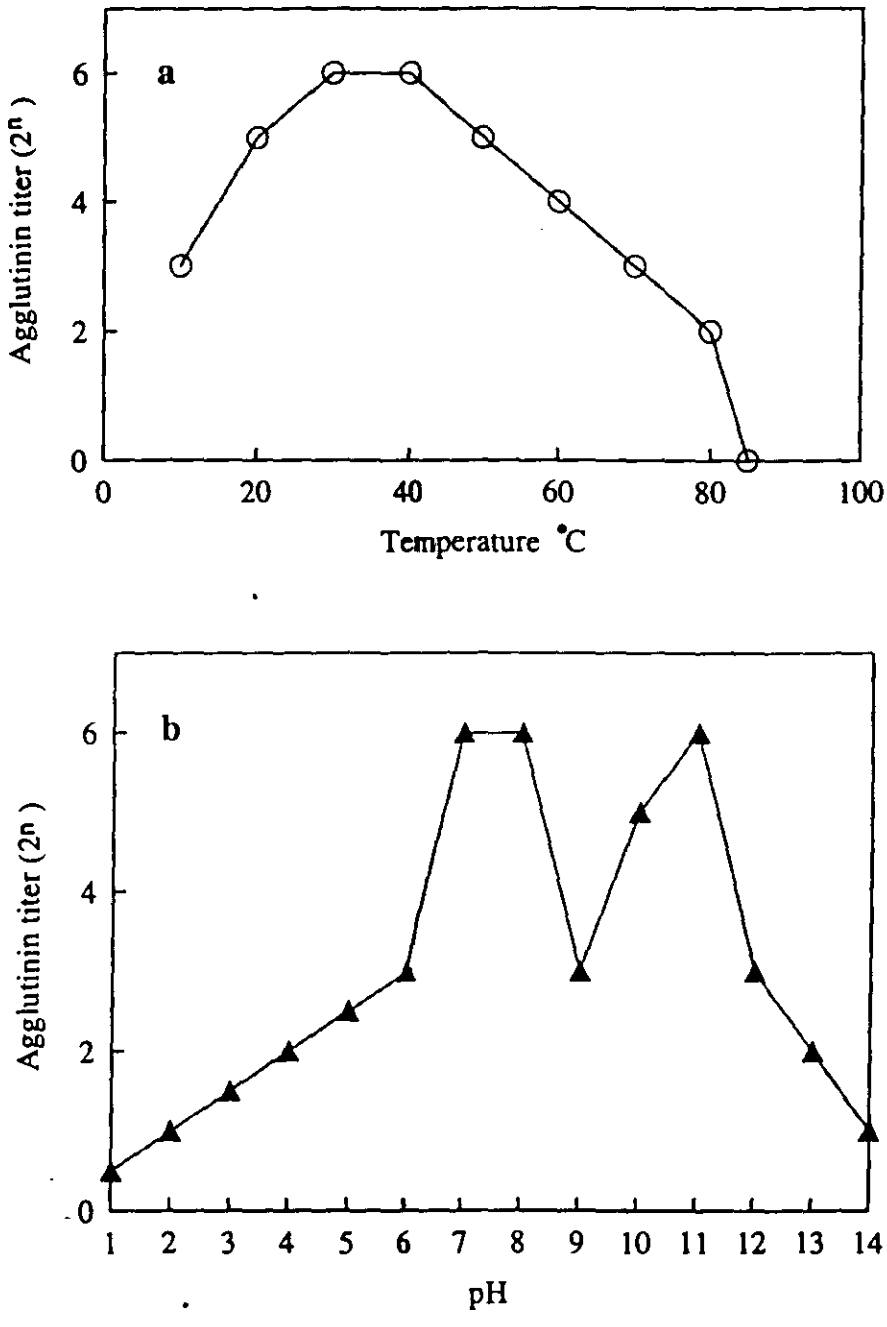


Fig. 3.1. The elution pattern of the *P. indicus* agglutinin through Sephadex G - 200 column. The serum after the removal of hemocytes was applied to the column. The elution buffer was phosphate buffered saline, pH 8.0 and the column size was 1.6 X 15 cm. Fractions of 1 ml were collected. Symbols used are: optical density at 280 nm, titers of agglutinin (2^n) against *vibrio* v-5 strain.



Figs. 3.2a & b. Influence of temperature and pH on agglutinin activity *in vitro* against *Vibrio* v-5 strain in the serum of *P. indicus*.

Table 3.3. Determination of physico – chemical stability and nature of the purified natural agglutinin in the hemolymph of *P. indicus*.

Experimental procedure	Complete inactivation of agglutinin	Partial inactivation	Agglutinin activity unaffected	Titer values against (v-5) bacteria *
Physical treatments				
Prolonged freezing (6 months)			+	64
Repeated freezing and thawing			+	64
Temperature –30 -40°C			+	64
Temperature 85°C	+			0
pH 1-5		+		32
pH 7-8			+	64
pH 9-12			+	64
Dialysis				
0.01M NaCl			+	64
Tris- HCl buffer			+	64
PBS			+	64
Chemical treatment				
Phenol	+			0
Chloroform	+			0
Toluene	+			0
Enzyme				
Trypsin	+			0

* titer 2^n

Table 3.4. Effect of sugars & $CaCl_2$ on agglutination activity of hemolymph agglutinin against *Vibrio* v-5 strain.

Sugars	Titer value [a]	Effect	Bacterial strain used
Control 0.85% NaCl	64	+ +	*Vibrio* v-5
Glucose	64	+ +	"
Galactose	64	+ +	"
Lactose	64	+ +	"
Maltose	128	+ + +	"
Cellobiose	64	+ +	"
Mannose	64	+ + +	"
Xylose	0	-	"
Raffinose	0	-	"
Trehalose	0	-	"
L - Arabinose	16	+	"
D - glucosamine	16	+ +	"
$CaCl_2$	128	+ + +	"

[a] Titer value (2^n); + + + Strong agglutination - stimulatory;
+ + Agglutination -stimulatory; + Partial agglutination; - Inhibitory.

Plate 3.1a. Phase contrast micrograph of Hyaline cell from the hemolymph of *P. indicus*. Note the characteristic spherical shape, centrally dispersed nucleus and absence of granules. x 400

b. Phase contrast micrograph of Dense granulocyte in the hemolymph of *P.indicus*. Note the oval shape, concentric kidney shaped nucleus, pseudopodia and dense granules. x 250

c. Phase contrast micrograph of Semi -dense granulocyte from the hemolymph of *P. indicus*. Note the ovoid shape, eccentric nucleus and semi -dense granules. x 250

Plate 3.2. Phase contrast micrograph showing clotting mechanism in the hemolymph of *P. indicus*. Note the densely coagulated hemolymph (C), fibrous network (F) and disintegrated hemolymph (H). x 650

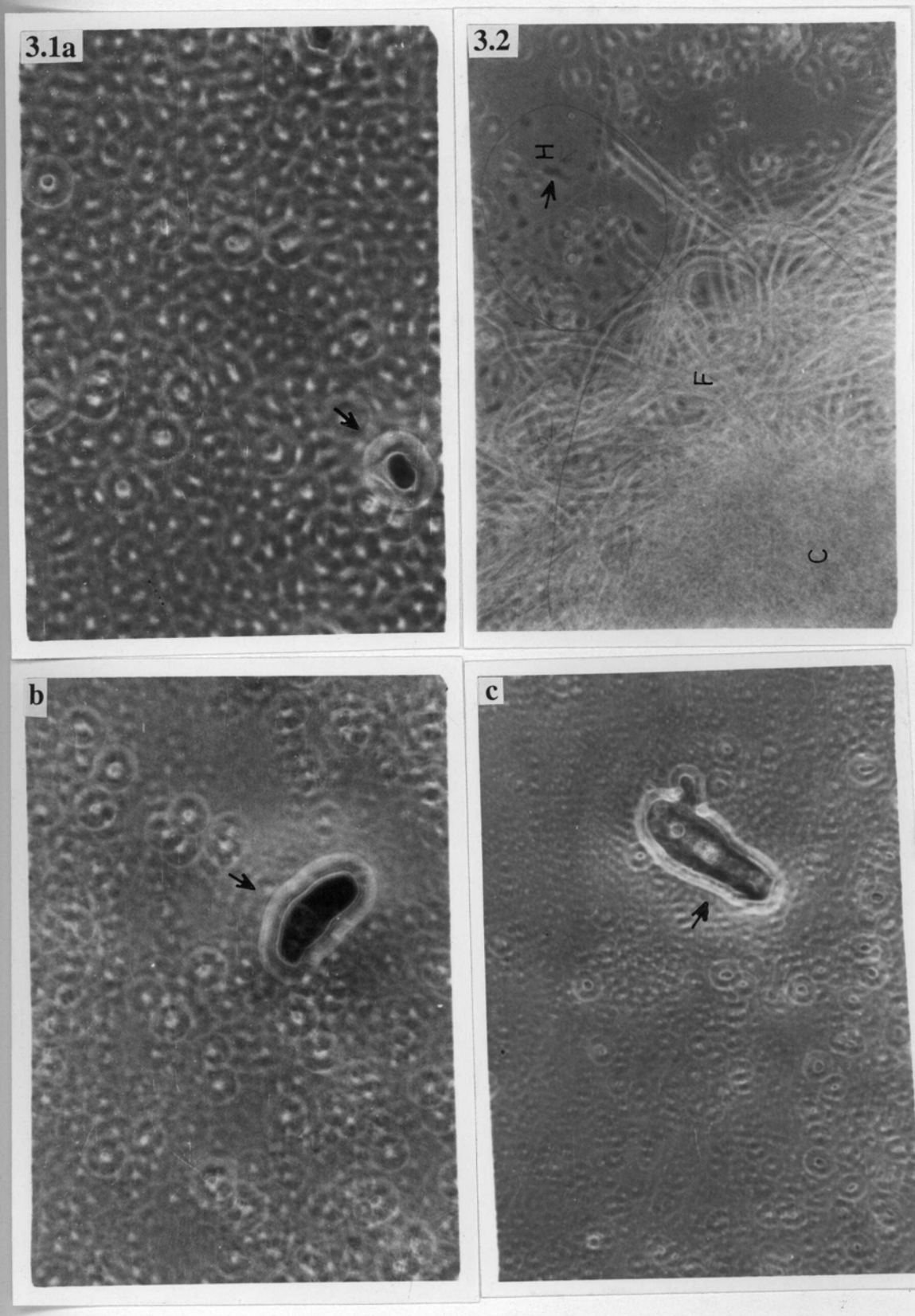
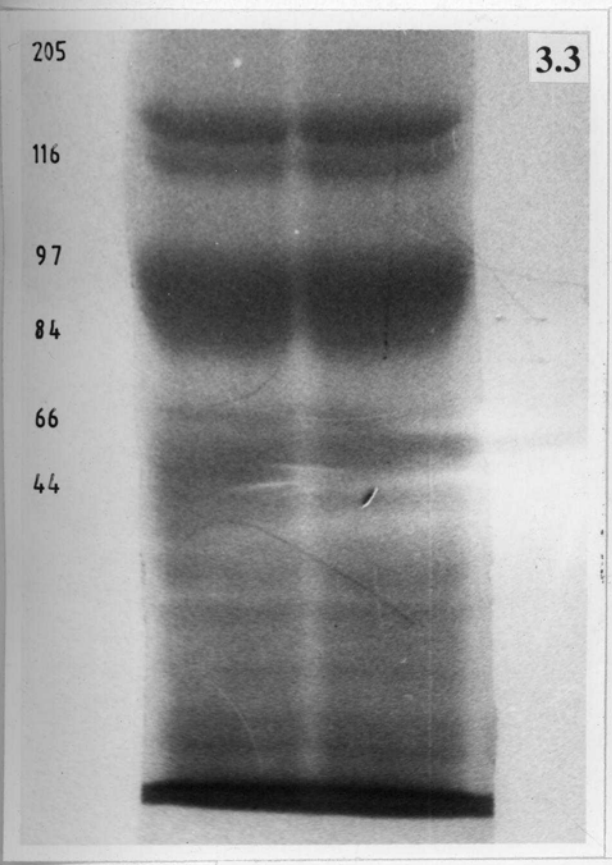
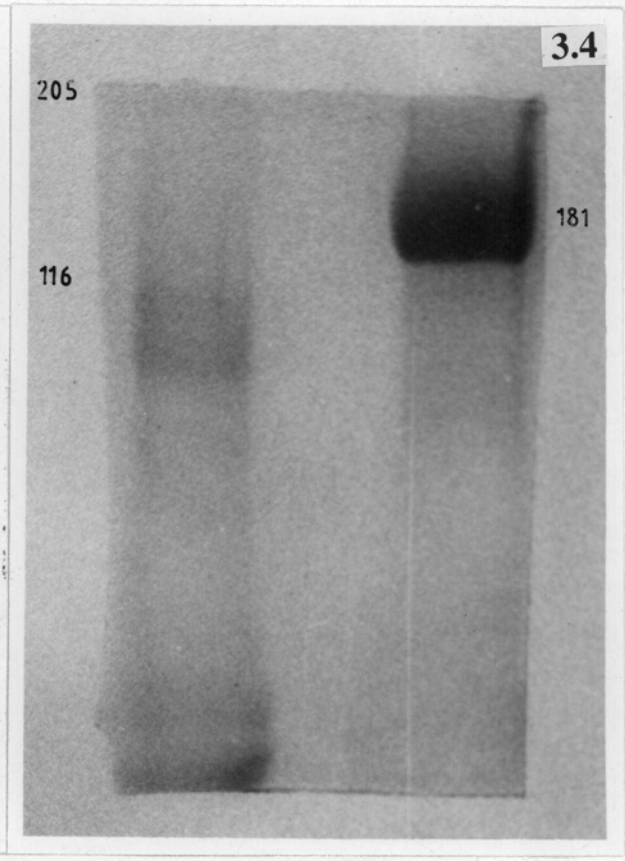

3.1a
3.2
H
E
C
b
c

Plate 3.3. Electropherogram of *P.indicus* crude hemolymph on polyacrylamide gradient gel (5%). Position of molecular weight markers are indicated at left.

Plate 3.4. Native PAGE (5%) electrophoresis of eluted agglutinin (7th fraction) from Sephadex G - 200 column chromatography, stained with Coomassie brilliant blue. Position of MW markers are indicated at left.

Plate 3.5. SDS - PAGE (7.5%) electrophoresis of purified electroeluted agglutinin from Native PAGE. Lane 1 & 3, agglutinin subunit A & B (97 kDa & 84 kDa). Lane 2 & 5, MW marker (Sigma) 97 kDa and 84 kDa.


205
116
97
84
66
44
3.3


3.4
205
116
181

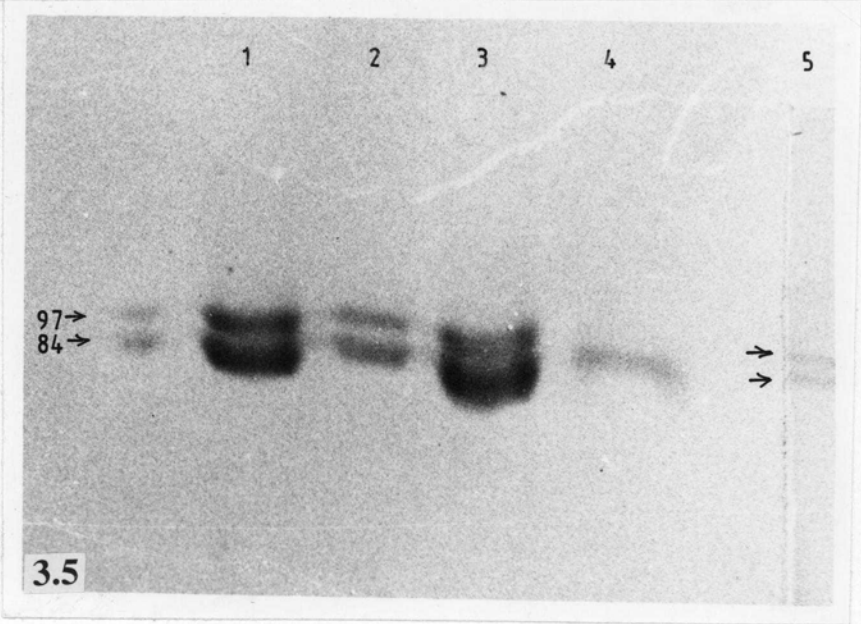
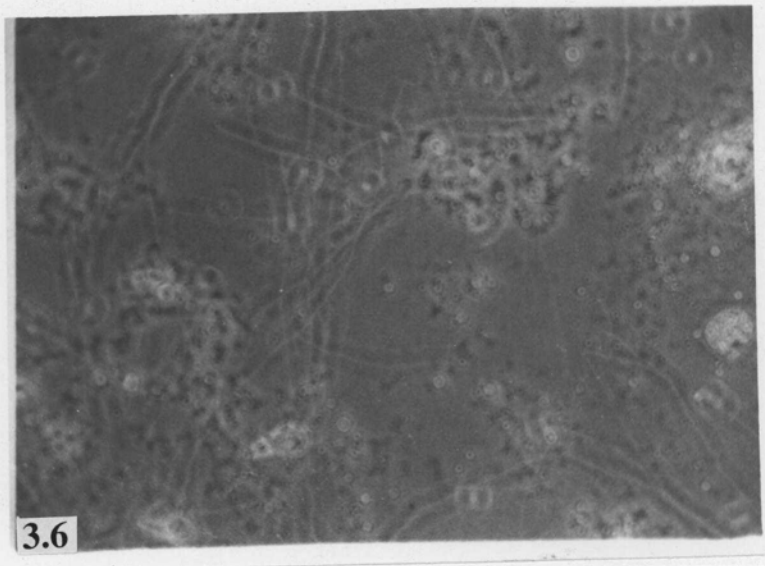

1
2
3
4
5
97
84
3.5

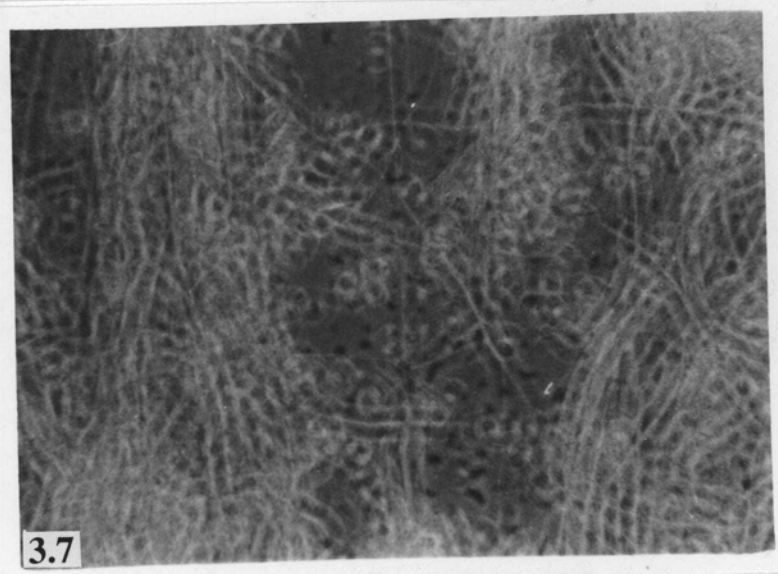
Plate 3.6. Phase contrast micrograph of agglutination occurred between *P. indicus* plasma and *Vibrio* v-5 strain. x 650

Plate 3.7. Phase contrast micrograph of agglutination occurred between *P. indicus* serum and *Vibrio* v-5 strain. x 650

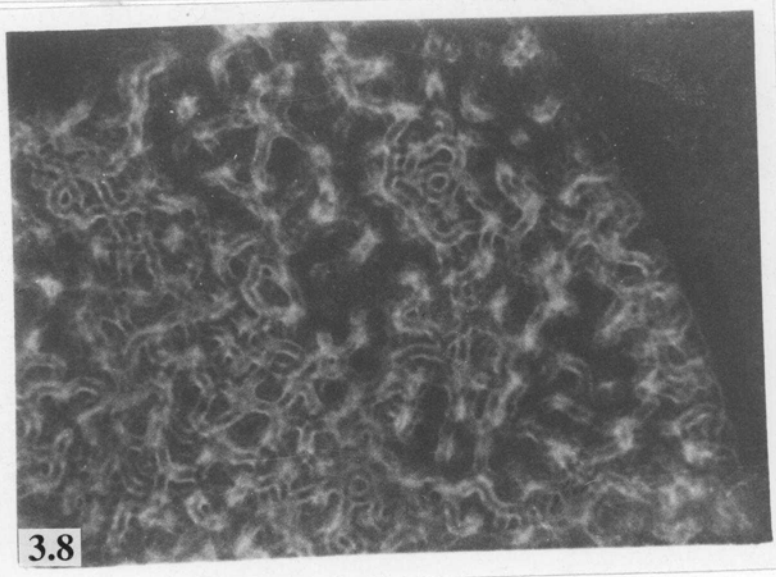
Plate 3.8. Phase contrast micrograph of agglutination occurred between *P. indicus* agglutinin and *Vibrio* v-5 strain. x 1250



3.6



3.7

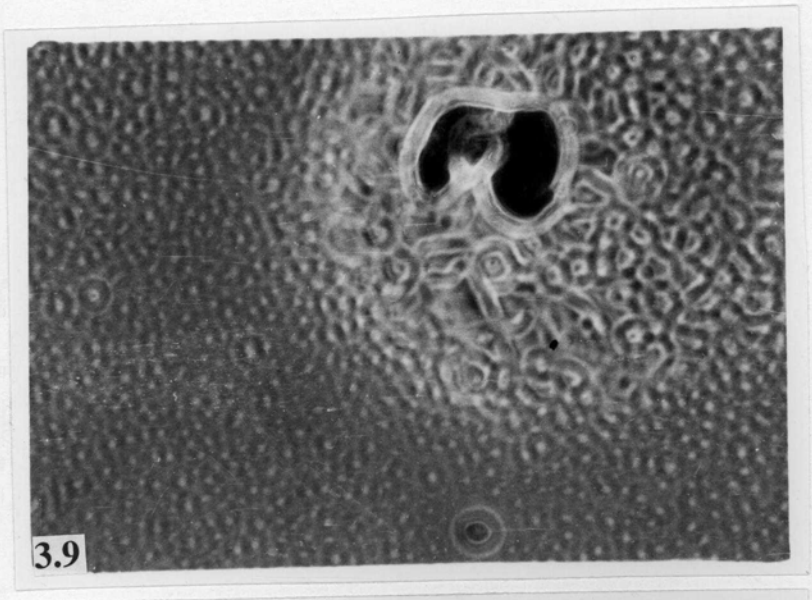


3.8

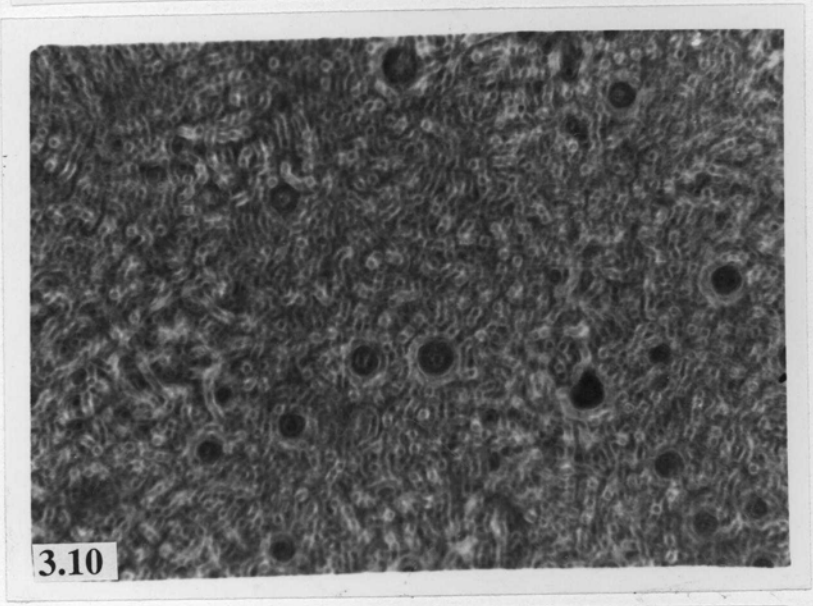
Plate 3.9. Phase contrast micrograph showing hemolysis and crosslinking between erythrocytes and plasma of *P.indicus.* x 650

Plate 3.10. Phase contrast micrograph showing hemolysis and crosslinking between erythrocytes and serum of *P.indicus.* x 650

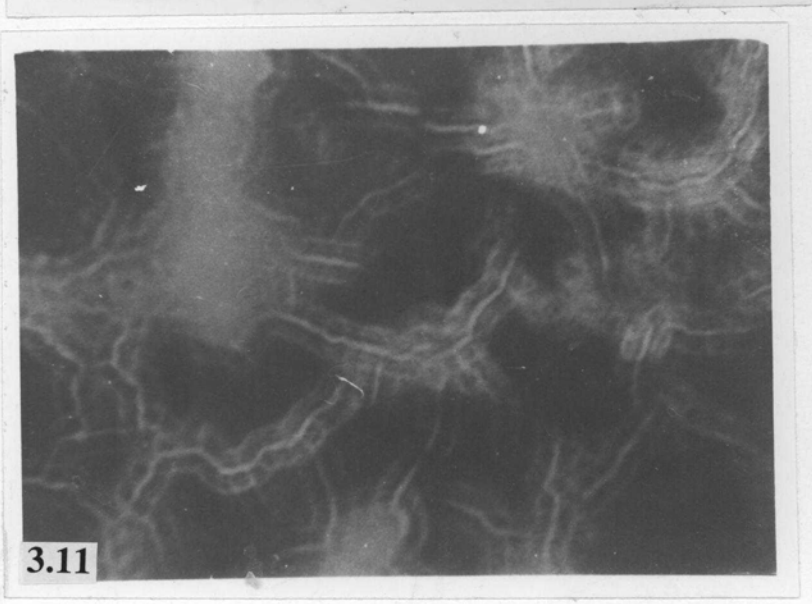
Plate 3.11. Phase contrast micrograph showing hemolysis and crosslinking between erythrocytes and agglutinin of *P.indicus.* x 650



3.9



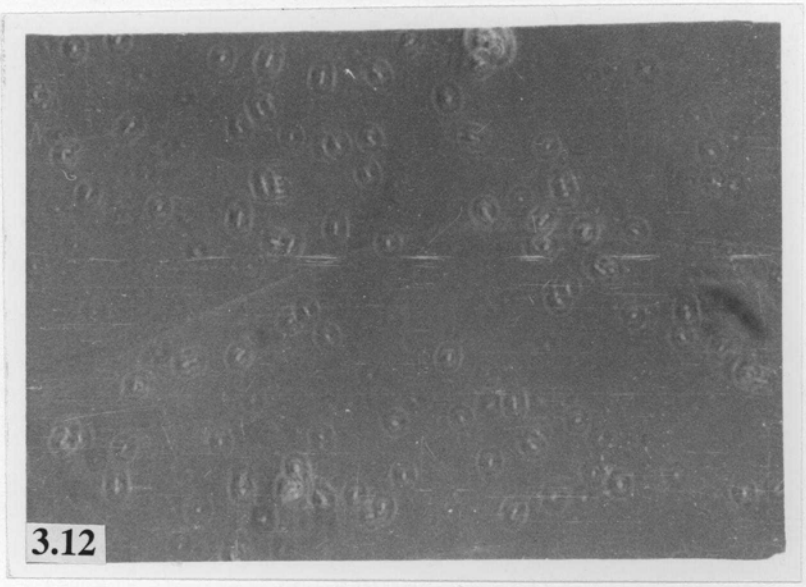
3.10



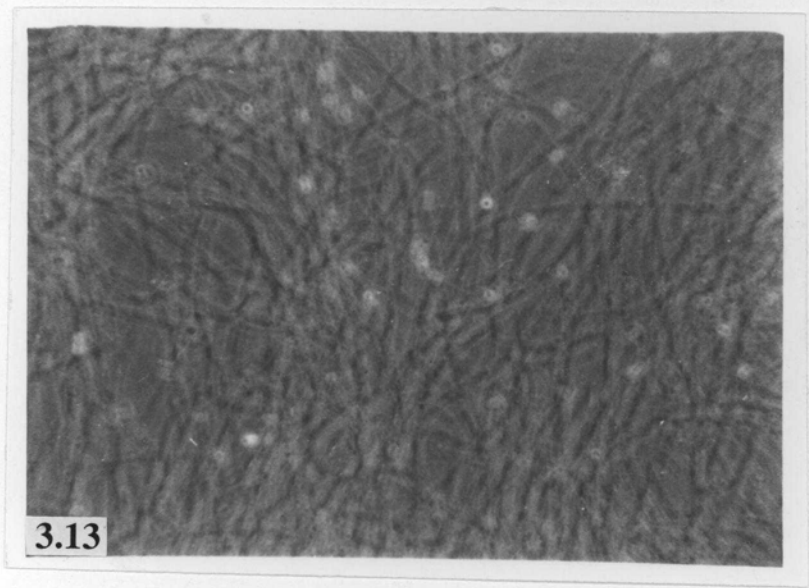
3.11

Plate 3.12. Phase contrast micrograph showing the inhibitory effect of Xylose on agglutination process against *Vibrio* v-5 strain in the hemolymph of *P. indicus.* x 250

Plate 3.13. Phase contrast micrograph showing stimulatory effect of Ca^{2+} on agglutination process against *Vibrio* v-5 strain in the hemolymph of *P. indicus.* x 650

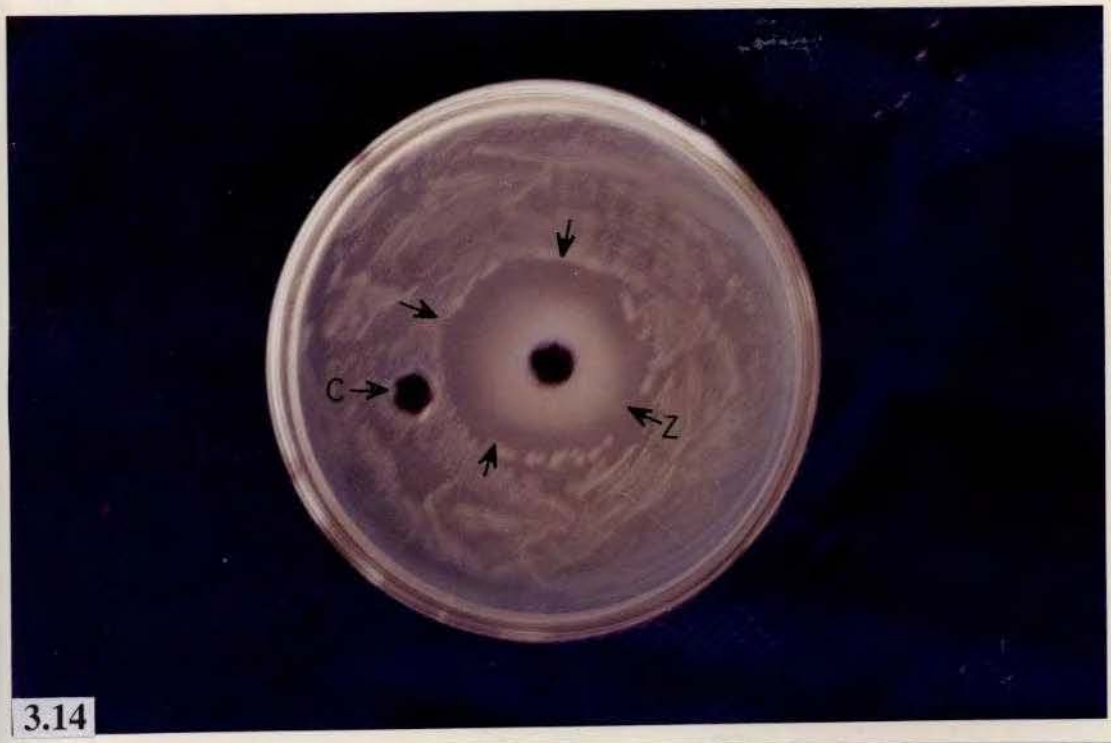


3.12



3.13

Plate 3.14. Demonstration of the presence of a natural antibacterial agglutinin in the hemolymph of *P. indicus*. Note the zone of clearance (**Z**) in the bacterial lawn produced around the well containing agglutinin, but not around the control well (**C**) inoculated with saline alone. The bacterial lawn was made of bacteria isolated from diseased prawn gut.



3.14

3.4. DISCUSSION

Many invertebrates, including the molluscs and crustaceans are now being extensively farmed and an understanding of their host defences would assist in avoiding and overcoming disastrous outbreaks of disease which may occur under stressful high density conditions of commercial cultures. All invertebrates recognize foreigness and exhibit phagocytotic and encapsulation responses as well as producing humoral immune factors. The humoral immune system acts synergistically with cellular immune system. The control of these processes is however, much more complex and depends upon intimate interactions between the humoral and cellular components and on co-operative events between different kinds of blood cells as if in concert.

In view of growing interest in hemocyte function which links morphology with physiology, three different types of hemocytes have been identified. Hyaline cells, which are circular in shape with a central nucleus comprises 50 to 60% of the circulating hemocytes in the hemolymph. The second category involve dense granulocyte with large number of dense granules, kidney shaped nucleus and pseudopodia. The third category, semi-dense granulocyte - an intermediate form. This classification was found to be in agreement with the scheme of hemocyte classification in prawn, *Sicyonia ingentis* (Hose and Martin, 1989). Previous reports of Martin *et al.* (1987) based primarily on cell morphology and staining techniques using light microscopic studies, divided hemocytes into granulocytes, semi- granulocytes

and hyaline cells. Where as this scheme works well for some species, difficulties arise when comparing cell types in different crustaceans. Studies on crustacean hemocyte functions become more sophisticated, it is essential to correlate information on physiology with cell morphology and incorporate this knowledge into classification scheme.

Functionally, hyaline cells are involved in clotting mechanism. Plate 3.2 shows shrivelled hyaline cells after clotting process in between mesh works. On the other hand dense granulocytes and semi- dense granulocytes are involved in phagocytosis of foreign particles. In addition, these granulocytes are reported to have enzymes such as phosphatase, esterases, glucoronidase and hyaluronidase that apparently degrade the microorganisms within the phagosomes (Hose *et al.*, 1987). The distinction of granulocytes into dense granulocytes and semi- dense granulocytes was in good agreement with the work of Martin and Graves (1985) in shrimp, *Sicyonia ingentis* and *Penaeus californiensis*. The hyaline cells of *P. indicus* which assist in coagulation are comparable to the hemostatic cells and granulocytes as phagocytic cells. Hemocytes are concerned with various physiological mechanisms of crustaceans. Alteration in the external environment alters the morphological and functional role of hemocytes. Change in pH and temperature alters the functional aspects of hemocytes (Ravindranath, 1975). Role of hemocytes in coagulation and phagocytosis will provide a chance to undergo further studies such as clotting mechanism and humoral defense mechanism of *P. indicus*.

Coagulation, a process occurred *in vitro* which resulted in the formation of extensive network of protoplasmic strands interconnecting with hemocytes. Anastomoses with other strands appeared to rejuvenate or increase the vigour of the strands and promotes continued formation. The shrivelled hyaline cells and adhered granulocytes were noticed among the crosslinks (Plate 3.2). The clotting mechanism observed in *P. indicus* was analogous to the general clotting mechanism of crustaceans (Durliat, 1985). *P. indicus* clotting process can be depicted as type B according to the broad classification of Tait (1911). In Macrura, plasma clotting results from the conversion of a plasma coagulogen into a gel which is crosslinked covalently by the action of a cellular transglutaminase (Lorand, 1972; Durliat, 1985). Different traumatism such as pathogens, change in external conditions and disease can damage or prevent these processes. During diseased conditions the clottable factors decreases to half and clotting mechanism was impaired in lobsters (Durliat and Vranckx, 1983b). According to Durliat (1985), a plasma clotting protein was involved for the clotting process and its soluble polymers converted to covalently crosslinked polymers of coagulin by Ca^{2+} dependent transglutaminase. Hence in Decapoda, clotting factors are found in both cell - free plasma and hemocyte compartments. Minnick *et al.* (1986) reported that agglutinin function as activator of hemocyte coagulation. The plasmatic clottable factors and heteroagglutinins are reported to perform clots in lobster, *Panulirus interruptus* (Tyler and Scheer, 1945). In majority of cases, invertebrate agglutinins are synthesized by blood cells (Coombe *et al.*, 1984).

Resistance in the form of bactericidal, lytic or agglutinating properties seem to be conferred on the hemolymph of invertebrates by the release of materials such as bactericidins, lysins and agglutinins from the hemocytes (Miller and Ratcliffe, 1994). In some invertebrates exposure to foreign particles may account for increased resistance to certain pathogens (McKay and Jenkin, 1969) where as few it impairs the defense system (Durliat and Vranckx, 1983b).

A heat - labile substance agglutinin from the hemolymph of *P. indicus* agglutinated in varying degrees to a broad range of marine bacteria and erythrocytes. This observation strengthen the role of agglutinin as non - self recognition, agglutinating and hemolytic molecule in the hemolymph of *P. indicus*. Bacterial strain, *Vibrio* sp. (v-5) isolated from diseased prawn gut exhibited maximum agglutinating property with agglutinin and less agglutination with *Pseudomonas* sp. *P. indicus* agglutinin agglutinated and lysed the erythrocytes of human, rabbit and rat. The range of reactivity and titer values of *P. indicus* agglutinin varies from the reports of Vasta *et al.* (1984) in oyster, Fisher and Dinuzzo (1991) in marine mollusc and Tripp (1992) in Hard clam. Comparison of plasma, serum and clottable protein for agglutinating activity against *Vibrio* sp. (v-5) strain and RBC resulted in maximum titer values of 256 with clottable protein (Table 3.1). From the observation it was confirmed that clottable protein agglutinin was dissolved in the hemolymph of *P. indicus*. Agglutinin functions as clottable protein in the hemolymph of *Astacus fluvialis* (Durliat and Vranckx, 1976), in spiny lobster

(Tyler and Scheer, 1945) and in Ascidia (Fuke and Sugai, 1972). The natural agglutinin in the hemolymph of *P. indicus* was similar in many of its properties when compared with other defense proteins investigated in crustaceans like *Procambarus clarkii* (Miller *et al.*, 1972), in lobster, *Homarus americanus* (Cornick and Stewart, 1973).

Zone of clearance (Z) in the bacterial lawn of *Vibrio* sp. (v-5) was observed around the well containing the *P. indicus* agglutinin while absent around the control well (C) inoculated with saline alone (Plate 3.14) which demonstrates the presence of antibacterial property of agglutinin molecule. The property of agglutinin was comparable to the antibacterial proteins in the coelomic fluid of *Glycera dibranchiata* (Chain and Anderson, 1983), in *Saccoglossus ruber* (Miller and Ratcliffe, 1987). Strong antibacterial activity against *Vibrio* (v-5) strain isolated from the diseased prawn gut was comparable with the result of Valembois *et al.* (1982) in Earthworm *Eisenia fetida andrei.* An optimal antibacterial titer was observed towards the bacteria residing in the natural fauna of animal where as higher titers were towards foreign bacteria (Valembois *et al.*, 1982). Lassègues *et al.* (1989) also observed bacteriostatic and agglutinating property of agglutinin in the hemolymph of earthworm. The antibacterial activity of agglutinin against bacterial fauna in the immediate environment can be considered as an innate defense mechanism results from the co -operative activity of agglutinin and complement system (Day *et al.*, 1970).

The agglutinating property of *P. indicus* agglutinin was found to be stimulated by sugars such as maltose, lactose, galactose, glucose, cellobiose, mannose and D - glucosamine while inhibited in the presence of Xylose, Raffinose and Trehalose. Maximum titer value of 128 was observed with Maltose and $CaCl_2$. The same pattern of stimulatory and inhibitory effect of sugars were observed in the hemagglutinating activity of sea urchin (Giga *et al.*, 1987). Lactose, sucrose and D - galactose resulted in the inhibition of agglutination in ascidian coelomic fluid (Coombe *et al.*, 1984). Hemagglutinating activity was found to be inhibited by galactose and lactose in *Sarcophaga peregrina* (Komano *et al.*, 1980). The agglutinating property of clam, *Mercenaria mercenaria* agglutinin was influenced by sugars, ion, temperature and surface receptor proteins (Tripp, 1992). Few sugars dissolved in the hemolymph enhance the property of agglutinin while a few others inhibit by masking the recognition sites.

The agglutination specificity of agglutinin may be due to the presence of specific binding sites for carbohydrate moieties (Candy, 1980). The surface structure of gram- negative bacteria is formed of alternating sequence of 'N - acetyl glucosamine (Glc NAc) and N - acetyl muramic acid, and several amino acids which were not found in proteins, namely diaminopimelic acid and the D-isomers of alanine and glutamic acid. These unique components and structure of bacteria along with reactions of cell wall synthesis, offer an "Achilles heel" to medical therapy (Schlegel, 1993). Agglutinins recognise the bacteria, agglutinates and result in lysis through complement (intermediate

pathway) activation (Weir and Stewart, 1993). Agglutinins recognize erythrocytes due to the presence of surface specific antigenic determinants such as H substance in O group, A substance in A group and B substance in B group (Candy, 1980).

The prawn agglutinin was non - dialysable and precipitable. Agglutinin protein precipitated using 45% ammonium sulphate and soluble only in 0.85% saline and these properties indicate the macromolecular nature of agglutinin. The agglutinating activity was associated with a distinct peak after separation by Sephadex G - 200, indicating a macromolecule of 181 kDa was involved with two subunits of 97 kDa and 84 kDa. The molecular weight of *P. indicus* agglutinin was comparable to the molecular weight of few other agglutinins isolated from the hemolymph of *Panulirus interruptus* (Doolittle and Fuller, 1972); oyster (Li and Fleming, 1967); sea hare (Pauley *et al.*, 1971) and balanus (Kamiya and Ogata, 1982). The agglutinin's sensitivity to TCA, phenol and chloroform extraction indicate that the activity may be due to a protein. Agglutinin is heat -labile and susceptible to pH extremes. The inactivation of agglutinin by heat is a characteristic property of proteins. McKay *et al.* (1969) reported that hemagglutinin of crayfish was a protein with enhanced adhesion and phagocytosis. Hemagglutinins of oyster was also reported as proteinaceous in nature by Acton *et al.* (1969). Complete inactivation of agglutinin protein was observed at 85°C which was higher when compared to previous reports in spiny lobster (Tyler and Scheer, 1945) and in crayfish (Miller *et al.*, 1972).

Agglutinins in marine invertebrates appear to play an important role in the defense mechanism against potential pathogens since they are living in an environment teeming with pathogenic microorganisms. Cornick and Stewart (1968) suggested that the lack of serum agglutinin in the lobster against *Gaffkya homari* may be the reason for the systemic infections in lobsters. The same pathogen resulted in the impairment of defense mechanism of lobsters due to pathogenic invasion (Durliat and Vranckx, 1983b). McKay and Jenkin (1970) found that most of the crayfish hemocytes were stimulated to phagocytose erythrocytes in the presence of an opsonic agglutinin *in vitro* and against pathogenic *Pseudomonas* sp. *in vivo.*

The agglutinating and antibacterial property simultaneously observed in *P. indicus* agglutinin is attractive and this activity may be due to the co - operative effort of hemolymph, agglutinin and complement system. Similar observation of agglutination and bactericidal property was observed in the coelomic fluid of mytillus against *Vibrio* sp. (Nottage and Birkbeck, 1990). Roch *et al.* (1981) isolated few simultaneous hemolytic and bacteriostatic agglutinin from the hemolymph of earthworm. Roch *et al.* (1984) isolated and characterized five agglutinins with agglutinating, hemolytic and antibacterial properties from the coelomic fluid of earthworm. The humoral defense mechanism require *de novo* synthesis of RNA and protein in response to pathogenic invasion. Antibacterial activity exists naturally at some basic level, involving regular translation of stable RNA's. When antibacterial activity

reaches its maximum after bacterial infection, proteins responsible for it undergo a turn over (Hirigoyenberry *et al.*, 1990). From the present study, our observation elucidated that a natural agglutinin, a polyfunctional protein of 181 kDa with antigenecity, agglutinating, hemolytic and antibacterial properties is present in the hemolymph of *P. indicus*. The possible involvement of agglutinin protein in the hemolymph is expected to perform selective protection against various pathogenic organism in *P. indicus*.

C h a p t e r I V

Immunological characterization of agglutinin using - Double Immunodiffusion and ELISA

4.1. INTRODUCTION

Over the past two decades, intensive interest has been generated in India to develop aquaculture in the coastal belts, but successful production has been increasingly hampered by many factors including environmental pollution, poor management and diseases. Of the infectious diseases, viral and bacterial infections manifest most of the production loss in different parts of the world (Wongteerasupaya *et al.*, 1995; Mohan *et al.*, 1998). The loss due to the recent out break of white spot disease in shrimp in different parts of India is estimated to be several million dollars and the causative agent has been found to be a baculovirus (Wongteerasupaya *et al.*, 1995). Vibriosis is one of the most serious problems in marine fish, shell fish and shrimp. Several species of *Vibrio*, to date, have been described as pathogens for aquacultural shrimp; *Vibrio alginolyticus*, *V. anguillarum* (Lightner, 1983), *V. fisheri*, *V. fluvialis* (Sakata and Taruno, 1987b), *V. parahaemolyticus* (Lightner, 1983) and *V. ulnificus* (Song *et al.*, 1990). Three types of baculovirus have been reported to infect penaeid shrimp (Momoyama, 1981). Recently two other viruses named yellow - head virus (YHV) and systemic ectodermal and mesodermal baculovirus (SEMBV) have been reported in *Penaeus monodon* (Wongteerasupaya *et al.*, 1995). Many invertebrates, including the molluscs

and crustaceans are now being extensively farmed and an understanding of their host defense mechanism would be helpful to avoid and overcome disastrous out break of diseases that may occur under stressful, high density conditions of commercial cultures. Infectious disease is the number one cause of mortality in aquatic organism in different parts of the world despite the armamentarium of antibacterial agents developed over the past half a century. Resistance to antibiotics takes many forms and is probably the consequence of its wide spread use and misuse. (Oppegaard and Sorum, 1994). Thus, the search for new antibacterials directed towards new target is not only a continuous process, but also at this time, an urgent necessity.

The marine invertebrates are obviously not immunologically naive, they have inhabited this hostile planet which abounds in potential pathogens too long and too successfully for that. They respond to foreign invasion not with the specific immunoglobulin competancies of vertebrates, but with an array of non- specific, even inducible defenses that permit survival. These defenses may be breached, however, just as they can be in vertebrates, by overwhelming infection pressure and by action of environmental stresses. In the invertebrates a natural immunity against the attack of many infective organisms and their noxious products is the first line of defense. This natural immunity is the result of complex processes, including phagocytosis and natural antibodies. Antibodies differ widely from the corresponding ones in

vertebrates, as exemplified by the bacteriolysins, agglutinins, lysins which are highly thermolabile with complementary action (Sindermannn, 1990). Phagocytosis of foreign particles by fixed and mobile cells in the crustaceans is augmented by naturally occurring bactericidins, lysins and agglutinins. The enhancement of titers of these humoral factors have been noticed during exposure to foreign proteins. Phagocytosis by fixed and mobile cells in the gills, pericardial sinus, and sinuses at the bases of appendages seems to be a principal cellular defense perimeter in many crustaceans. Cellular defense factors and humoral factors may often interact or even share components to protect the individual species from the invasion of potential pathogens.

Immunity in invertebrates have been proved to involve both humoral and cellular defense mechanisms (Sindermannn, 1990). Species across the evolutionary scale from invertebrates to vertebrates use peptides to combat bacteria (Nagaraj, 1997). Agglutinin (Ag) is one of the humoral factors that agglutinates foreign particles including bacteria, viruses and RBC's, can be considered as endogenous antibacterial peptide (Ratcliffe *et al.*, 1985). Natural antibacterial activity can be enhanced after injecting pathogenic bacteria; gram- negative, *Aeromonas hydrophila;* and gram- positive, *Bacillus megaterium* (Lessègues *et al.*, 1989a). Endogenous molecular armaments that insects, amphibians and mammals use to counter microorganisms are peptides which are composed of 20 - 50 amino acids (Boman, 1995). At this juncture,

quantification of the agglutinin in the hemolymph of *Penaeus indicus* is very essential to assess the resistance power of prawn against potential pathogens. Few antibacterial peptides (*e.g.* RWJ - 49815) isolated are bactericidal, which inhibits the autophosphorylation of Kinase A of the Kin A:: Spo OF two - component signal transduction system *in vitro* (Barret *et al.*, 1998).

Lee *et al.* (1997) isolated and characterized the female specific protein (vitellogenin) in the hemolymph of fresh water prawn, *Macrobrachium rosenbergii* through developing ELISA. Chang *et al.* (1994) characterized vitellogenin in the hemolymph of *Penaeus monodon* through developing ELISA. Liang *et al.* (1980) carried out immunological studies for the detection and characterization of membrane -associated endotoxin binding protein in *Limulus.* Komano *et al.* (1980) isolated and immunologically detected the presence and increase in titers of hemagglutinins in the hemolymph of *Sarcophaga peregrina* larvae on injury and pathogenic infection. A dimeric protein (180 kDa) vitellogenin of Gilhead seabream, *Sparus aurata* was characterized through developing ELISA (Mosconi *et al.*, 1998). An indirect ELISA test was developed for titrating vitellogenin in the hemolymph for quantitative comparison along the reproductive cycle (Derelle *et al.*, 1986). Quackenbush and Fingerman (1985) have developed an ELISA for the quantification of Black pigment dispersing hormone from the fiddler crab, *Uca pugilator.* ELISA was used in shrimp, *Penaeus vannamei* to study the gene -

product release (Bradfield, 1989). Song *et al.* (1993) developed an immunological method for the detection of *Vibrio damsela* infection in shrimp, *Penaeus monodon.* Hameed *et al.* (1998) developed a nitrocellulose-enzyme immunoblot (NC - EIB) method and competitive ELISA to isolate and purify the systemic ectodermal and mesodermal baculovirus (SEMBV) pathogenic to *P. indicus* and *P. monodon* in the hemolymph, eyestalk, gill, head and soft tissues.

From the above survey of literature it was evident that immunological characterization of *P. indicus* agglutinin was scanty. Of all the other methods, ELISA was opted for the characterization of agglutinin due to its high sensitivity, specificity and reproducibility. Even nanogram (ng) increase in concentration of agglutinin in the hemolymph can be monitored using ELISA. This chapter deals with the production of polyclonal antibodies (anti -Ag), characterization using double immunodiffusion, conjugation of antibody with enzyme and the subsequent development of ELISA. In the present study, immune serum was specific against Ag and was used to develop a direct enzyme - linked immunosorbent assay (ELISA). The assay was specific, sensitive and suitable for quantifying Ag in the hemolymph. It is anticipated that ELISA quantification will prove useful in future investigations of agglutinin against potential pathogens.

4.2. MATERIALS AND METHODS

4.2.1. Experimental animals

A. Two New Zealand white male rabbits of 4 months old purchased from the Veterinary College, Kerala Agricultural University, Mannuthy were used for the immunological studies. The rabbits were acclimatised in laboratory conditions and were given food and water *ad. lib.*

B. *P. indicus* of both the sexes collected from Vallarpadam area of Cochin backwaters, Kerala were used for immunological studies.

4.2.2 Production of polyclonal antibodies

Polyclonal antibodies were raised in New Zealand white male rabbits according to the method of Harlow and Lane (1988) using electroeluted agglutinin.

One week before immunization, blood was withdrawn via the marginal ear vein for preparation of preimmune sera. First dose were four injections given intramuscularly with 500 µl of electroeluted agglutinin plus 500 µl Freund's complete adjuvant (Sigma). The 2nd, 3rd and 4th booster doses were

given with 500 µl electroeluted agglutinin emulsified with an equal volume of Freund's incomplete adjuvant (Sigma) at four different places at intervals of 2 weeks. Two weeks after the booster dose (8th week) blood was collected and allowed to clot. Clotted blood was kept at 4°C overnight, then centrifuged at 10,000 X g, 10 min. The serum was anti – Ag Ig G and used for further studies.

4.2.3. Purification of antibodies by using DEAE - matrix.

Ig G fractions were purified by batch method of Harlow and Lane (1988) using DEAE- cellulose column (Sigma).

Principle

Antibodies have a more basic isoelectric point than other serum proteins; ion -exchange chromatography is a useful method for purifying these molecules. The pH is kept below the isoelectric point for most antibodies, and therefore, will not bind to an anion exchange resin such as DEAE - cellulose. Therefore antibodies remain in solution. This is particularly a quick method, because the binding is done in bulk. More precise elution can be achieved by following standard chromatographic procedures.

Procedure

Pre-treated the DEAE - matrix with 0.5N HCl and 0.5N NaOH for several hours. Equilibrated the DEAE - matrix 3 to 4 times with 0.01M

phosphate buffer until the pH reaches 8.0. The buffer was drained off and stirred with dialysed serum (polyclonal antibody) for 1 hr continuously at room temperature (30°C). The supernatant was collected after centrifugation at 10,000 X g for 10 min. at 4°C. The purified Ig G was used immediately. Concentration of Ig G was determined according to the method of Lowry *et al.* (1951).

4.2.4. Double Immunodiffusion

Double immunodiffusion test was performed following the method of Ouchterlony and Nilsson (1978) and Chang *et al.* (1993).

Principle

Double immunodiffusion tests (DD) consists of agar gel wherein preparations of antigens and specific antibodies are placed in adjacent sample wells. DD is a quantitative test that has been used successfully to show antigenic relatedness, to confirm identity and to clarify taxonomic position. The number of precipitin bands produced between opposing wells of reactants corresponds to the number of antigenic components in a given gel antigen - antibody system. Equal concentration of antigen and antibody are required to develop precipitin bands.

Procedure

3 mm circular wells (6 peripheral and 1 central) were punched in 0.9% agarose (Sigma) plates. 80 µl of anti -Ag immune serum was added to the centre well and the surrounding wells were filled with crude prawn serum,

agglutinin and agglutinin free prawn serum (80 µl). Plates were incubated overnight in a humid chamber at 30°C. The agarose gel was washed with PBS, pH 7.0 followed by distilled water. After drying with blotting paper, the gels were stained with Coomassie brilliant blue R-250 and destained several times to visualise the precipitin lines.

4.2.5. Conjugation of Ig G with HRP – Periodate: Oxidation method.

Conjugation of Ig G with horseradish peroxidase (HRP) was done following the method of Hampton *et al.* (1990).

Principle

Periodate - oxidation method takes advantage of the fact that vicinal hyroxyl groups present on the carbohydrate moiety of the enzyme can be oxidized, by sodium periodate ($NaIO_4$), to reactivate aldehyde groups which are then susceptible to subsequent nucleophilic attack by free amino groups on the surface of the antibody molecule. The resulting Schiff's base linkage is then reduced by reaction with ascorbic acid. The reverse approach has also been utilized, whereby the carbohydrate moiety present on the Fc region of Ig G is first oxidized with periodate prior to coupling with amino groups of the enzyme. Self coupling of activated enzyme molecules can be prevented by blockage of free amino groups of the enzyme by maintaining the oxidized enzyme solution at pH 4-5 prior to addition of antibody.

Reagents

1. Horseradish peroxidase (HRP)

   HRP solution was prepared by dissolving 4 mg of HRP in 1 ml of distilled water.

2. 0.1M $NaIO_4$

   2.14 g $NaIO_4$ dissolved in 50 ml and made upto 100 ml with distilled water.

3. 0.01M carbonate buffer, pH 9.6.

   1.59 g Na_2CO_3 and 2.92 g $NaHCO_3$ dissolved in 100 ml and made upto 1 litre by adjusting the pH to 9.6.

4. PBS, pH 7.4 , 1.42 g Na_2HPO_4,

   1.56 g NaH_2PO_4 and 9 g NaCl dissolved in 100 ml and made upto 1 litre by adjusting the pH to 7.4.

5. Ammonium sulphate (Enzyme grade, SRL).

6. Ascorbic acid.

Procedure

200 µl of 0.1M sodium periodate ($NaIO_4$) was allowed to react with 4 mg/ml of HRP for 1 hr at room temperature in dark. The HRP solution was dialysed against 2 - 3 changes using acetic acid, pH 4.5 for 12 - 16 hrs at 4°C. 8 mg of purified antibody was dissolved in 50 µl of 0.01M carbonate buffer (pH 9.6) and incubated with the HRP solution at room temperature with shaking. Then 0.8 ml of freshly prepared ascorbic acid was mixed and allowed

to react at 4°C for 12 - 16 hrs. The conjugated antibody with HRP was precipitated using ammonium sulphate and centrifuged at 5, 000 rpm, 4°C and dissolved in 4 ml of PBS buffer, pH 7.4. The enzyme - antibody was dialysed against PBS buffer (pH 7.4) for 2 - 3 times, 12 - 16 hrs at 4°C. The conjugated antibody after dialysis was kept at 4°C for further use.

4.2.6. Direct enzyme - linked immunosorbent assay (ELISA)

ELISA was carried out according to the method of Hampton *et al.* (1990) and Chang and Shih (1995).

Principle

A solid phase read out system for the detection of the antigen – antibody reaction is the ELISA assay. Antigen or antibody is covalently coupled to the enzyme so that bound enzyme activity is measured. Antigen is attached to the ELISA plates by passive adsorption. Horseradish peroxidase (HRP) enzyme with high turnover numbers was conjugated with antibody. Periodate oxidation of the extensive carbohydrate group of HRP produces aldehyde groups that will combine with the amino groups of immunoglobulin to produce schiff base conjugates that can be stabilized by reduction with ascorbic acid. When the enzyme couples with the colourless substrate (OPD) in the presence of H_2O_2, a coloured product (green, orange, yellow) will be formed. The amount of test antibody is measured by assessing the amount of coloured end product by optical density staining of the plates in ELISA reader.

Reagents

1. ELISA coating buffer (sodium carbonate buffer), pH 9.6.

   1.59 g of Na_2CO_3 and 2.92 g of $NaHCO_3$ dissolved in 100 ml and made upto 1000 ml by adjusting the pH to 9.6.

2. Washing solution , PBS - Tween

   0.2 ml of Tween 20 dissolved in 400 ml of PBS - Tween, pH 7.4.

3. ELISA buffer (PBS - Tween - BSA).

   0.5 mg Tween 20 and 2 g BSA was dissolved in 100 ml and made upto 1000 ml using PBS buffer, pH 7.4.

4. Substrate buffer (Phosphate citrate buffer), pH 5.0.

   A 19.21 g citric acid dissolved in 1000 ml of distilled water.

   B 28.40 g Na_2HPO_4 dissolved in 1000 ml of distilled water.

   24.3 ml of solution A was mixed with 25.7 ml of solution B and diluted to 100 ml.

Procedure

ELISA plates (96 -well, flat bottom, NUNC, Maxisorp, Denmark) were incubated 2 hrs at 37°C with antigen (purified electroeluted agglutinin 8900 ng from SDS gel) diluted with ELISA coating buffer, pH 9.6. After incubation, ELISA plates were washed 4 to 5 times with PBS- Tween and allowed the plates to dry. Then, 100 µl of anti -Ag Ig G - horseradish peroxidase (enzyme conjugated antibody) diluted in ELISA buffer (1:100) was added to each well. Plates were incubated at 37°C for 3 hrs. The ELISA plates were washed with PBS- Tween 4 to 5 times and allowed to dry. After washing, 100 µl of substrate was added to each well (O -phenylene diamine (OPD) 3.4 mg/10 ml in the presence of H_2O_2 prepared in substrate buffer). The plates were kept under dark for 60 min. The reaction was stopped by adding 50 µl of 2M sulphuric acid (H_2SO_4). Photometric data were read at 492 nm using an automated ELISA Reader (SPAN, India). Standard curve was constructed using the O. D value of known agglutinin concentration.

4..2.7. Statistical Analysis

A correlation coefficient was calculated to assess the relationship between optical density (O.D) and the amount of purified Ag. A one -way analysis of variance (ANOVA) was carried out to determine the effect of sample dilution on the concentration of Ag in the hemolymph sample (Shukla and Gulshan, 1986).

4.3. RESULTS

Characterization of anti - Ag immune sera.

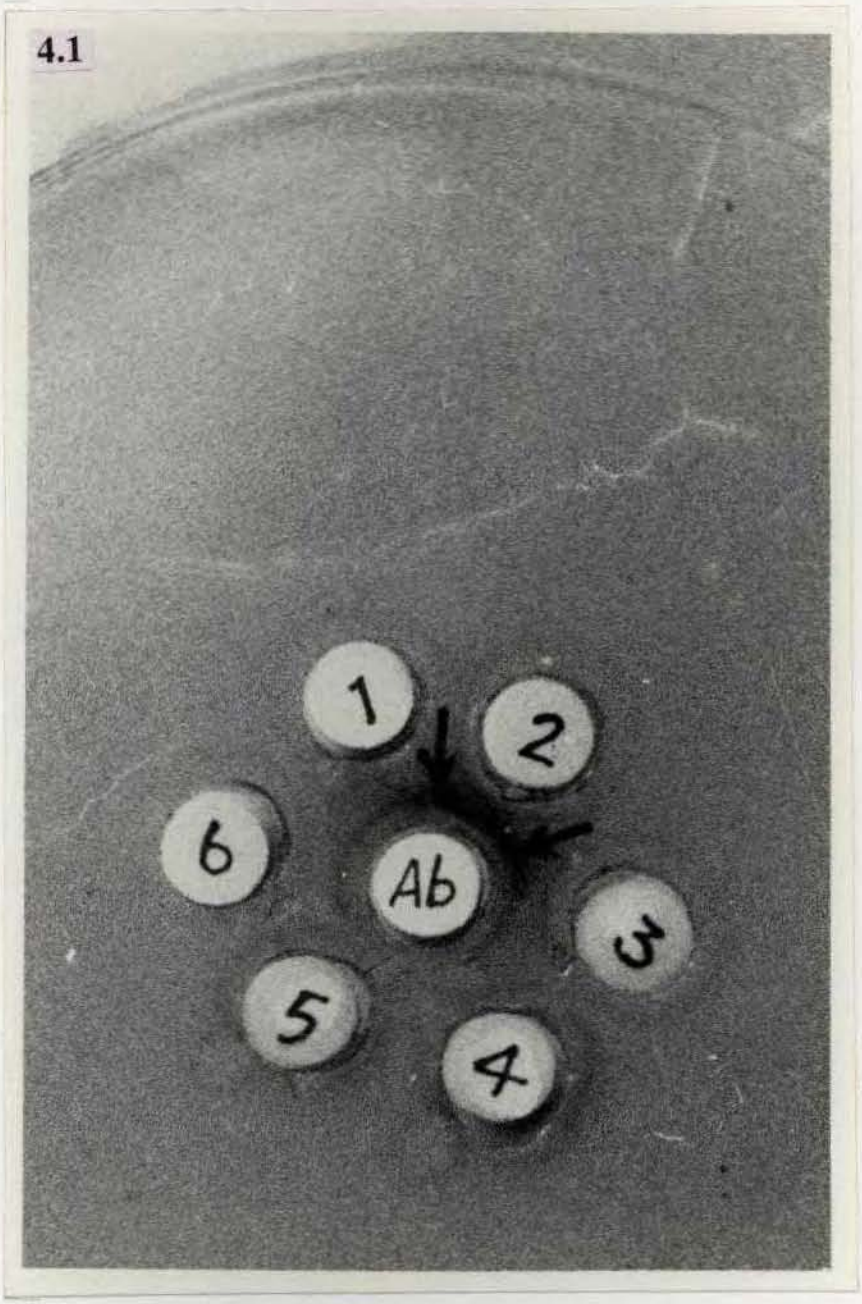
Immunoserum raised against purified Ag was characterized by double immunodiffusion test. As adjusted by the formation of single precipitin lines, anti - Ag immune serum (centre well) precipitated purified Ag (peripheral wells 2 & 3). Narrow precipitin lines were also observed between the centre well and peripheral well containing crude hemolymph (peripheral wells 1 & 4) indicating the presence of trace amounts of agglutinin in the crude sample. While anti - Ag immune serum did not precipitate any antigen from the control well (wells 5 & 6) (Plate 4.1).

ELISA

Anti -Ag serum was used to develop a direct ELISA. Fig. 4.1 shows a typical standard curve using purified Ag as the reference antigen. The standard curve was linear over the range 17.8 ng - 8,900 ng (correlation coefficient r = 0.99, B = 0.14). The sensitivity of the assay (*i.e.* the lowest concentration of Ag giving an O. D significantly greater than background) was 178 ng/ml. To demonstrate the specificity of the assay, serially diluted agglutinin free hemolymph was used as a negative antigen (Fig. 4.2). The O. D generated by agglutinin free hemolymph was comparable to background values (the ratio of mean agglutinin free hemolymph O. D to mean background O. D was 1.08). To determine whether the ELISA is suitable for quantifying agglutinin in

Plate 4.1. Analysis of anti - Ag immune serum by double immunodiffusion test in *P. indicus*. To the centre well of an agarose gel plate, 80 µl (0.5 mg/ml) anti - Ag immune serum was added. Peripheral wells were filled with crude hemolymph (1 & 4); purified electroeluted Ag from SDS - PAGE (2 & 3); agglutinin free fraction of hemolymph as control (5 & 6). Gels were stained with Coomassie brilliant blue R - 250. Arrow indicates precipitin line formed between anti - Ag and agglutinin.

4.1



hemolymph, agglutinin rich hemolymph were serially diluted and titrated by ELISA (Fig. 4.2). The agglutinin rich hemolymph curve was parallel to the curve generated with agglutinin standard (Fig. 4.1). (*i.e.* One- way ANOVA indicated no significant difference in slope *(P >0.05)*. Concentrations of hemolymph agglutinin calculated from the Ag standard curve and corrected for the dilution are shown in Table 4.1. Dilution of samples had no effect on the corrected concentration of Ag *(P >0.05)*.

Table 4. 1. Effect of sample dilution on calculated concentration of Ag in *P. indicus*

Sample	Dilution factor	Calculated Ag concentration (ng/ml)
Agglutinin	10	8840.77
	100	884.94
	1,000	84.76
	2,000	42.06
	4,000	21.26
	5,000	15.437

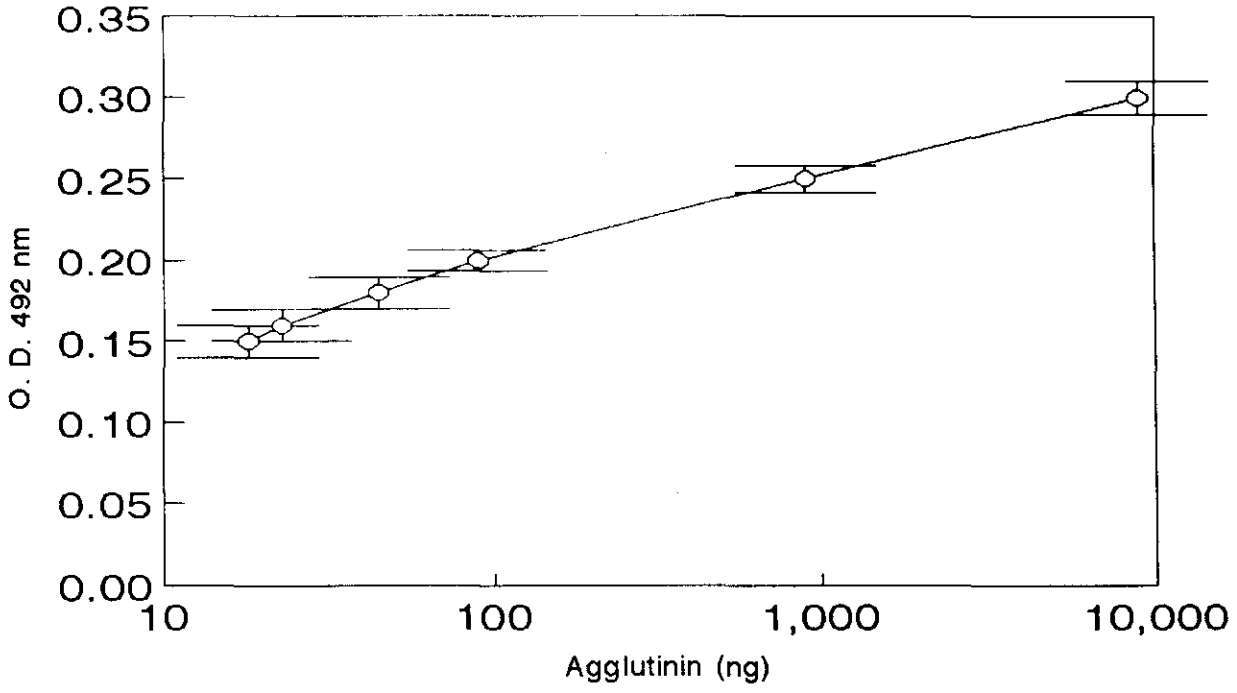


Fig. 4.1. ELISA standard curve of *P. indicus*. Each point represents a mean ± SD of triplicate analysis.

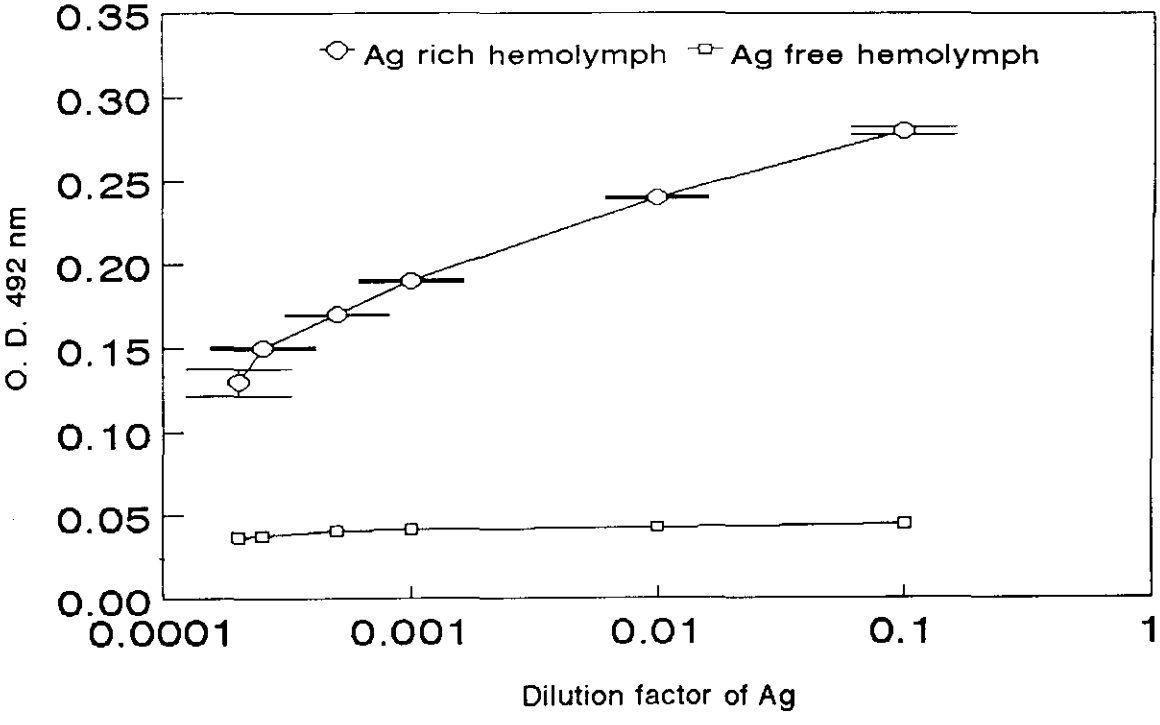


Fig. 4.2. Dose - response titration curve of agglutinin - rich hemolymph and agglutinin free hemolymph of *P. indicus*. Each point represents a mean ± SD of triplicate analysis.

4.4. DISCUSSION

An ELISA was developed to study the level of agglutinin in the hemolymph during normal growth process, stress conditions and pathological invasions. Properly designed immunoassays are rapid, precise and reproducible and have advantage over other assays (Chard, 1987). Assay of agglutinin was required for the investigation of antibacterial activity against a variety of gram- negative, gram- positive microorganisms and fungi. In the present study, precipitin curve developed through double immunodiffusion between anti - Ag and agglutinin of *P. indcus* conforms the antigenic property of agglutinin (Plate 4.1) and a single precipitin line obtained in DD proves agglutinin is formed of a single protein. Liang (1980) used double immunodiffusion method in *Limulus* to detect an amoebocytic bound protein of mol. wt. 80 kDa which recognize endotoxins. Presence of vitellogenin in the hemolymph was first detected through double immunodiffusion in *Penaeus monodon* (Chang *et al.*, 1994). Lee *et al.* (1997) first identified the immunoreactive 700 kDa vitellogenin in the hemolymph of prawn, *Macrobrachium rosenbergii* through double immunodiffusion test. Bradfield *et al.* (1989) used immunoblot assays for the detection of cortical granule polypeptides in *Penaeus vannamei.* Using double immunodiffusion for

detection and quantifying the concentration of immunoreactive proteins are possible only to a limited extent. Hence more precise and sensitive method such as ELISA have been adopted to monitor the antibacterial peptide.

ELISA was validated for the measurement of agglutinin during growth process, under stress and pathogenic conditions. Linear absorbance was obtained from 17.8 - 8,900 ng/ml of agglutinin (Fig. 4.1) and sensitivity of the ELISA was 178 ng/ml. In the present study, ELISA standard curve was linear across a ~ 445 fold increase in agglutinin concentration and was highly reproducible (correlation coefficient r = 0.99, N = 6). The sensitivity of the ELISA, 178 ng/ml was comparable to the sandwich ELISA (100 - 250 ng/ml) of Derelle *et al.* (1986) in *Macrobrachium rosenbergii* while studying the variation in vitellogenin and vitellin during reproductive maturation. Komano *et al.* (1980) immunologically characterized an insect lectin, a protein of mol. wt. 190 kDa released during injury, functions as hemagglutinating and antibacterial assumed to be participating in immune surveillance. Quackenbush (1989) developed an ELISA to quantify the 97 kDa yolk protein production during ovarian maturation in prawn, *Penaeus vannamei.* The standard curve of the 97 kDa yolk protein was linear from 1.25 - 1,200 ng using ELISA (r = 0.97). Mosconi *et al.* (1998) validated ELISA for a rapid and reliable measurement of plasma vitellogenin (180 kDa protein) changes related with those of estradiol 17β in female brood stock of seabream (*Sparus aurata),*

standard curve ranged between 3.9 to 2,000 ng/ml and sensitivity by 8 mg/ml. In Fiddler crab, *Uca pugilator* Quackenbush and Fingerman (1985) obtained ELISA standard curve that ranges between 6.6 to 25,000 ng of black pigment dispersing hormone. In order to validate the ELISA for sample titration, it is essential to establish that standard and sample titration curves are parallel (Fieldkamp and Smith, 1987; Peterman and Butler, 1989). The specificity (178 ng/ml) of the ELISA reported in the present study was demonstrated by titration of agglutinin free hemolymph and had a protein profile similar to that of agglutinin rich hemolymph, produced background O. D in the ELISA. In the present study, it was demonstrated that the slopes of the sample *(P >0.05)* titration curves were not significantly different from that of Ag standard curve (Fig. 4.1 & 4.2). Consequently, concentrations of Ag determined from Ag standard curve and corrected for dilution were not significantly different. Competitive ELISA was used by Hameed *et al.* (1998) for the detection of systemic ectodermal and mesodermal baculovirus (SEMBV) infection in shrimps. ELISA techniques has been used widely in diagnostic test both human and animal viruses (Nadala *et al.*, 1992; Lu *et al.*, 1996). Lee and Watson (1994) developed an ELISA to study the level of vitellogenin and vitellin during reproductive phase of *Callinectes sapidus*. ELISA kits developed will be helpful in monitoring the health conditions of organisms.

Much progress has been made in understanding the cellular events and regulation of agglutinins in defense mechanism (Miller and Ratcliffe, 1994). However, important question regarding the synthesis and effects of environmental cues and pathogenic stresses on agglutinin turnover remain unresolved. It is anticipated that the ELISA developed in the present study will be useful in future investigations of these processes and to monitor the health conditions of *P. indicus*. Determination of primary structure and engineering of agglutinin for the synthesis of analogues are promising alternative to currently used drugs against potential pathogens.

C h a p t e r V

Amino acid composition of agglutinin

5.1. INTRODUCTION

Invertebrates includes a vast diversity of animal species from the unicellular protozoans to the more complex echinoderms and prochordates. Organisms posses an array of defense mechanisms that enables them to withstand many forms of adversity. Like vertebrates, invertebrates have very effective physico- chemical barriers as a first line of defense. In addition or complementary to the highly specific cell mediated immune response, vertebrates and other invertebrates have a defense system made up of distinct groups of broad spectrum agglutinins (Miller and Ratcliffe, 1994) and antibacterial peptides (Boman, 1995). In contrast to vertebrates, invertebrate immunity is not based on immunoglobulins and interactive lymphocyte sub populations, but it is extremely efficient, utilizing interactive cellular and humoral components. Natural antibody like humoral substances agglutinins, lysins and antimicrobial factors capable of agglutination, hemolysis and antibacterial properties have been reported in the body fluids of many invertebrates (Miller and Ratcliffe, 1994; Boman, 1995). Opsonizing and agglutinating factors of crustacean hemolymph can be separated quantitatively (Goldenberg and Greenberg, 1983). Komano *et al.* (1980) found that agglutinin synthesis was induced by wounding which is composed of α and β subunits in the fly *Sarcophaga peregrina.* Synthesis of agglutinin has also been reported in the fat body of insects and in the blood cells of invertebrates (Ratcliffe *et al.*, 1985).

The amino acid sequence of the *Limulus* amoebocyte bound agglutinin have been studied by Liang *et al.* (1980). Complete amino acid composition and sequence of Echinoidin, an agglutinin of sea urchin, *Anthocidaris crassispina* was characterized by Giga *et al.* (1987). Suseelan *et al.* (1997) characterized the amino acid composition of bifunctional dimeric lectin of mol. wt. 183 kDa from *Vigna mungo.* Roch *et al.* (1984; 1986) isolated five agglutinins with agglutinating, hemolytic and antibacterial properties from the earthworm coelomic fluid and its amino acid composition were determined and compared with agglutinins of other zoological groups. Several short antimicrobial peptides (~ 40 amino acids and less) have been isolated and characterized from insects (Andreu *et al.*, 1983; Boman, 1995). Amino acid sequence of few insect antibacterial peptides such as Cecropins, Andropin, Defensins have been studied (Steiner *et al.*, 1981). Positively charged polypeptides which adopt amphipathic α helical structure were found to be cytotoxic to both bacteria and erythrocytes. The antibacterial activity was attributed through destroying the energy metabolism of the target organism by increasing the permeability of energy transducing membranes (Shai and Oren, 1996). Nagaraj (1997) engineered few endogenous antimicrobial synthetic peptides, which have broad spectrum of antibacterial activities against a variety of gram- negative, gram- positive microorganisms and fungi.

Agglutinins of crustaceans characterized include, lobster *Homarus americanus* (Cornick and Stewart, 1973), crayfish *Procambarus clarkii* (Miller

et al., 1972), crabs *Ranina ranina, Playgusia dentipes, Chaybdis japonica* and *C. acuta* (Ueda *et al.*, 1991). Amino acid composition of hemolymph protein such as vitellin (Vn) and vitellogenin (Vg) of *P. monodon* and *Macrobrachium rosenbergii* have been characterized (Chang *et al.*, 1994; Lee *et al.*, 1997). Neutboom *et al.* (1989) and Lassalle *et al.* (1988) determined the amino acid composition of hemocyanin and hemolymph lysozyme protein. Amino acid composition helps to determine the species anatomy and function. Individual protein molecules are documents of evolutionary history and their study leads to molecular evolution. Molecular biologists are modifying existing proteins through genetic manipulations and tries to assemble in a changed fashion to build a new protein. After determining amino acid composition of organisms, comparisons were done to find out the similarity and differences between them. Similarity indicates structurally homologous proteins and a common ancestry. By making such comparisons systematically, the apparent pattern of evolution of the molecules can be traced.

The *P. indicus* agglutinin was found to be a protein of mol. wt 181 kDa with antigenecity, agglutinating, hemolytic and antibacterial properties. This chapter reports the amino acid composition of agglutinin subunits A & B and their compositional similarity between these two. Similarity between other proteins belonging to different zoological groups was carried out using the method of Marchalonis and Weltman (1971) equation.

5.2. MATERIALS AND METHODS

5.2.1. Purification and biological activities

Agglutinin was isolated and purified using Spehadex G - 200 column chromatography. The fraction was subjected to electrophoresis (Native PAGE) and their subunits were resolved under dissociating SDS - PAGE. The agglutinin subunits A & B were eluted using electroelutor and dialysed against distilled water for several hours. Biological activities of agglutinin subunits (A & B) were carefully analysed.

5.2.2. Determination of protein content

The method of Lowry *et al.* (1951) was used with bovine serum albumin as standard. The concentration of the purified agglutinin protein subunits were adjusted to 330 μg/ml (0.33 mg/ml) with distilled water and used immediately.

5.2.3. Amino acid analysis

Quantitative amino acid analysis was done with Shimadzu High - Performance Liquid Chromatography (HPLC).

Principle

Primary structure can be deciphered through amino acid composition. HCl hydrolyses the protein into its constituent amino acids. Orthophthaldehyde

(OPA) converts these amino acids into derivatives which are short lived. The derivatised amino acids are detected using FLD - 6A detector of HPLC.

Procedure

Sample of purified agglutinin protein subunits A & B 0.33 mg/ml each were hydrolysed at 110°C for 24 hrs in 10 ml of 6N HCl in a hard glass tubes (15 X 50 mm) sealed under vacuum with nitrogen gas. The tubes were cooled, broke opened and transferred the contents quantitatively into a flask. The hydrolysed protein was flash evaporated to remove HCl using a rotatory flask vacuum evaporator. The dried hydrolysate was dissolved in 10 ml of 0.05M HCl. 20 µl of this solution was used for amino acid analysis in a Shimadzu High - Performance Liquid Chromatographic system (HPLC) on an ion-exchange resin column. Elution was carried out with a gradient mixture of sodium citrate buffer of pH 3.25 and 10. Post column derivatisation of the eluted amino acids were done using Orthophtalaldehyde (OPA) and detected in FLD - 6A fluorescence meter. The response of each amino acid was integrated by an integrator C - R6A. In order to quantify the amino acids, a standard chromatogram was run using a standard mixture of protein amino acids having a concentration of 0.005 µmol of each amino acids (Sigma). Ion-exchange resin of HPLC analyses the relative percentage of each amino acids in a protein. As fractions emerge, each amino acid concentration is measured by a reaction that yield a colour, the depth of which is proportional to amino acid concentration.

5.2.4. Estimation of relatedness among proteins

Statistical comparisons of amino acid composition within the agglutinin subunits A and B with various other known proteins listed in Table 5.3 were carried out by the S Δ Q method of Marchalonis and Weltman (1971). Briefly, S Δ Q = Σ (XiA - XiB)^2, where XiA and XiB are the percentage of a given amino acid of type 'i' in protein A and B respectively. In our computation, we considered only sixteen different amino acids (Table 5.1). Data concerning amino acid composition of various non- penaeus proteins were found in literature as mainly gross composition or sometimes complete sequence. Prior to computation, these data were converted to residues per hundred as indicated by Darcel and Kaldy (1983). A difference of *<100* S Δ Q units indicates possible relationship between the two considered proteins when lowest is the S Δ Q value, highest is the change of relationship. According to Cornish - Bowden (1983), an S Δ Q *<50* units provides a significant relationship excluding coincidental occurrence.

5.3. RESULTS

5.3.1. Amino acid composition of *P. indicus* agglutinin protein

The amino acid composition of the *P. indicus* agglutinin subunits A & B is presented in Table 5.1, Figs. 5.1 and 5.2. Analysis was made on purified agglutinin. No particular assay was made to preserve Cysteine during hydrolysis. All the amino acids were determined by extrapolation on 24 hr hydrolysis.

Table 5.1. Biological activities, amino acid compositions and molecular weight of agglutinin subunits (A & B) isolated from *P. indicus* hemolymph.

Biological activities	Agglutinin			
	A		B	
Agglutination	+ + +		+ + +	
Hemolysis	+ +		+ + +	
Antibacterial (bactericidal)	+ + +		+ +	
Amino acids	Number of residues/ molecule		Molar % of total residue	
	A	B	A	B
Aspartic acid	89.5	78.40	13.56	13.35
Threonine	32.3	28.14	4.80	4.79
Serine	31.0	30.36	4.60	5.17
Glutamic acid	98.6	88.84	14.94	15.12
Proline	34.2	28.96	5.18	4.93
Glycine	33.7	32.03	5.10	5.45
Alanine	33.8	32.32	5.12	5.50
Valine	32.0	28.12	4.84	4.78
Methionine	2.78	3.21	0.408	0.55
Isoleucine	31.1	27.55	4.71	4.69
Leucine	55.9	49.60	8.47	8.44
Tyrosine	25.3	23.82	3.83	4.05
Phenylalanine	38.4	34.16	5.81	5.82
Histidine	46.6	39.61	7.06	6.74
Lysine	26.6	20.41	4.03	3.47
Arginine	48.2	42.12	7.30	7.17

Calculated MW of agglutinin subunit A = 92.52 kDa. Measured MW of agglutinin subunit A ≅ 97.0 kDa.
Calculated MW of agglutinin subunit B = 81.96 kDa. Measured MW of agglutinin subunit B ≅ 84.0 kDa.
Calculated MW of agglutinin = 174.47 kDa. Measured MW of agglutinin ≅ 181 kDa.

Agglutinin subunit A is characterized by a high amount of glutamic acid (14.94%) and aspartic acid (13.56%). In agglutinin subunit B, it was remarkable to find a high amount of glutamic acid (15.12%) and aspartic acid (13.35%). In both of these A & B subunits Cysteine is characteristically absent. Based upon these data, calculated relative molecular weight of agglutinin subunits were found to be 92.52 kDa and 81.96 kDa, respectively (Table 5.1). The calculated MW of subunits are in good agreement with previous values obtained in SDS gel electrophoresis . *i.e.* 97 kDa and 84 kDa respectively. Both the subunits predominates acidic in nature. Figs. 5.1 & 5.2 shows each peak represents one amino acid emerging from a column at a characteristic place. The area under the peak for each amino acid is proportional to its prevalence in the original protein (it gives the number of residues of each amino acid).

5.3.2. Biological activities

Both the subunits of agglutinin protein A & B exhibits agglutination, hemolytic and antibacterial properties. Subunit A is more antibacterial and less hemolytic when compared to subunit B, which is more hemolytic and less antibacterial.

5.3.3. S Δ Q values within agglutinin subunits

Agglutinin subunits A and B show compositional similarities with 1.096 units as maximum S Δ Q value (Table 5.2). Both the subunits were found to be similar which is associated with hydrogen bonds to form a dimmer.

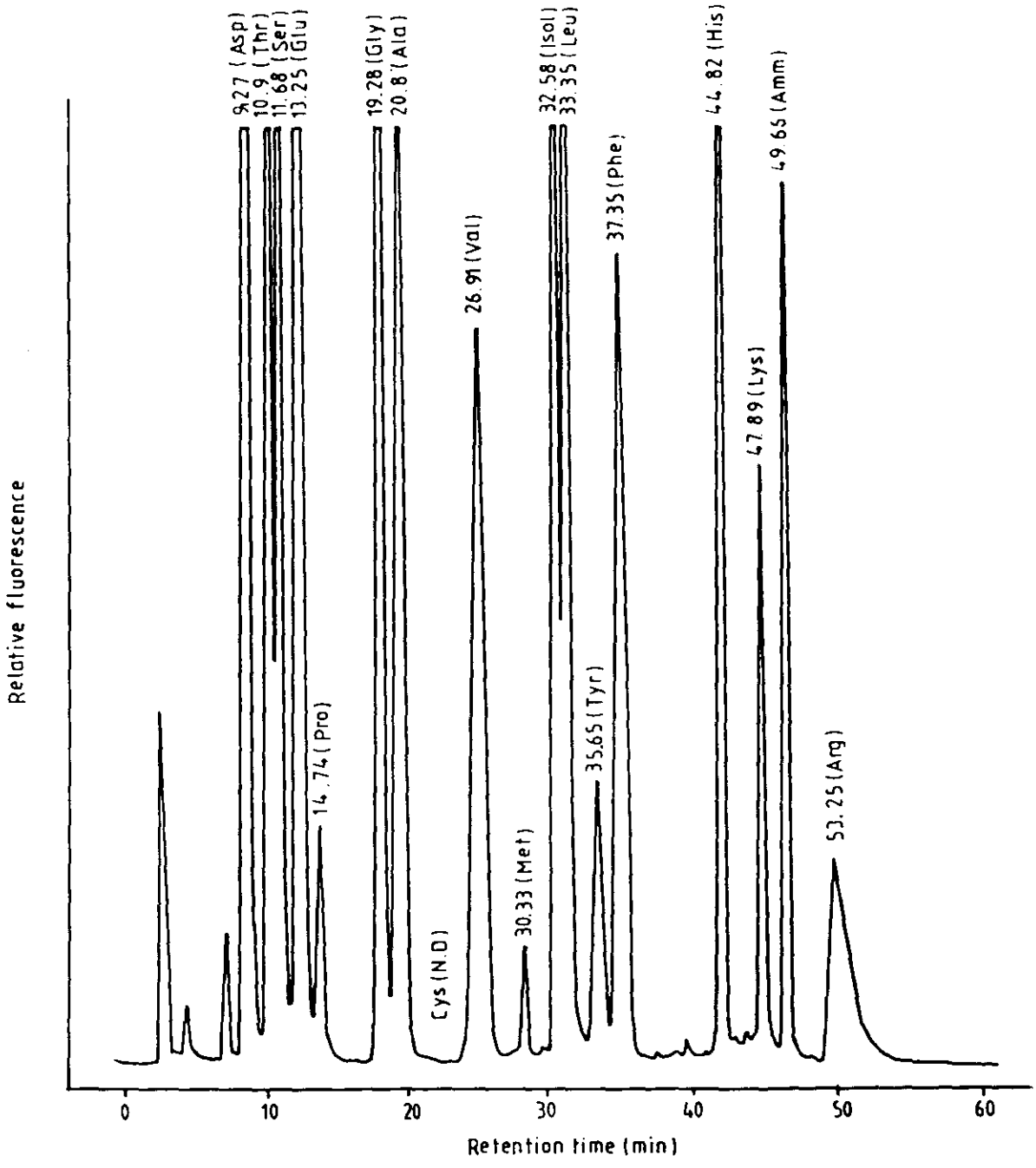


Fig. 5.1. Amino acid analysis of agglutinin ***subunit A*** using high - performance liquid chromatography (HPLC) using ion exchange resin. Elution was carried out with a gradient mixture of sodium citrate, pH 3.25 & 10. Post column derivatisation of the amino acids were done using Orthophthalaldehyde (OPA). The detector used was FLD - 6A fluorescence meter. Base line corresponds to elution time.

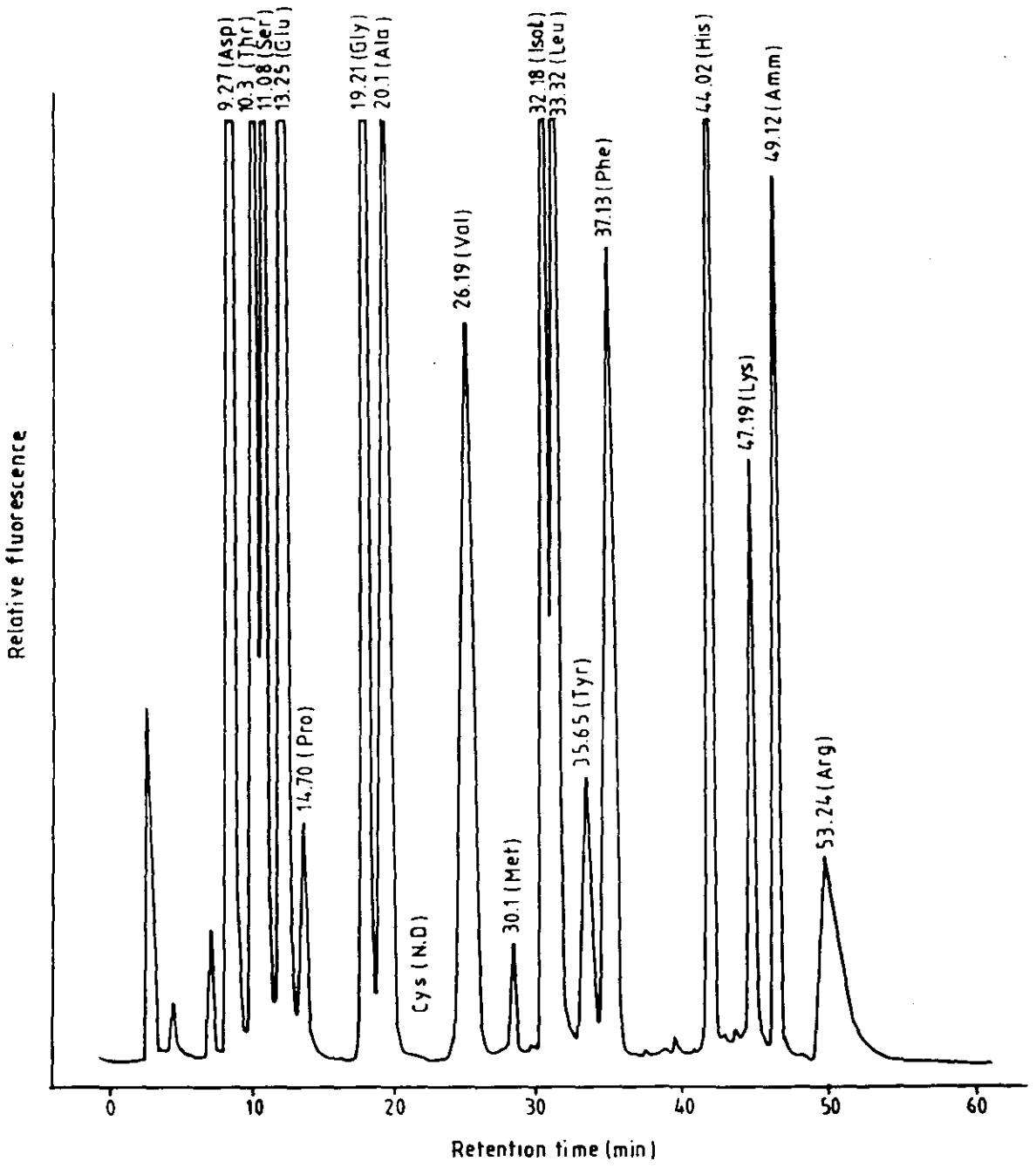


Fig. 5.2. Amino acid analysis of agglutinin ***subunit B*** using high - performance liquid chromatography (HPLC) using ion exchange resin. Elution was carried out with a gradient mixture of sodium citrate, pH 3.25 & 10. Post column derivatisation of the amino acids were done using Orthophthalaldehyde (OPA). The detector used was FLD - 6A fluorescence meter. Base line corresponds to elution time.

Table 5.2. Estimation of compositional similarities within the two subunits of agglutinin protein of *P. indicus*.

Agglutinin	Agglutinin protein *		Similarity
	A	B	
Subunit A	0	1.096	Related
Subunit B	1.096	0	Related

* S Δ Q value of each comparison was calculated according to the method of Marchalonis and Weltman (1971). $S \Delta Q = \Sigma (XiA - XiB)^2$

5.3.4. Estimation of compositional similarities between *P. indicus* agglutinin and proteins from other zoological groups based on S Δ Q values

Compositional similarity were evidenced between *P. indicus* agglutinin subunits (A & B) and Earthworm defense protein δ and ε in the order 98.83, 96.35, 68.98 and 65.69 as S Δ Q values (Table 5.3). Agglutinin of *P. indicus* also exhibits relatedness with *Limulus* agglutinin in the order 68.5, 66.25, 65.11 and 63.83 as S Δ Q values. Subunit B was found to be more related to Earthworm (δ & ε) and Limulus (α & β) agglutinin. *P.indicus* agglutinin shows unrelatedness to other proteins listed in Table 5.3 with high S Δ Q values *(>100)*. From the calculation it was found that *P.indicus* agglutinin was a specific defense protein showing relatedness with proteins of its own kind having properties like agglutination, hemolytic and antibacterial nature.

Table 5.3. Estimation of compositional similarities between *P. indicus* agglutinin and some other protein based on S Δ Q values.

Name of other proteins	*P. indicus* agglutinin	
	A	B
Sea urchin [a]	19.8	195
Earthworm agglutinin (α) [b]	318.1	320
Earthworm agglutinin (β) [c]	109.87	105
Black gram lectin [d]	312	314
Earthworm defense [e] protein (agglutinating, hemolytic and antibacterial) (δ)	**98.83**	**96.35** *
Earthworm defense [f] protein (agglutinating and antibacterial) (ε)	**68.98**	**65.68** *
Limulus agglutinin (α) [g]	**68.53**	**66.25** **
Limulus agglutinin (β) [h]	**65.11**	**63.83** **
P. indicus muscle protein [i] (collagen)	276.32	283
P. monodon vitellogenin (Vg) [j]	343	342
" vitellin (Vn) [k]	111.6	112
Macrobrachium rosenbergii - vitellogenin (Vg) [l]	324	328
" vitellin (Vn) [k]	100	101
Cecropin A [n]	318	318.31
Attacin [o]	200	200.84

[a] Giga *et al.*, 1987; [b, c, e, f] Roch *et al.*, 1986; [d] Suseelan *et al.*, 1997; [g, h] Liang *et al.*, 1980; [i] Sivakumar *et al.*, 1997; [j, k] Chang *et al.*, 1994; [l, m] Lee *et al.*, 1997; [n] Andreu *et al.*, 1983; [o] Painter *et al.*, 1998.

* The values enclosed in dotted box represent proteins (agglutinating, hemolytic and antibacterial) determined as probably 'related'.

** The values enclosed in heavy box represent proteins (agglutinin) of Arthropoda are 'related' to *P. indicus* agglutinin.

5.4. DISCUSSION

The best way to investigate protein phylogeny is to compare the corresponding nucleotide sequence at the DNA level. Partial or complete amino acid sequences are not available for *P. indicus* agglutinin. In the present study we have isolated hemolymph agglutinin and complete amino acid composition of agglutinin protein was analysed. The best way to unravel the marvellous message of defense protein agglutinin is to find out the amino acid composition of subunits. The biological property of *P. indicus* agglutinin subunits were found to be agglutinating, hemolytic and antibacterial.

The complete amino acid composition of agglutinin subunit A and B was analysed using HPLC. Both the subunits predominate in acidic residues. Amino acid composition of subunit A and B are presented in Table 5.1. Majority of the amino acid in subunit A was comparable to the subunit B (Table 5.1 and 5.2). Subunit A predominate in Aspartic acid (13.56%), Glutamic acid (14.94%), Leucine (8.47%) and Arginine (7.3%). Subunit B was rich in Glutamic acid (15.12%), Aspartic acid (13. 35%), Leucine (8.44%), Arginine (7.17%). Both subunits (A & B) predominates in acidic residues with hemolytic, agglutinating and antibacterial properties (Table 5.1). The calculated mol. wt. of subunit A, 92.52 kDa was comparable to the measured mol. wt. of 97.0 kDa using SDS - PAGE and calculated mol. wt. of subunit B, 81.96 kDa which again approximates the measured mol. wt. of 84.0 kDa. Undetectable levels of Cysteine and low level of Methionine was observed.

Absence of Cysteine helps easy separation of polypeptide subunits via SDS - PAGE. The binding of polypeptide subunits of agglutinin is therefore considered as a non- - disulfide bond.

Chang *et al.* (1994) and Lee *et al.* (1997) observed the subunits of vitellogenin and vitellin are held together by non- - disulfide bonds. Agglutinins are usually composed of two or multiples of two subunits held together by non- - covalent (hydrogen) or disulfide bonds. The actual number of subunits and their arrangement may vary with different agglutinin molecules. For example, the agglutinin from the sea urchin, *Anthocidaris crassispina* comprises between 18 and 20 polypeptide chains bonded in pairs by disulfide bonds (Giga *et al.*, 1987). Where as agglutinin from the giant silk moth, *Hyalophora cecropia* contains two subunits A chain (Mr 38 kDa) and B chain (Mr 41 kDa) which combine non - covalently in different tetrameric proportions (*i.e.* A_4, A_3B, A_2B_2, AB_3, B_4) to form isolectins (Castro *et al.*, 1987). The blackgram lectins (183 kDa) was reported to be formed of two subunits 89 kDa and 94 kDa held together by non - covalent forces (Suseelan *et al.*, 1997). The mol. wt. and subunit structure of *P. indicus* agglutinin was comparable to few isolated agglutinins from invertebrates. A hemagglutinin Limulin from *Limulus polyphenus* of MW 400 kDa formed of 18 polypeptide chains of uniform size have been isolated by Machalonis and Edelman (1968). The hemolymph of octopus, *Octopus vulgaris* contains a lectin of Mr 260 kDa made up of similar subunits of Mr 30 - 32 kDa with differing isoelectric points (6.4, 6.6, 7 and 7.3) (Rögener *et al.*, 1985).

Biological activities of both A and B subunits of agglutinin was tested against bacteria and erythrocytes. Agglutinin subunit A and B exhibits agglutinating, hemolytic and antibacterial properties. The agglutinin A was found to be more antibacterial than subunit B (Table 5.1). This may be due to the predominance of acidic amino acids, Proline and Leucine. Acidic amino acids, Proline and Leucine enhanced antibacterial activities (Andreu and Merrifield, (1985). Net positive charge was found to be a prerequisite for antibacterial activity (Shai and Oren, 1996). Agglutination of non- self materials resulting in bactericidal or hemolytic activity may be due to complement lysis (Weir and Stewart, 1993). A molecule with these properties may likely to function as host defense protein. The difference in biological activities may be due to different arrangement of amino acids at N - terminal and C - terminal region of a protein and difference in the pattern of amino acids. This type of difference was studied by Fink *et al.* (1989) in Cecropin A and D. Change in position of amino acid alters the composition of agglutinin, which helps in the preparation of analogues. Protein rich in Glutamic acid, Leucine, Proline etc. shows hemolytic and antibacterial properties (Sitaram and Nagaraj, 1990). *P. indicus* agglutinin subunits rich in Glutamic acid, Leucine and Proline are likely to function as hemolytic and antibacterial protein.

Amino acid compositions cannot lead to give precise conclusions as sequence arrangement. But they are by no means empty of information, if

interpreted with care they will provide indication of relatedness. According to the polymorphism and common allelic origin of invertebrate agglutinins (Miller and Ratcliffe, 1994), it was decided to compare the different amino acid compositions within the isolated agglutinin subunits as well as between agglutinins from different organisms coming under different zoological groups. Both the subunits of agglutinin A and B shows close relatedness with S Δ Q units 1.096. This clearly indicates that subunit A and B differ in its amino acid composition only at 1.096 units. Our result was comparable to the S Δ Q unit of 1.02 relatedness obtained for subunit A and B of insulin (Cornish - Bowden, 1983). Table 5.3 summarises the result of relatedness calculated using S Δ Q equation. The compositional similarities between *P. indicus* hemolymph agglutinin and other proteins of amino acid composition belonging to other zoological groups can be arranged into two groups. The first group involves possible similarities between earthworm agglutinins δ and ∈. The 2nd group of possible similarities concerns the agglutinins of the same phyla, *Limulus* agglutinins α and β. The above mentioned four agglutinins exhibited compositional similarities with *P. indicus* subunits A and B. No similarities was observed between other agglutinins, lysozymes, antibacterial peptides and other hemolymph proteins such as Vn and Vg (Table 5.3). *Limulus* agglutinin α differs from *P. indicus* agglutinin subunit A in 68.53 amino acids and 66.25 amino acids with subunit B. *Limulus* agglutinin β differ in 65.11 and 63.83 amino acids from *P. indicus* agglutinin A and B. This relatedness will help to define the nature of protein. The result was comparable

to the S Δ Q values of insulin with sheep insulin, S Δ Q = 3 for the chain A and S Δ Q = 1 for the B chain. The S Δ Q units will be helpful in predicting the relatedness between proteins more efficiently with subunits (Cornish - Bowden, 1983). The agglutinin of *P. indicus* shows compositional similarity with agglutinins of same phyla (Arthropoda). *i.e.* with ***Limulus*** agglutinin (Table 5.3, heavy box). The compositional similarity of ***P. indicus*** agglutinin with agglutinin of earthworm with agglutinating, hemolytic and antibacterial activity was remarkable (Table 5.3, dotted box). Dissimilarity with other proteins were also explainable.

Invertebrates are a polyphyletic assemblage of animals which have evolved independently in response to different environmental pressures. So great heterogeneity in agglutinin structure and physico- chemical properties are possible. Differences are seen not only between agglutinins from members of diverse phyla, but also within phyla. Five different kinds of agglutinins with agglutinating, hemolytic and bactericidal properties have been isolated from the coelomic fluid of earthworm (Roch *et al.*, 1986). Variations have also been reported in the size and shape of agglutinins like that of sponge, *Axinella polyploides*, 15 and 21 kDa (Bretting and Kabat, 1976), agglutinin of *Biomphalaria glabrata*, a protein of mol. wt. 10^6 dalton (Boswell and Bayne, 1984). The majority of invertebrate agglutinins are naturally occurring in the hemolymph and its role in defense mechanisms have been studied in flesh fly, *Sarcophaga peregrina*, 190 kDa (Komano *et al.*, 1980). Agglutinin functions

as recognition molecules in defense reaction have been reported by Renwrantz (1983) and its role in facilitating hemolysis by Komano and Natori (1985).

Amino acid composition of prawn agglutinin shows relatedness to *Limulus* agglutinin from amoebocyte membrane which functions as agglutinating, antibacterial factor and its concentration increases due to injury (Liang *et al.*, 1980). A large number of true antibacterial peptides have been isolated from the hemolymph of invertebrates (Steiner *et al.*, 1981; Azumi *et al.*, 1990; Xylander and Nevermann, 1990). Primary and secondary structure of few isolated and sequenced antibacterial peptides have been engineered which are helpful in defense mechanism (Nagaraj, 1997). Chemical synthesis of Cecropin A and D, insect antibacterial peptide and its analogues with enhanced antibacterial activity have been reported (Merrifield *et al.*, 1982; Andreu and Merrifield, 1985; Fink *et al.*, 1989). A large number of antibacterial chemotherapeutants have been in use in Finfish Aquaculture (Stoffregen *et al.*, 1996).

Aquaculture industry in India is already well established in the area of prawn and mussel culture. Increased domestic production can be successfully achieved through advances in nutrition and efficient use of disease resistant mechanisms. Diagnosis and prevention of diseases through adding antibiotics are now replaced by the addition of relatively less expensive but more reliable antibacterial substances derived from insect antibacterials such as Cecropins and Attacins.

In the present study, we have isolated and purified a natural agglutinin in the hemolymph of *P. indicus* with antigenecity, agglutinating, hemolytic and antibacterial properties. Complete amino acid composition and biological activities of both the subunits were analysed. *P. indicus* agglutinin showed similarity with those proteins with similar biological properties like hemolysis, agglutinating and antibacterial nature. It was concluded that *P. indicus* agglutinin was a specific defense protein found naturally dissolved in the hemolymph. The arrangement of amino acid will be helpful in the synthesis of new antibacterial analogues which can be used against disease causing organisms. Further work has to be done in the field of sequencing, preparation of analogues so that it can supplement with food of prawns. At large extent, new cDNA can be structured, cloned in *E. coli* and the synthesized agglutinin can be lyophilized and used as antibacterial agents against potential pathogens. Further investigations in this field will be fruitful not only for its applied purposes but also in basic science concerning the relationship between agglutinin and its role in defense mechanism.

Summary

SUMMARY

Isolation and characterization of Agglutinin in the hemolymph of *Penaeus indicus* H. Milne Edwards have been studied under five major sections in the thesis.

1. Relationship between growth rate, nucleic acid, Protein and body length with reference to Nervous system, Eyestalk and Muscle tissue in *P. indicus.*

2. Biochemical analysis of hemolymph.

3. Isolation, partial characterization of agglutinin in the hemolymph, study of hemocytes and clotting mechanism.

4. Immunological characterization of agglutinin through double immunodiffusion and ELISA.

5. Amino acid composition of agglutinin.

RNA, DNA and protein contents were measured in Nervous system, Eyestalk and Muscle tissue; percentage growth rate (%G/ day) and specific growth rates (SGR) (changes in protein or nucleic acid/ day) were calculated with changes in body length to assess the growth rate and differential gene

action during ontogenesis. DNA, RNA and protein concentration of nervous system decreased from G I to G VIII, which reflects a negative correlation with increase of body length. In eyestalk and muscle tissue, DNA and RNA concentration decreased from G I to G VIII, where as protein content increased significantly with respect to increase of body length.

Percentage growth rate (%G/d) of *P. indicus* decreased significantly as a linear function of increase in body length from G I to G VIII with maximum growth rate in G II prawns. $RNA/_{DNA}$ ratio and specific growth rate (in terms of RNA, DNA, $RNA/_{DNA}$ and protein) in three regions, showed cyclic fluctuation in the order G II > G VII > G V *($P < 0.05$)* reflects differential gene action due to molting process.

Increase of band numbers observed from G I – G VIII electropherogram of three regions exemplifies growth process. The intensity of protein bands in the electropherogram of nervous system decreased from G I to G VIII, where as intensity and density increased from G I to G VIII in the case of eyestalk and muscle tissue. The phenomenon occurred in nervous system might be due to the transport of various synthesized products to the hemolymph where as in eyestalk and muscle tissue synthesis and accumulation of protein occurs.

The quantitative data of nucleic acid and protein reveals that growth being maximum in G II prawns and % G/d decline from G I to G VIII.

Electropherogram of three regions and $RNA/_{DNA}$ ratio predicts growth resulted through the synthesis and accumulation of protein as flesh in a discontinuous pattern due to differential gene action.

Biochemical constituents in *P. indicus* with respect to developmental stages and influence of environment on the hemolymph constituents was compared among the samples collected from three localities (Cherai, Azhikode and Chettuva).

Total protein, agglutinin, total free amino acid and proline concentration in the hemolymph exhibited an increasing trend from G I to G VIII. Significant variation was observed among 8 size groups *($P < 0.01$)* as a function of growth and maturation. The same trend was observed in total protein, agglutinin, total free amino acid and proline content in the hemolymph of sample collected from Chettuva, Azhikode and Cherai. The hemolymph of prawn from the mud bank (Chettuva) recorded maximum concentration of protein, agglutinin, total free amino acid and proline. This may be due to the impact of mud bank environment on the hemolymph. Cations such as Sodium, Potassium, Calcium and Magnesium content in the hemolymph exhibited a linear increase from G I to G VIII.

Prevalence of four cations was observed in the order $Na^+ > K^+ > Ca^{2+} > Mg^+$. High sodium index and low magnesium index was observed. Significant variation was observed in cation concentration in the hemolymph between

Chettuva, Azhikode and Cherai. The order of prevalence was same as above, $Na^+ > K^+ > Ca^{2+} > Mg^+$. The *P. indicus* hemolymph from Chettuva (mud bank) recorded higher levels of Na, K, Ca, and Mg. Mud bank formation in the Arabian Sea during the south-west monsoon is a temporary phenomenon. The increased concentration of cations observed might be due to the augmented secretion of hormones during stress conditions, for that prawns osmoregulate and establish mineral balance. The mud bank (Chettuva) with high productivity, low salinity and stressful conditions lead to the increased concentration of total protein, agglutinin, total free amino acid, protein and cations.

Naturally occurring agglutinin in the hemolymph of *P. indicus* was isolated and characterized. The hemocytes of *P. indicus* were classified and clotting mechanism studied.

The hemocytes of *P. indicus* were studied based on their functions and are classified into hyaline cells, semi -dense granulocytes and dense granulocytes. Hemocytes attributes cellular defense through the formation of clots and secretes humoral substance such as agglutinins to the hemolymph. Clotting mechanism in *P. indicus* was depicted as type (B) where coagulation was initiated through hemocyte agglutination followed by gelation of plasma. Hyaline cells release clotting enzymes. Hemolymph contains dissolved fibrinogen like factor, which during clotting is polymerised to covalently crosslinked gel by catalytic action of transglutaminase in the presence of Ca^{2+}.

Agglutinin present in the plasma, serum and clottable solution agglutinated to varying degrees to *Vibrio* sp. isolated from diseased prawn gut (Titer 64, 64 & 128). Natural agglutinin in the hemolymph resulted in hemolysis and crosslinking with erythrocytes of Rat, Rabbit and human (Titers 128, 128 & 256). Agglutinin, a naturally dissolved humoral factor in the hemolymph of *P. indicus* recognizes foreign particles such as bacteria and erythrocytes through agglutination. Natural agglutinin also functions as antibacterial agent against *Vibrio* sp. isolated from diseased prawn and with *E. coli.*

Agglutinin was isolated through chromatographic procedures (Sephadex G - 200) by monitoring its biological properties. Purity of agglutinin was tested by running through Native PAGE and molecular weight subunits were resolved under SDS - PAGE. Agglutinin was found to be a macromolecule with 181,000 Dalton with two subunits (97 kDa and 84 kDa).

The physical and chemical stability of agglutinin was studied. Agglutinin was heat -labile and inactivated at 85°C. *P. indicus* agglutinin was stable to pH values ranging from 7-8, 10-11 and gets reduced, but never completely inactivated at extreme acid or alkaline values. *P. indicus* agglutinin was stable to dialysis, storage at low temperature, repeated freezing and thawing. All these characterization proves proteinaceous nature of agglutinin. Agglutinating property was stimulated by few sugars such as glucose,

galactose, lactose, maltose and cellobiose and inhibited by Xylose, Raffinose and Trehalose. Ca^{2+} ions released from $CaCl_2$ stimulate the agglutinating property of agglutinin. Purified naturally occurring agglutinins in the hemolymph of *P. indicus* was conformed as a polyfunctional protein, a dimmer of molecular weight 181,000 Dalton (97 kDa & 84 kDa) with antigenecity, agglutinating, hemolytic and antibacterial properties.

Immunological characterization of agglutinin was carried out through developing double immunodiffusion and ELISA. Polyclonal antibodies (anti - Ag) were raised in New Zealand white male rabbits against purified agglutinin of *P. indicus*. Single precipitin line formed between agglutinin and anti - agglutinin confirmed the antigenecity of agglutinin and presence of anti - agglutinin. Purified Ig G fraction from anti- Ag immune serum was used for ELISA development.

The agglutinin standard curve was linear over a range of 17.8 ng - 8,900 ng. The sensitivity of the ELISA was 178 ng/ml. Agglutinin free hemolymph yielded background optical density values, confirming the specificity of the assay. Serial dilutions of agglutinin rich hemolymph produced sample titration curves that are parallel to the Ag standard cure. Thus ELISA is suitable for quantification of Ag in the hemolymph. Naturally occurring agglutinin in the hemolymph varies in its concentration according to external physiological state (stress, pH, nutrient status etc.) and susceptibility

of the species to various diseases. Thus ELISA developed will be helpful in health monitoring studies of prawn, *P. indicus*.

Amino acid composition of agglutinin subunits A & B involved in various defense processes were established. Agglutinin subunit A was characterized by high amount of glutamic acid (14.94%) and aspartic acid (13.56%). Subunit B was characterized by high amount of glutamic acid (15.12%) and aspartic acid (13.35%). Cystine was absent in both the subunits. Calculated molecular weight of agglutinin subunits A & B, 92.52 kDa and 81.96 kDa was comparable to the measured mol. wt. of 97 kDa and 84 kDa in SDS – PAGE. Both the agglutinin subunits exhibited agglutination, hemolytic and antibacterial properties. Subunit A is more antibacterial and less hemolytic when compared to subunit B, which is more hemolytic and less antibacterial.

Agglutinin subunits A & B show compositional similarities with 1.096 units as maximum S Δ Q value proves common allelic origin. Compositional similarities were evidenced between *P. indicus* agglutinin subunits (A & B) and Earthworm defense protein δ and ε, with corresponding S Δ Q values of 98.83, 96.35, 68.98 and 65.69. *P. indicus* agglutinin also exhibits relatedness with *Limulus* agglutinin in the order 68.5, 66.25, 65.11 and 63.83 as S Δ Q values. From the relatedness calculation it was evident that *P. indicus* agglutinin was a specific defense protein showing relatedness to protein having agglutinating, hemolytic and antibacterial properties.

In the present study, we have isolated and purified a natural agglutinin in the hemolymph of *P. indicus* with antigenecity, agglutinating, hemolytic and antibacterial properties. The influence of growth and environmental parameters on the level of agglutinin in the hemolymph was studied. Agglutinin concentration during normal growth process was compared. The agglutinin concentration in the hemolymph was quantified through developing ELISA, which is helpful in health monitoring studies of individual species. Complete amino acid composition of both the subunits of *P. indicus* agglutinin were analysed. *P. indicus* agglutinin showed similarity to those proteins having antigenecity, hemolytic and agglutinating properties. Hence, agglutinin was considered as a natural defense protein in the hemolymph of *P. indicus* responsible for immune surveillance. The humoral defense mechanism of agglutinin was a co-operative effort with hemocytes and complement system. The composition of isolated agglutinin of *P. indicus* amino acids will be helpful in the synthesis of new antibacterial analogues which can be used against disease causing organisms.

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* *Original not seen.*