PARTIAL PROTEASE CHARACTERIZATION, *IN VITRO* PROTEIN DIGESTIBILITY AND UTILIZATION OF DIETARY SOYBEAN IN GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* (DE MAN)

by

MD. AYAZ HASAN CHISTY

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

May 2005

ACKNOWLEDGEMENTS

All praise for Allah, the most gracious, the most merciful, Whose blessings make me able to submit this thesis. At first, I'd like to express great honor, profound thanks and deepest appreciation to my supervisor, Prof. Dr. Roshada Hashim, for her great supports, thoughtful guidance, continuous encouragement and constructive criticisms during the course of the study. I would like to express my gratitude to Dr. Alexander Chong whose valuable advice and kind permission of his lab access helped me a lot for the study. I'd also like to take the opportunity to acknowledge Dr. Sudesh for using his lab facilities.

I extend my heartfelt thanks to all my fellow researchers and colleagues of the Aquaculture Research Group, School of Biological Sciences, USM, for their kind cooperation and amicable association throughout the study period. Special thanks to Mr. Jamil and Auntie Anna for their kind assistances and cooperation throughout the experimental period.

I wish to thank all my friends and colleagues in Khulna University, Bangladesh and USM for their best wishes, especially to Mahbub for his sovereign behave and accompany. I must express my gratefulness to all my family members and relatives for their prayers and continuous inspiration all the time. I express profound gratefulness to my parents for their prayer, patience and untiring support in every way of my life and gratitude to my wife and daughter.

The study was funded by Support for University Fisheries Education and Research (SUFER) Project, a joint research project between University Grants

Commission of Bangladesh (UGC) and Department for International Development (DFID), UK.

Finally, I'd like to acknowledge the authority of Khulna University, Bangladesh for allowing me the study leave, the dean and all other officials of the School of Biological Sciences and IPS for their kind approval and arrangement to study here in Universiti Sains Malaysia.

Md. Ayaz Hasan Chisty

TABLE OF CONTENTS

CHAPTER 6: SUBSTITUTION OF FISHMEAL PROTEIN WITH SOY MEAL IN DIETS FOR THE CULTURE OF GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* (*DE* MAN) IN EARTHEN PONDS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviations	Full name
BAPNA	Benzoyl-Arg-*p*-nitroanilide
COC	Coconut oil cake
DMSO	Dimethylsulfonyl oxide
EDTA	Ethylene-diamine-tetra-acetic acid
ExSM	Extracted soybean meal
FCR	Food conversion ratio
FM	Fishmeal
GE	Gross Energy
MOC	Mustard oil cake
PER	Protein efficiency ratio
PMSF	Phenylmethylsulfonyl fluoride
RPD	Relative protein digestibility
SAPNA	Succinyl-$(Ala)_2$-Pro-phe-*p*-nitroanilide
SGR	Specific growth rate
SOC	Sesame oil cake
TLCK	Tosyl-lysinechloromethyl ketone
TPCK	Tosyl-phenylalanine chloromethyl ketone
ZPCK	Carbobenzoxy-Phe-*p*-chloromethyl ketone

LIST OF APPENDICES

SEBAHAGIAN PENCIRIAN PROTEASE, PENGHADAMAN PROTEIN SECARA *IN VITRO* DAN PENGGUNAAN DIET BERASASKAN KACANG SOYA DALAM UDANG GALAH, *MACROBRACHIUM RESENBERGII* (DE MAN)

ABSTRAK

Pemerhatian secara asai biokimia dan elektroporesik dengan menggunakan SDS-page telah dijalankan sebagai sebahagian daripada pengenalpastian dan pencirian proteases dalam sistem penghadaman udang galah (Macrobrachium resenbergii). pH optima bagi ekkstrak kasar enzim udang menunjukkan 3 puncak aktiviti iaitu pada pH 3.0, 6.0 dan 9.0. Proteinase berasid dan trypsin menunjukkan aktiviti yang lebih tinggi pada pH 2.5 dan 9.0 selepas 3 dan 5 jam selepas pemberian makanan terakhir. Kehadiran protenease dari kelas serine telah dikenal pasti dengan menggunakan PMSF telah mengurangkan 33% daripada keseluruhan aktiviti proteolitik. Pengurangan aktiviti sebanyak 82% dan 55% apabila menggunakan perencat khusus TLCK dan TPCK, masing-masing telah mengesahkan kehadiran trypsin dan kimotrysin manakala EDTA telah mengurangkan 34% aktiviti yang seumpamanya dengan kehadiran metallo-proteinase. Pencirian proteinases beralkali oleh 12% SDS-PAGE telah menunjukkan 6 jalur aktif pada 13 hingga 136kDa jisim molar selepas inkubasi ekstrak sama ada dengan kehaditan mahupun tanpa kehadiran perencat khusus. 2 jalur tripsin pada 13 dan 136 kDa, 3 jalur kimotripsin pada 23, 47 dan 73 kDa dan 1 jalur metallo-proteinase pada 136 kDa telah dikenalpasti daripada zimogram. Ini umpamanya memperlihatkan bahawa pepsin yang bertanggungjawab bagi proses awal penghadaman protein manakala tripsin dan kmotripsin merupakan enzim yang memainkan peranan bagi proses yang seterusnya.

Dua teknik in vitro iaitu kaedah Lazo yang menggunakan satu enzim iaitu tripsin lembu dan asai enzim udang digunakan bagi mengukur keterhadaman protein relative (RPD) bagi bahan mentah diet iaitu serbuk ikan (FM), ekstrak serbuk kacang soya (ExSM), kek minyak mustard (MOC), kek minyak bijan (SOC)dan kek minyak kelapa (COC) dan

7 diet praktikal. RPD bagi bahan mentah dan diet yang menggunakan kaedah Lazo ialah 44-61% dan 42-53% manakala RPD dengan menggunakan enzim udang adalah 34-82% dan 74-84% masing-masing. Ini menunjukkan keputusan yang bertentangan di antara 2 kaedah ini. Pada perbezaan secara statistic ($p<0.05$). maka boleh dianggap bahawa enzim udang dapat memberikan petunjuk yang lebih baik dalam menentukan RPD. Kesan perencatan in vitro bagi bahan mentah pada aktiviti protease udang adalah pada julat yang besar iaitu 4-56%. Kesan FM pada pengurangan aktiviti adalah terendah (4%) apabila larutan protein dari Fm diinkubasi dengan perut udang dan ekstrak hepatopankreas. Secara perbandingan kesan yang lebih tinggi dipantau pada COC dan SOC (56% dan 52%) dalam asai manakala ExSM dan MOC mengurangkan aktiviti masing-masing sebanyak 19% dan 5%. Kehadiran perencat protease dalam bahan mentah dilihat sebagai kelesepan secara sebahagian atau keseluruhan jalur berbanding zimogram yang tidak direncat dari SDS-PAGE.

Tujuh diet isonitrogenus (protein 35.44 ±0.73%) dan isokalorik (GE: 17.88 ± 0.37 kJ g^{-1}) dirumuskan dengan penggantian protein FM dengan ExSM (20-70%) digunakan sepanjang 3 bulan kajian pertumbuhan *M. resenbergii* dalam akuarium. Parameter pertumbuhan udang yang diberi makan diet berasaskan FM (kawalan) adalah tidak berbeza secara signifikan ($p<0.05$) dalam pertumbuhan berat badan (0.53-0.76g), SGR (3.0-3.41), FCR (1.92-3.2), PER (0.96-1.37) dan kemandirian (53- 78%) berbanding diet yang lain-lain. Berikutan itu, pertumbuhan sepanjang 95 dan 118 hari dijalankan dalam kolam bermusim (kolam kajian 1 dan 2) dengan menggunakan 5 diet isonitrogenus (protein 25.73 ± 0.65%) dan isokalorik (GE: 18.04 ± 0.16 kJ g^{-1}) dengan penggantian protein FM kepada Ex SM pada 0, 30, 40, 50 dan 80% (kawalan, diet 1 hingga diet 4). Prestasi pertumbuhan didapati lebih baik pada udang yang diberi rawatan diet 4 dalam kolam eksperimen 1 dengan pencapaian FCR yang lebih rendah (1.09) dan PER, SGR dan tuaian yang lebih baik (masing-masing 3.43, 2.6% dan 653 kg ha-1 95 hari-1) juga menunjukkan perbezaan yang signifikan ($p<0.05$) berbanding kawalan. Prestasi pertumbuhan yang sama juga diperhatikan pada udang yang diberi makan diet 4 dalam kolam eksperimen 2 dengan pencapaian nilai FCR yang lebih rendah (2.97) dan nilai PER, SGR dan tuaian serta kemandirian yang lebih baik (masing-masing 1.51, 1.31%, 382 kg ha-1 118 hari-1 dan 72%). Namun tiada perbezaan yang signifikan ($p<0.05$) dengan kawalan. Maka bagi kos penyediaan diet yang efektif , 80% protein FM dapat digantikan dengan ExSM bagi ternakan *M. resenbergii* dalam kolam.

PARTIAL PROTEASE CHARACTERIZATION, *IN VITRO* PROTEIN DIGESTIBILITY AND UTILIZATION OF DIETARY SOYBEAN IN GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* (DE MAN)

ABSTRACT

Biochemical assays and electrophoretical observations using SDS-PAGE was conducted for partial identification and characterization of proteases in the digestive system of giant freshwater prawn (*Macrobrachium rosenbergii*). pH optima of the crude enzyme extracts of prawn showed 3 peaks in activity at pH 3.0, 6.0 and 9.0. The acid proteinase and trypsin at pH 2.5 and 9.0 showed higher activity respectively at 3 and 5hrs after the last feeding. Presence of serine class proteinase was identified using PMSF that reduced 33% of the total proteolytic activity. Reduction in activity by 82% and 55% using specific inhibitors TLCK and TPCK confirmed the presence of trypsin and chymotrypsin respectively while EDTA reduced 34% activity resembles the presence of metallo-proteinase. Characterization of the alkaline proteinases by 12% SDS-PAGE, after incubation of extract with or without specific inhibitors, produced six active bands of 13 to 136 kDa molar mass. Two trypsin bands of 13 and 36 kDa, three chymotrypsin bands of 23, 47 and 73 kDa and one metallo-proteinase of 136 kDa were identified from zymogram. The results suggest that an acid protease responsible for the initial digestion of proteins and trypsin and chymotrypsin are the main enzymes responsible for the rest.

Two *in vitro* techniques, viz. Lazo single enzyme assay using bovine trypsin and prawn crude enzyme assay, were used to measure the relative protein digestibility (RPD) of five feed ingredients namely fishmeal (FM), extracted soybean meal (ExSM), mustard oil cake (MOC), sesame oil cake (SOC) and coconut oil cake (COC) and seven practical diets. The RPD of feed ingredients and diets in Lazo single enzyme assay ranged between 44-60% and 42-53% and that of prawn crude enzyme were 34-82% and 74-84%. However the results between these two methods were contradictory in that the RPD values were found to be higher in prawn enzyme but lower when Lazo

enzyme system was used. Thus it can be assumed that prawn crude enzyme assay method gives a better indicator in determining the RPD. *In vitro* inhibitory effects of different food ingredients on prawn protease activity varied from 4-56%. The effect of FM on the reduction in activity was lowest (4%) when the protein solution from FM was incubated with prawn stomach and hepatopancreas extract. Comparatively higher affect of COC and SOC was observed (56 and 52%) in the assay while ExSM and MOC reduced the activity by 19% and 5% respectively. The presence of protease inhibitors in the ingredients were visualized by partial or complete disappearance of bands comparing with the zymograms of no inhibition from SDS-PAGE.

Seven iso-nitrogenous (protein 35.44 ± 0.73%) and iso-caloric (GE: 17.88 ± 0.37 kj g^{-1}) diets, prepared by substituting FM protein with ExSM (20-70%), were used for a 3 month study in *M. rosenbergii* under aquarium condition. The growth parameters of prawns fed on FM based diet (Control) were not statistically different ($P > 0.05$) in weight gain (0.53-0.76g), SGR (3.0-3.41), FCR (1.92-3.20), PER (0.96-1.37) and survival (53-78%) with other diets. A follow up study based on a 95 and 118 days trial in seasonal ponds (pond experiment-1 and 2) using five iso-nitrogenous (protein: 25.73 ± 0.65 %) and iso-caloric (GE: 18.04 ± 0.16 kj g^{-1}) diets, substituting the FM protein with ExSM by 0, 30, 40, 50 and 80% (control, diet-1 to diet-4) was conducted. Better growth performances of prawns fed on diet-4, in pond experiment-1, were observed by achieving lower FCR (1.09) and higher PER, SGR and yield (3.43, 2.60% and 653 kg ha^{-1} $95days^{-1}$ respectively) that showed significant differences ($P < 0.05$) with control. Similar growth performance of prawns in pond experiment-2 was evident by the lower FCR (2.97) and higher PER, SGR, yield and survival (1.51, 1.31%, 382 kg ha^{-1} $118days^{-1}$ and 72% respectively) when diet-4 (80% replacement) was used, however this was not statistically different ($P > 0.05$) with the control. For the cost effective diet preparation, 80% FM protein can be substituted with ExSM for *M. rosenbergii* culture in ponds.

CHAPTER ONE
GENERAL INTRODUCTION

1. 0 General Introduction

The wealth of aquatic resources has always been assumed to be an unlimited gift of nature. Hence, with increasing knowledge and the dynamic development of fisheries after the Second World War, this myth has since faded. In the face of reality, the aquatic resources although renewable, are not infinite and need to be properly managed, if their contribution to the nutritional, economic and social well-being of the growing world's population is to be sustained (FAO, 1996). World fisheries have undergone rapid changes during the last decades. New technologies, creation of Exclusive Economic Zone (EEZ), the 1982 UN convention of the Law of the Sea (UNCLOS) and other development have brought about drastic changes in the fisheries management and enhanced access, and significant expansion of effort and production (Ahmed *et al.*, 1999).

It is widely acknowledged that fish supplies from traditional marine and inland capture fisheries are unlikely to increase substantially and that the projected shortfalls in fish supply will probably be met mainly from the expansion within the aquaculture sector (FAO, 2002a). Aquaculture is one of the fastest growing food-producing sectors, providing an acceptable supplement to and substitute for wild fish and plants.

To meet the challenges of food security and to generate the employment and foreign exchange, aquaculture has clearly demonstrated its potentiality by the rapid expansion of this sector, which has grown at an annual percent rate (APR) of almost 10 since 1984 compared with 3 for livestock meat and 1.6 for capture fisheries production (Rana, 1997). Much of the reported increase originated from the low-income food deficit countries, in particular China, Indonesia, India, and Bangladesh and reflects the increasing trend in these countries by increased use of aquatic resources to further diversify food production (FAO, 1997). Regional, cultural and historic attributes have played major roles in influencing both the production base and rate of expansion of aquaculture. The historic tradition of growing fish in Asia, which is well documented in countries such as China, India, Cambodia and Indonesia, has played a significant role in maintaining Asia's dominant role in aquaculture. Production of finfish, shellfish and plants from culture in Asia accounted for over 90% of world output (FAO, 1997). Production areas of these countries are being expanded by mobilizing land resources unsuited to agriculture (saline soils, waterlogged areas, etc.), under-utilized water bodies (for cage and pen culture), seasonal water bodies, and rain-fed ponds, and by use of irrigation systems and integration with agriculture.

Varieties of cultural, socio-economical and institutional settings together with the diversified set of aquatic species and farming practices has given a multidimensional identity in the sector of aquaculture. The vast majority of aquaculture practices around the world have previously been pursued with significant nutritional and social benefits, and generally with little or no environmental costs (FAO, 1997). Issues of sustainability can be expected to change in perceptions of desirable forms of aquaculture development and management (Roberts and Muir, 1995); new ways of farming that strike a balance between food security and the environmental and resource costs of production will have to be adopted (Roberts and Muir, 1995; Pillay, 1996).

Aquaculture has become recognized as an economic important sector in many developing countries and has been able to attract the attention of many private and public sectors. Development plans of most producing countries are aimed at increasing fish supplies from aquaculture for the local and export markets, and at increasing the sector's contribution to food security in rural areas.

The rapid growth in aquaculture production has made the sector important to many countries and, in the case of some traded aquatic products; the sector has become either an important source of supply or the main supplier. In these cases, fluctuation in production of farmed products has significant impact on price trends. Crustacean, the most prominent product from aquaculture in international trade, is marine shrimp and aquaculture has been the major force behind increased shrimp trading during the past 7-8 years. Shrimp is already the most traded seafood product internationally, and in 1996 about 25% or 700,000 mt came from aquaculture (Rosenberry, 1996). Since the late 1980s, farmed shrimp have tended to act as a stabilizing factor for the shrimp industry. Therefore, the major crop failures in Asia and Latin America during the past few years have had an impact on overall supply, demand, prices and consumption trends. For example, shrimp consumption declined in the US in 1995 due to lower imports caused by declining supplies from Asian countries (FAO, 1997).

The major markets of shrimp and prawn are Japan, the USA and, to a lesser extent, the European Union (EU). The largest exporters of farmed shrimp are Thailand, Ecuador, Indonesia, India, Mexico, Bangladesh and Vietnam (Branstetter, 1997). Demand for shrimp and prawn is expected to increase in coming years. Asian markets such as China, the Republic of Korea, Thailand and Malaysia, may expand as local economies grow and consumers demand more seafood. This trend is already reducing the availability of shrimp to traditional importers and will eventually put upward pressure on prices if supplies do not expand. Increase in prices will encourage new entries into shrimp farming, if sustainable methods of production are practiced that would help to avoid production crashes.

Worldwide production of the freshwater prawn, *Macrobrachium rosenbergii*, increased 636% between 1989 and 1998 while its value increased 957% during the same period (New, 2000). This is partially due to several biological characteristics which are positive attributes for their aquaculture production. Freshwater prawns do not appear to be susceptible to most of the viral diseases which have impacted marine (penaeid) shrimp production (Wang et al., 1998). Also, being freshwater species, they can be produced in inland locations in closer proximity to large urban markets. Freshwater prawns produce large individual sizes which can command high prices in the market. In addition, prawn production tends to be more environmentally sustainable. This is largely due to their more territorial nature, which limits the biomass densities in grow out ponds compared to penaeid species (New, 2000). However, these lower production levels have also been a major constraint on their commercial development compared to several penaeid species. Prawns also tend to have greater size variation at harvest, which can negatively impact marketing. To increase the commercial viability of prawn production, it is important that production rates be increased, large average sizes maintained, and the magnitude of size variability decreased, without sacrificing environmental sustainability.

There has been a growing trend within most developing countries and many developed countries (e.g. Japan) toward the increased use of artificially compounded feeds (aqua-feeds) for farmed finfish and crustaceans. This trend has been particularly apparent within developing countries with the progressive intensification of farming systems, and in particular within the main low-income food deficit countries for the production of both lower-value staple food fish species (mainly freshwater finfish such as carp, tilapia and catfish) and higher-value cash crop species for luxury or niche markets (mainly marine and diadromous species such as shrimp, salmon, trout, yellowtail, sea bass, sea bream and grouper). In fact, the production of aqua-feeds has been widely recognized as one of the fastest expanding agricultural industries in the world, with annual growth rates in excess of 30% per year (Rabobank, 1995; Tacon, 1996). On the basis of different categories of species, 25% of total aqua-feed production was for shrimp.

At present, nearly all farming operations for carnivorous freshwater fish, marine fish and crustaceans, which are based upon the use of aquafeeds, are net fishery resource 'reducers' rather than 'producers'. The quantity of inputs of dietary fishery resources, in the form of fishmeal, fish oil, crustacean by-product meals, `trash fish', etc. has exceeds outputs of farmed fishery products by a factor of 2 to 3. Production of the 3 million mt of farmed marine/diadromous finfish/crustacean species (wet basis) in 1995 would have required over 1.5 million mt of fishmeal and fish oil (dry basis) or the equivalent of over 5 million mt of pelagic fish (wet basis; assumes a pelagic to fishmeal conversion factor of 5:1) (FAO, 1997).

Despite the superior nutritional and economic merits of feeding regimes based upon the use of fishery resources as feed inputs for carnivorous fish and marine shrimp, the future availability and cost of these feed ingredients are both uncertain and unstable. Despite optimistic projections made by the fishmeal and fish oil manufacturing industry concerning the availability and use of these fishery products for animal feeds (including aqua-feeds) over the next decade, there are increasing doubts regarding the long-term sustainability of farming systems based entirely upon these finite and valuable fishery resources, in particular concerning the efficiency and ethics of feeding potentially food-grade fishery resources back to animals (including fish) rather than feeding them directly to humans (Best, 1996a; Hansen, 1996; Pimentel *et al.*, 1996; Rees, 1997).

The efforts must be placed on the use of by-products from the much larger and faster-growing terrestrial agricultural production sector, including: 1) terrestrial animal by-product meals resulting from the processing (i.e. rendering) of non-food grade livestock by-products; 2) plant oilseed and grain legume meals; 3) cereal by-product meals; and 4) miscellaneous protein sources such as single-cell proteins, leaf protein concentrates, invertebrate meals, etc. However, the eventual success of these potential feed resources as fishmeal replacement in aquafeeds will depend upon the further development and use of improved techniques in feed processing/manufacture (Riaz, 1997; Watanabe and Kiron, 1997) and feed formulation, including the increased use of specific feed additives such as feeding stimulants, free amino acids, feed enzymes, probiotics and immune-enhancers (De Vresse *et al.*, 1997; Feord, 1997; Hardy and Dong, 1997).

Moreover, efforts should be made whenever possible to upgrade, through the use of improved processing methods, and the increased usage of locally available feed ingredient sources so as to reduce the current dependence of imported sources (Rabobank, 1995; Best, 1996b). The local production of farm-made aquafeeds by small-scale farmers plays an important role in that it facilitates the use of locally available feed ingredient sources and agricultural by-products that would otherwise not be used (New *et al.*, 1995).

In semi-intensive and intensive culture of shrimp species, artificial feeds are an important nutrient source. The feeds must be nutritionally adequate and economical for the system of culture. Feed is the major cost variable in shrimp aquaculture representing up to 60% of total costs (Akiyama *et al.*, 1992; Sarac *et al.*, 1993). Since protein is the most critical ingredient in shrimp diets from standpoint of cost (New, 1976; Akiyama and Dominy, 1991) and growth response (Andrews *et al.*, 1972; Balazs *et al.*, 1973; Lim *et al.*, 1979; Alava and Lim, 1983), research in feed formulation is currently concentrating on investigations of various economical protein sources. Ideally these should be readily available at low cost and have high nutritional quality

(D'Abramo and Lovell, 1991; O'Sullivan and Watson, 1991). The diets should preferably be formulated from locally available feed ingredients to make the formulation easier and keep costs low (Rajyalakshmi *et al.*, 1986). New (1976) in a review of dietary studies with shrimp concluded that the source, dietary level and amino acid composition of proteins are the most important criteria for prawn nutrition.

According to Tacon (1997a), if the finfish and crustacean aquaculture sector is to sustain its rapid growth rate (11.7% per year since 1984), the aquafeed manufacturing sector will have to compete successfully with other users, including humans and the much larger animal livestock production sector for available feed resources. Therefore, the aquaculture sector will have to base its feeding regimes upon the use of feed ingredient sources whose global production and availability can keep pace with the increasing needs of a growing and hungry world. For example, in terms of global protein supply, soybean meal production has been growing more than four times faster than has fishmeal production: an average annual growth rate between 1984 and 1995.

The quantity and quality of dietary protein are primary factors influencing prawn growth. In commercially manufactured feeds for prawn culture, marine protein sources fish, shrimp and squid meals are one of the primary protein sources being included in the feed. Marine protein sources are often utilized in aquatic feeds because they are an excellent source of indispensable amino acids, essential fatty acids, vitamins, minerals, and generally enhance palatability. Marine by-products such as scallop waste, lobster waste, squid viscera and shrimp head meal have been evaluated as alternative marine protein sources (Sudaryono *et al.*, 1995). However, fish meals and marine by-products are commodities for which supplies are limited and demand is expected to continue to increase. Hence, maintenance of the economical viability of commercial aquaculture will require the replacement of expensive marine proteins, with lower cost ingredients for which production is not limited. Feed costs constitute 40-60% of operational costs in production of the freshwater prawn *Macrobrachium rosenbergii* (D' Abramo and Sheen, 1991). Initial efforts to reduce feed costs for aquaculture species often include replacement of expensive animal protein with plant protein meals, which are generally less expensive (Piedad-Pascual *et al.*, 1990; Tidwell *et al.*, 1993), or elimination of excess vitamin and mineral premixes from the diet (Triño *et al.*, 1992).

Protein sources that can be utilized to replace marine protein sources, either partially or completely, include both terrestrial plant and animal sources that are either locally available or traded on the world market. Replacement of marine proteins in shrimp feeds has met with different degrees of success. Considerable attention has been devoted to the evaluation of plant proteins such as: soybean meal (Lim and Dominy, 1990; Piedad-Pascual *et al.*, 1990; Tidwell *et al.*, 1993; Sudaryono *et al.*, 1995), solvent-extracted cottonseed meal (Lim, 1996), lupin meals (Sudaryono *et al.*, 1999), various legumes cowpea, green mungbean, rice bean and leaf meals (Eusebio, 1991; Eusebio and Coloso, 1998). Because of their low price and consistent quality, plant proteins are often an economically and nutritionally viable source of protein. However, problems persists with deficient levels of indispensable amino acids e.g., lysine and methionine, anti-nutrients and poor palatability. Different protein sources are used for commercial feed production to reduce costs. Soybean protein is currently used to supplement feeds for cultured shrimp to reduce costs. In commercial diets for shrimp, a combination of different meals (fish, shrimp, squid and soybean) are traditional sources of dietary protein. Usually the marine animal/fishmeals are expensive fractions and should be utilized economically and be incorporated into diets at minimum levels to reduce the cost. Full fat soybean can make a valuable contribution to the diets of *Macrobrachium rosenbergii* due to its high oil and protein content.

The modified extensive culture system involves growing natural food organisms during pond preparation and throughout the culture period, stocking at I -7.5 m^{-2}, and providing a formulated feed. At present, shrimp cultured by this system are fed commercial feeds containing high vitamin and mineral supplementation. The vitamin and mineral supplements can account for 20 to 23% of the total feed cost (Pascual and Catacutan, 1990; Triño *et al.*, 1992). Vitamins and minerals are essential nutrients for the tiger shrimp and prawn. The dietary requirements are low and may be obtained not only from the vitamin and mineral supplements but also from other feed ingredients, from microbes, natural food organisms and other sources (Phillips, 1984; Akiyama *et al.*, 1991; Lovell, 1991).

The costs of formulated feed and labour associated with feeding are a major component of the cost of cultured shrimp production. Optimizing the feeding strategy is a prime consideration in intensive shrimp pond management, and involves nutrition, processing and feed management. It is well established that the nutrient content of the feed will influence growth, survival and the amount of metabolic and excreted waste products entering the system. However, processing also plays a critical role as it influences stability of the feed and hence availability of the feed over time. These factors have a substantial effect on the amount of waste produced through pellet fragmentation, leaching loss, residual feed and undigested material. Feeding strategies also influence water quality and crustacean health. Feeding time is very important to ensure rapid consumption of the feed by prawn and shrimp, thereby minimizing the loss of nutrients and resulting in an improved growth rate.

Success of prawn culture depends on the use of supplementary feed in combination with natural feed. Biota in the pond is an important food source for prawn. Phytoplankton is not directly consumed by prawn but it can be eaten_adhered to detritus. The abundance of important communities, such as zooplankton and benthos depend on phytoplankton productivity. Some organisms from the zooplankton and benthic community are effectively consumed by prawn and contribute significantly to their nutrition (Allan and Maguire, 1993; Shishehchian and Yussof, 1999). Copepods, larvae of polycheates, juvenile and adult polycheates, larvae of insects and molluscs, ostracods, rotifers and other crustaceans have been considered most important feed for prawn (Martinez- Cordova *et al.*, 1998a; Pena-Messina, 1999). Despite these important findings no significant advantage of natural food in commercial farms has yet been documented. The main problem is the deficiency in maintaining a high biomass of these organisms during the whole farming period (White, 1986). Usually most of them are grazed during the first weeks and their abundance declines consistently over time (D'Abramo and Conklin, 1996; Martinez- Cordova *et al.*, 1998b).

Economical sound footing of an aquaculture industry or farm depends on the optimum production with the involvement of lowest possible cost. The success of prawn culture depends on providing a cost-effective, nutritionally balanced, water-stable formulated diet. One major problem identified with regard to culture of crustaceans is their slow, intermittent feeding response (Marchetti *et al.*, 1999). In the culture of *Macrobrachium rosenbergii* juveniles, they do not always feed immediately after a diet is introduced; the diet sometimes remaining untouched even for hours. Upon exposure of a diet to water, loss of nutritional and attractive properties occurs. A nutritionally balanced diet might exhibit poor water stability and quickly become nutritionally inferior as a result of nutrient loss through leaching. Cuzon *et al.* (1982), for example, demonstrated a loss of 19% dry matter, 11% protein and 8% carbohydrate from a shrimp diet after 1 hr immersion in seawater. Water-soluble vitamins were the most vulnerable to leaching with an 89% loss of vitamin C within the first hour and 99% after 3 h. Thus, a diet needs to be stable in water for several hours before ingestion.

Digestibility is clearly important in determining the nutrients available to fish for growth. The diet value is therefore highly dependent on digestibility and availability of nutrients in components. Consequently, information on digestibility will provide an indication of the potential of a feed ingredient for supporting growth. Based on the nutrient digestibility of the components, a system could be developed to formulate fish diets. Feedstuff digestibility assessment in fish and shell fish is an essential prerequisite in determining nutrient requirements, for screening the potential nutritive value of alternative feed ingredients and in the development of nutritionally adequate diets at least cost (Hajen *et al.*, 1993).

Soybean meals (SBM) are widely used as the most cost-effective alternative for high-quality fish meal in feeds for many aquaculture animals because of high protein content, relatively well-balanced amino acid profile, reasonable price and steady supply of soybeans. Many studies have been carried out to examine the effects of the partial or total replacement of fish meal by SBM. Most results showed that incorporation of high levels of SBM in fish diets has frequently leaded to reduced growth performance and feed utilization efficiency. Reasons for poor utilization of SBM could be attributed to a number of factors such as an improper balance of essential nutrients (amino acids, energy and minerals), lower digestibility, reduced diet palatability and the presence of anti-nutritional factors such as trypsin inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins and allergens (Tacon, 1994). Trypsin inhibitors reduce feed efficiency and survival of some fish species. Heat treatment applied during commercial processing of SBM inactivates most of the trypsin inhibitors and other heat-sensitive anti-nutritional factors.

Bangladesh is one of the leading countries that export giant freshwater prawn. Traditional extensive or improved-extensive culture practice in *Gher* (embankment of low lying paddy field) farming system mostly depended on natural productivity of the

Gher. Hence, to increase the production of prawn, some of the farmers use commercial prawn feed that involve comparatively higher investment. To raise the production, most of the farmers, now a day, use varieties of feed supplements on the basis assumption and previous experiences rather knowing the nutritional demand. The most commonly used feedstuffs are different oilseed cake (mustard oil cake, sesame oil cake, and coconut oil cake), pulse (in raw or boiled form), bran and polishing (rice, wheat), cereal (cooked rice and wheat) and snail meat. Some of the government and non-government organizations are training up the farmers to use farm-made feed that eventually lower the feeding cost up to 30% (Abedin *et al.*, 2001). Soybean meal could be a suitable among the ingredients that lower the feeding cost (Chisty *et al.*, 2000).

Numerous researches have been carried out on feed development of finfish and penaeid shrimp. Due to the world wide susceptibility of disease, though penaeid shrimp culture is popular, production over the last decade has been decreased and is still threatened. Further, shrimp culture requires coastal saline water. On the other hand, being primarily freshwater organisms, *Macrobrachium rosenbergii* can be produced farther inland areas. Large individual sizes of this species have a high demand in the market. In addition, freshwater prawn production tends to be more environment-friendly (compared with saltwater shrimp production) because they are more territorial in nature, and are stocked at lower densities. Due to its large socio-economical potentials it recently has been emphasized as an important species by the nutritionists. Thus the aquaculture development of *Macrobrachium rosenbergii* would among other factor, depend on the use of environment friendly and low cost feeds.

1.1 Issues for research in giant freshwater prawn

Feed costs can be minimized via the use of non-conventional protein sources to replace the more costly fishmeal. However, the suitability, in terms of the year round availability, nutritional values, antinutritional substances and digestibility of the new feedstuffs as the ingredients for *Macrobrachium rosenbergii* diets have to be determined. Digestion of different foodstuffs is governed by a series of bio-physico-chemical process of which enzymes play an important role. For a better understanding, the enzymes that take part in the digestion of these feedstuffs have to be identified and characterized.

1.2 Objectives of study

Considering the socio-economical potentials of *Macrobrachium rosenbergii*, this research has been taken with the following objectives to:

1. Prepare cost-effective diets by substituting the fishmeal protein by soy meal protein.

2. Identify and characterize the different protease enzyme in prawn.

3. Study the digestibility of different formulated diets and locally available feed ingredients that has a great importance in feed manufacturing.

4. Detect the inhibitory effects of different protein rich feed ingredients on the proteases activity of prawn.

CHAPTER TWO
LITERATURE SURVEY

2.1 Classification:

Class- Crustacea

Subclass- Malacostraca

Order- Decapoda

Super Family- Palaemonoidea

Family-Palaeomonidae

Genus- *Macrobrachium*

Species- *M. rosenbergii*

2.2 Natural history

The giant freshwater prawn is also known as the giant river or Malaysian prawn. is native to the tropical Indo-Pacific region. The species requires brackish water (dilute seawater) at the early stage of its life cycle. Prawns breed and spawn in warm freshwater.

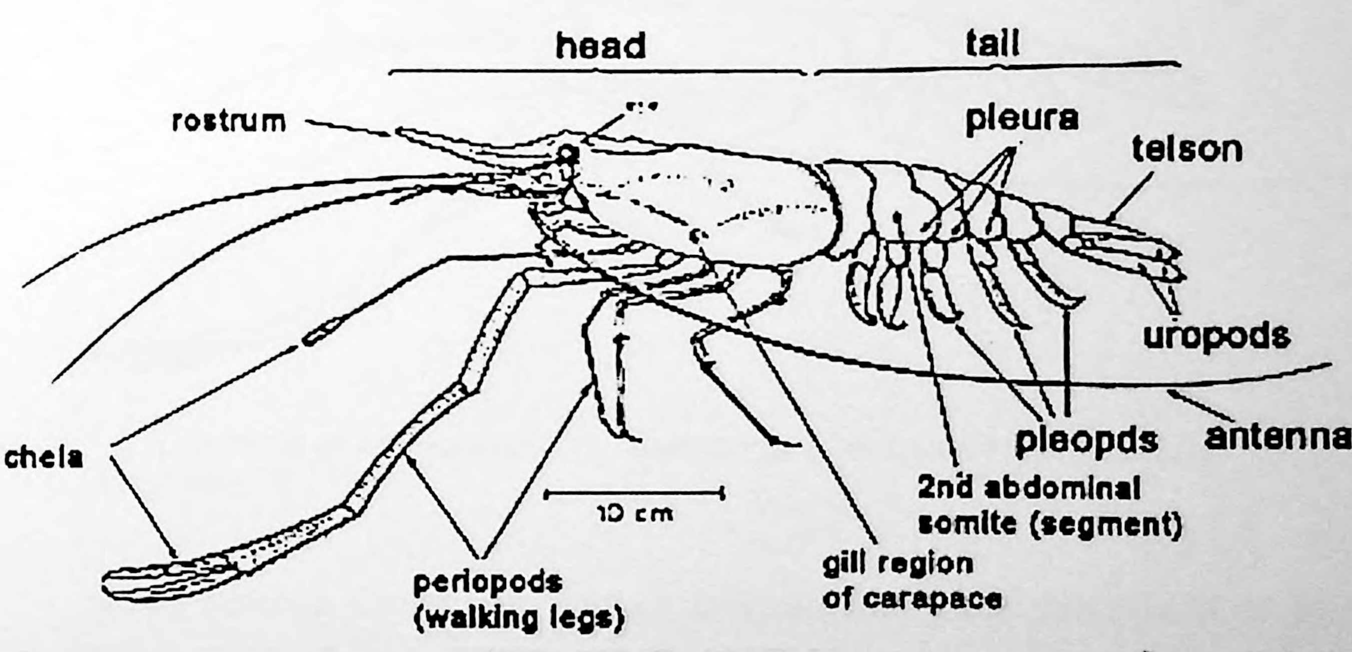

Figure 1.1: Morphological features of *Macrobrachium rosenbergii* (Courtesy: New, M.B and Singholka, S., 1982)

Mating takes place between a soft-shell female which has molted her shell and a hard-shell male. The male stimulates the female by using its long claws and protects her for 1-2 days until the shell hardens. The male deposits sperm between the walking legs of the female. A few hours after mating, the female lays the eggs that are fertilized by the stored sperm. The female attaches her eggs to the underside of abdomen between the space of the swimming legs (plepods) where they are incubated. Pleopods (paddle-like abdominal appendages) circulate water over the egg mass to provide oxygenated water and to remove debris. At first, the egg mass is a bright yellow-orange color, but it turns to brown near the hatching time. A 30 g female may produce 10,000 to 20,000 larvae 4-5 times a year. Mating typically occur throughout the year when water temperatures are above 70° F. Hatching occurs in approximately 3 weeks when the water temperature is 80°F. The entire brood hatches in 1 or 2 nights and the larvae are dispersed by the female (KSU AP, 2002).

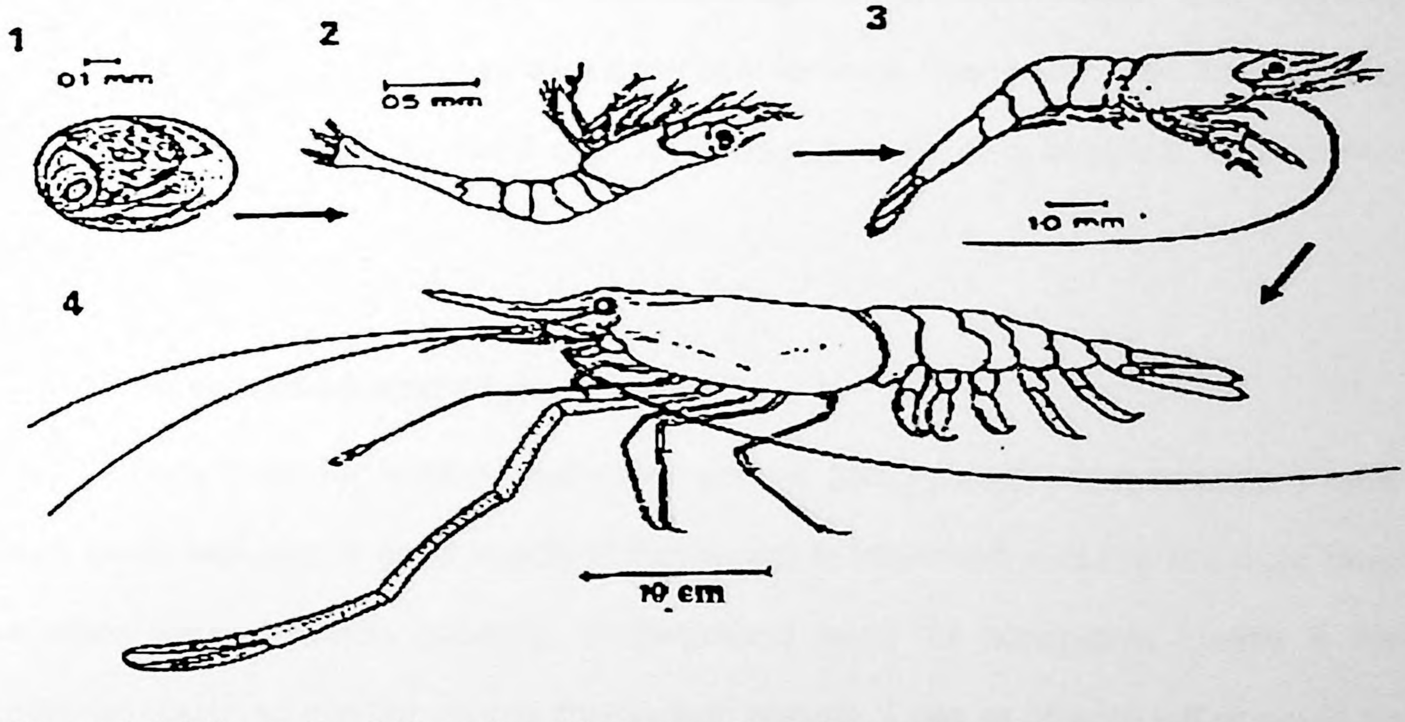

Figure 1.2: Life cycle of *Macrobrachium rosenbergii* (Courtesy: New, M.B and Singholka, S., 1982)

Newly hatched larvae must require brackish water with salinities of 10 to 14 parts per thousand (ppt) within 2 days of hatching, or they will not survive. In nature, prawn therefore breeds near the estuary or the down stream from which place the newly hatched larvae can be drifted to the optimum saline zone. At this stage, larvae swim upside down and tail first. They feed on zooplankton (microscopic animals), worms, and the larvae of other aquatic organisms. To reach the post-larval stage, the larvae must undergo 11 molts in approximately 30 days. Postlarvae resemble adult prawns and are about 0.3 - 0.4 inches in length. At this stage, postlarvae typically crawl along the bottom, but also can swim in a forward direction, against current, right-side up. It can also move backward rapidly by contracting the abdominal (tail) muscles. As postlarvae reach the juvenile stage, their bodies become a blue or brown color similar to adults (KSU AP, 2002).

2.3 Culture of giant freshwater prawn *Macrobrachium rosenbergii*

Giant freshwater prawn, *Macrobrachium rosenbergii* (De Man) inhabits in both freshwater and low saline estuarine water but it never goes to the marine water. It is a typical palaemonid which spawns and develops in brackish water, and following metamorphosis, the postlarvae move upstream towards freshwater. This species is of commercial importance for food due to its fast growth in subtropical and tropical regions.

2.4 Site selection and pond design

Ponds used for raising freshwater prawns (*Macrobrachium rosenbergii*) have many basic features. A good supply of freshwater is important, and the soil must have excellent water-retention capacity. Underground water of acceptable quality is the preferred water source for raising freshwater prawns. Lake or stream water could be the source of water subjected to use after water treatment. Surface runoff water from rivers, streams and reservoirs can be used, but quality and quantity can be highly variable and subject to uncontrollable change. The quality of the water source should be evaluated before any site is selected. Ponds should be located in areas that are free from the problem of periodic flooding. Before building ponds specifically for producing freshwater prawns, it is preferred to check the soil for the presence of pesticides. Prawns are sensitive to many of the pesticides used on agricultural crops. Soil should be analyzed for the presence of residual pesticides. Ponds that are subject to drift from agricultural sprays or to runoff water that might contain pesticides should be avoided.

The surface area of grow-out ponds ideally should range from 1 to 5 acres, but larger ponds have been successfully used. The pond should be rectangular in shape to facilitate distribution of feed across the entire surface area. The bottom of the pond should be completely smooth and free of any potential obstructions to seining. Ponds should have a minimum depth of 2 to 3 feet at the shallow end and a maximum depth of 3.5 to 5 feet at the deep end. The slope of the bottom should allow for rapid draining (D'Abramo and Brunson, 1996)

2.5 Water quality

Specific information on water quality requirements of freshwater prawns is limited. Although freshwater prawns have been successfully raised in the following conditions as mentioned by different author

2.5.1 Temperature

Macrobrachium rosenbergii do best in water of 26 to 30 °C. (79 to 86 °F). They can survive from 22 to 32 °C. (71 to 90 °F.), but growth and activity becomes at best sluggish at the ends of their range (Daniel, 1981). Temperatures of 26-31°C are considered satisfactory for prawn growth (Sandifer and Smith, 1985) with 29-31°C considered optimal (New, 1990). However, most studies on temperature tolerance and requirements are conducted on larvai stage animals. Feed conversion of *Macrobrachium rosenbergii* has reported to be more efficient at 25°C than 30°C

(Farmanfatmaian and Moore, 1978). New (1995) reported that *Macrobrachium rosenbergii* can be reared at temperatures ranging from 14 to 35°C and the optimal temperature range is 29-31°C.

Larger animals may respond differently to culture temperatures (Silverthorn and Reese, 1978). Tidwell *et al.* (1994) reported that prawns cultured in ponds with water temperatures averaging 25°C had higher production rates than those reported by D'Abramo *et al.*, (1989) for prawns cultured at 29°C. Both studies were conducted under similar conditions of stocking size, density, and diet. They also reported that prawns cultured under cool water conditions had a population structure that differed from those cultured at higher water temperatures. These lower culture temperatures appeared to increase both total production and the percentage of market-size prawns. Buck *et al.* (1981) reported that prawns raised in Illinois had production rates of 6.7 kg/ ha/day, despite water temperatures exceeding 27°C and 24°C for only 22 days and 68 days, respectively.

Lumare *et al.* (1993) found that the mean growth rate of the tropical black tiger shrimp *Penaeus monodon* cultured in a cold temperate climate (21-27°C) was similar to that reported for the same species in tropical and subtropical areas. They also identified three main periods during grow out of *P. monodon.* During the initial period, growth rates increased rapidly to a maximum despite low temperatures (23°C). During the second phase growth rates were high and did not increase as temperature increased. Only at larger sizes (Phase III) growth rate was decreased by decreasing temperature. Wang (1983) reported that the redtail shrimp, *P. penicillatus,* is a warm-water species with optimum growing temperatures of 25-30°C. However, Chen *et al.* (1988) reported that the red-tail shrimp grew faster in coldwater than warm water.

Tidwell *et al.* (1996) identified two major periods in culture of *M. rosenbergii* and reported that during Phase I, prior to animals achieving sexual maturation, temperature differences had no negative effect on prawn growth but in lower mean temperature prawn growth was slightly higher. In favour of these findings, they explained that higher growth rates at lower temperatures (24-26°C) could possibly be due to decreased maintenance requirements at the lower temperatures and a resulting increase somatic for growth. And in Phase II, prawns attain sexual maturity; temperature differences had a large impact on population structures and growth was higher at lower temperature. A greater proportion of prawns of both sexes became sexually mature earlier at 30°C than at 24-26°C, especially pronounced in females. Whereby, the attainment of sexual maturity in females normally results in decreased growth, as energies are redirected to egg production (Karplus *et al.*, 1986). The additional energy, demand for gonadal development, decrease the somatic growth in prawns. By maturing later in low temperature, females get longer period of somatic growth. They also stated that juvenile prawns under production conditions may have lower temperature optimum than of larvae and post-larvae.

Johnson (1967) found that natural populations of *M. rosenbergii* in Malaysia occur at a temperature range of 24.5 to 25.5°C. Justo *et al.* (1991) found that *M. rosenbergii* raised under controlled temperatures and photoperiods typical of higher temperate latitudes (28ºC and 15 h light, 9 h dark) had a reduced frequency of reproductive molts, increased frequency of common (growth) molts, and greater growth after reproductive molts than prawns raised under conditions typical of tropical zones (32°C and 12 h light, 12 h dark). The authors stated that increased reproductive activity under tropical conditions caused a considerable draw on energy reserves and resulted in an antagonism between reproduction and somatic growth.

2.5.2 Dissolved oxygen

Dissolved oxygen (DO) is particularly important, and a good oxygen monitoring program is necessary to achieve maximum yields. Routinely checking and monitoring dissolved oxygen in the bottom 1 foot of the pond which the prawns occupy is recommended. It is important the dissolved oxygen concentration in the bottom 1 foot of water should be equal or above 3 ppm. Dissolved oxygen concentrations of 3 ppm are stressful, and lower concentrations can be lethal. Chronically low levels of dissolved oxygen result in less than anticipated yields at the end of the growing season (D'Abramo and Brunson, 1996).

Good aeration is important for prawn rearing. Although there are indications that *M. rosenbergii* is tolerant of low oxygen for short periods of time, extended hypoxia seems to decrease their appetite. Overall, they become less active, but often more aggressive towards their fellow prawn, resulting in cannibalism in their effort perhaps to "weed out the competition" (Daniel, 1981).

According to other reports, incidents of dissolved oxygen deficit in extensive systems in Khulna, Bangladesh might have contributed to stressing conditions (Wahab, et al., 2003). In Indonesian *tambaks*, early morning dissolved oxygen concentrations between 3 and 5 mg l^{-1} were considered favorable for growth without causing stress in *P. monodon* (Hariati *et al.*, 1996). According to Hall and Van Hamm (1998), dissolved oxygen concentrations for juvenile *P. monodon* needs to maintain "above 4 mg l^{-1} during the grow-out period if repeated bouts of metabolic stress to the prawns are to be avoided."

At night, respiration by fish, plants, and other pond organisms causes decline in DO concentrations. Higher temperature also decreases the oxygen saturation levels of water body. Thus, during warm months, night-time DO concentrations in ponds often are below saturation. In production ponds, DO may vary 5–10 mg l^{-1} at night, and in un-aerated ponds, DO concentrations at sunrise may be less than 2 mg l^{-1} (Boyd, 1990). Such low DO concentrations can cause stress or mortality in culture species.

2.5.3 pH

The effect of pH on crustacean physiology has been seldom studied and mainly on freshwater species. Prawns need somewhat alkaline water, pH 7.2 to 8.4. At pH's below 7.0, prawns have a difficulty hardening properly after a molt. The carapace of intermolt prawns also becomes weak providing a site for infection (Daniel, 1981). In freshwater ponds, pH levels can fluctuate from 6.6 to 10.2 because of the removal of carbon dioxide due to photosynthesis by plants during daytime and the release of carbon dioxide by both plants and animals during the night. A high pH can cause mortality through direct pH toxicity, and indirectly because a higher percentage of the total ammonia in the water exists in the toxic, unionized form. Although freshwater prawns have been raised in ponds with a pH range of 6.0 to 10.5 with no apparent short-term adverse effects, it is recommended to avoid a pH below 6.5 or above 9.5, if possible (Boyd, 1990). New (1995) reported that *M. rosenbergii* can be reared at optimal pH range 7.0-8.5. Constantly high pH stresses the prawns and reduces growth rates (D'Abramo and Brunson, 1996).

In a study conducted by Allan and Maguire (1992), low pH water (5.9) has been reported to cause decreased growth in *P. monodon*. Wickins (1984) showed that decreasing pH from 7.9 to 6.7 induced carapace weight loss, increased magnesium content and decreased strontium content. The minimum acceptable pH levels in *Macrobrachium rosenbergii* were 6.2 and 7.4 based on growth and feeding, respectively (Chen and Chen, 2003). The effect of pH on crustacean physiology has been seldom studied and mainly on freshwater species. Low pH disturbed ion regulation in crayfish and acid–base imbalance in crayfish and freshwater shrimp

(Morgan and McMahon, 1982; Chen and Lee, 1997). The exposure of crayfish to low pH results in a net loss of Cl^- and Na^+ (Zanotto and Whealthy, 1993).

2.5.4 Alkalinity and hardness

Alkalinity and hardness are both important components of water quality in prawn production ponds.

- Total alkalinity indicates the quantity of base present in the water bicarbonates, carbonates, phosphates and hydroxides.
- Hardness represents the overall concentrations of divalent salts present (calcium, magnesium, and iron) but does not identify which of these elements is/are the source of hardness.

Alkalinity measures the total amount of base present and is the indicator of the pond's ability to resist large changes in pH (or buffering capacity). The concentration of total alkalinity should be no lower than 20 mg l^{-1}. Pond pH can swing widely, from 6 to 10, when alkalinity concentrations are below this level. The suggested range of total alkalinity concentrations for prawn farming is 50-150 mg l^{-1} (KSU AP, 2002).

Hardness also is important for prawn culture. Calcium and magnesium are the most common sources of water hardness. The critical component is the calcium concentration, referred to as "calcium hardness." Calcium is essential in the biological processes of aquatic animals. It is also important in the molting process of shrimp and can affect the hardening of the newly formed shell. It has been reported that freshwater prawns can tolerate a wide range of calcium hardness concentrations, but the suggested range is from 50-150 mg l^{-1} (KSU AP, 2002).

Successfully raised of *Macrobrachium* in soft water (5 to 7 ppm total hardness), a softening of the shell was noticed. Hard water, 300-plus ppm, has been implicated in reduced growth and lime encrustations on freshwater prawns. Therefore, use of water with a hardness of 300-plus ppm is not recommended (D'Abramo and Brunson, 1996).

2.5.5 Nitrogen compounds

Nitrites at concentrations of 1.8 ppm have caused problems in hatcheries but there is no definitive information as to the toxicity of nitrite to prawns in pond situations. High levels of un-ionized ammonia, above 0.1 ppm, in fish ponds can be detrimental. Concentrations of un-ionized ammonia as low as 0.26 ppm at a pH of 6.83 have been reported to kill 50 percent of the prawns in a population in 144 hours(D'Abramo and Brunson, 1996). Crustaceans are generally considered to be ammoniotelic mainly converting the end products of nitrogenous catabolism to ammonia. Environmental factors like temperature, salinity and ammonia have been reported to affect ammonia excretion of crustaceans and were reviewed by Regnault (1987) and stated that in decapod crustaceans, nitrogen is mainly excreted as ammonia (60-70%), with small amounts of amino acid, urea and uric acid (Regnault, 1987).

2.6 Stocking of juveniles

Juvenile prawns must be gradually acclimated to conditions in the grow-out pond to prevent temperature and pH shocks or other types of stress. Water in which postlarvae and juveniles are transported should be gradually replaced by the water in which they will be stocked. This acclimation procedure should not be attempted until the temperature difference between the transport and culture water is less than 6 to 10°F. It has been recommended to maintain the temperature of the pond water at least 68°F (20°C) at stocking to avoid stress because of low temperatures. Juvenile prawns appear to be more susceptible than adults to low water temperatures. Juveniles, preferably derived from populations that have been size graded, ranging in weight from 0.1 to 0.3g, should be stocked at densities of 12,000 to 16,000 per acre. The size grading results in more uniform growth and helps to reduce the percentage of smaller individuals. Lower stocking densities yield larger prawns but lower total harvest (D'Abramo and Brunson, 1996). Tidwell *et al.* (1995) reported to stock 39,520 juvenile per hector for semi-intensive culture for *M. rosenbergii*.

In aquarium culture of prawn Daniel (1981) reported that prawns cannot be overcrowded and expected to do well. For young *Macrobrachium*, one can almost cover the bottom with them. But once they reach a 5 centimeter (2") body size, their claws are strong enough to defend a territory. At this age prawns should be given at least 600 square centimeters of bottom area each, or about 120 square centimeters for each centimeter of body length. Sexually mature adults, 12 centimeters (4.5") in body length or larger, need at least a 20 gallon tank. Overcrowding, poor water quality, or a poor diet all seem to make prawns more aggressive, leading to injury or death, especially during molting. D'Abramo *et al.* (2000) found better growth rate of juvenile *Macrobrachium* while they were provided sufficient bottom surface area. They also reported to observe reductions in weight gain began to occur when a critical biomass density of approximately 500 mg/l was attained.

It has been reported that space limitation can be suspected as a factor causing reduced rate of growth in crustaceans. As for example, the American lobster *Homarus americanus* was cultured under space limited condition and an increase in the duration of intermolt and a decrease in the weight gain per molt were observed by Templeman, (1948). Later investigations associated with the culture of the same species showed that weight gain of juveniles was significantly reduced under conditions of reduced culture space (Shleser, 1974). The observed reduction in weight gain occurred before movement was constrained by the dimensions of the holding area. The reduction in growth of space-restricted juvenile American lobsters was attributed to both reduced weight gain per molt and a prolonged molt interval (Van Olst and Carlberg, 1978).