CHAPTER I

GENERAL BACKGROUND

1. Introduction

The black tiger shrimp (*Penaeus monodon*) culture has been the most economic important aquaculture developed in Thailand. In 2000, penaeid shrimp production of 249,632.90 metric tons and exported value of 107,890.44 million bahts was recorded. The production in 2003 was reduced to 234,277 metric tons and exported value 71,847 million bahts (Department of Internal Trade, 2003). The penaeid shrimp aquaculture production has considered to be the most society and economic importance in Thailand due to penaeid shrimp aquaculture production sector has effected the worker labor more than 1.5 million persons (Wiwattanachisat, 2002). In the study and research about the diet requirements and immunostimulants of *Penaeus monodon* were expected the permanent developments for maximal production of penaeid shrimp culture in Thailand. In present, some efforts have been made to increase productivity per unit space. Overcrowding was often caused adversely effect the health of cultured shrimp, due to unsuitable water quality (low pH, high turbidity, high ammonia and nitrite, etc.) and surface area of pond bottom (accumulation of bottom sludge), these conditions tend to produce poor physiological environment for shrimp and increase susceptibility to infections. Various chemotherapeutics have been used to treat bacterial infection in shrimp culture for about the last 20 years. The incidence after chemical and antibiotic treatment was found that drug-resistant bacteria and drug-residual antibiotic in shrimp has become a major problem in shrimp culture. In the current status, we put efforts on research to decrease using of chemotherapeutic agents. The study on using of immunostimulants is also an effective means of increasing the immunocompetency and disease resistance of shrimp (Flegal et al., 1992; Flegel et al., 1995).

Polysaccharides are the most abundant organic molecules in nature. The majority of these substances contained long chain units of monosaccharide which has a simple elemental formula of $C_n(H_2O)_n$. They have been adapted for a wide variety

of biological function, which including energy sources and structural elements (Mckee Trudy, and Mckee James, 1999). It has long been learned that the types and levels of carbohydrate in the diet have effected on growth and survival rate of *Penaeus monodon* juveniles. Pascual et al. (1983) has reported significant differences between types and concentrations of carbohydrates in the diets on a survival rate of *P. monodon* juvenile shrimps. Fox (1993) has reported that increasing levels of dietary chitin did not significantly effect individual weight gain, specific growth rate, feed conversion ratio (FCR), survival or production ($P>0.05$) in *P. monodon* at semi-defined diets containing levels of chitin between 0 and 16%, in 50 days growth. Oral admistration of β-1,3-glucan, at an optimal level of 10 g per kg diet for 20 days effectively enhanced the immune system and improved the survival of white sport syndrome virus (WSSV) infected *Penaeus monodon* (Cheng et al., 2004). Crude fucoidan extracted from brown algae *Sargassum polycystum* has reduced the impact of white sport syndrome virus infection in *Penaeus monodon* and also inhibited the growth of *Vibrio harveyi* (Chotigeat et al., 2004).

Shrimp possess immune systems both humoral and cellular responses, they are less specialized than vertebrate immune responses. Hemocytes play an important role in the cellular immune response, including encapsulation, cytotoxicity and cell-to-cell communication (Söderhäll 1999). Clotting of hemolymph is a critical mechanism to protect shrimp from excessive loss of body fluids, as well as to sequester and immobilize invading microorganism. Certain foreign substances such as lipopolysaccharide (LPS) and β-1,3-glucan cause hemocyte lysis, which relases enzymes that initiate clotting through the prophenoloxidase (proPO) system (Söderhäll et al., 1996). Humoral factors (antimicrobial peptides, lectins, and other defensive enzymes) are then released from granules contained in the hemocytes that effect killing of the pathogens prior to phagocytosis. Once an invader is phagocytized and/or encapsulated, the process of melanization renders it. Thus, shrimp use a combination of both humoral and cellular responses in their defense mechanism against microbial invaders.

Polysaccharide gel (PG) extracted from fruit-hulls of durian (*Durio zibethinus* L.) have been found to be useful in preparation of food, cosmetics and pharmaceutical

products such as jelly, tablet, suspension, emulsion, film dressing, cow teat dip, mouth refreshing film, antiacne and antiseptic gel (Pongsamart et al., 1989a; Pongsamart, 1989b; Umprayn et al., 1990abc; Lertchaiporn et al., 2003; Nakchat et al., 2003; Pongsamart et al., 2003; Pongwiwatana et al., 2003; Chansiripornchai et al., 2004ab; Maktrirat et al., 2004; Paphattarapong et al., 2004ab; Pongwiwatana et al., 2004; Siripokasupkul et al., 2004ab). The composition of sugars and properties of PG have been previously described (Pongsamart, 1998; Girddit et al., 2001; Hokputsa et al., 2004). Toxicity test of PG was determined, a high oral dose (2 g/kg) did not induced severe toxicity in male mice and rats (Pongsamart et al., 2001a). No toxic effects were observed in subacute treatment in male mice (Pongsamart et al., 1989) and subchronic studies in male and female mice confirmed the consumptive safety of PG (Pongsamart et al., 2001b).

A recent interesting study has revealed that polysaccharide gel extracted from fruit-hulls of durian (*Durio zibethinus* L.) have antibacterial activity (Pongsamart et al., 2003) and also have some influence on the complement system (Hokputsa et al., 2004). Since, the black tiger shrimp culture (*Penaeus monodon*) has expected to be the most aquaculture of commercial importance in Thailand. The study and research about the requirement of diet compositions and immunomodulators for *Penaeus monodon* has potential to be the long-term sustainability developments of penaeid shrimp culture and avoiding antibiotic treatment as well. Use of immunostimulants is also an effective means of increasing the immunocompetency and disease resistance of shrimp (Söderhäll et al.; 1996; Song et al., 1997; Vargas-Albores, 1997; Söderhäll, 1999).

The purpose of this study was to assess both growth stimulating and immunomodulating effect of polysaccharide gel from durian fruit-hulls as additive diet, a potential diet ingredient to increase nutritional value, for *Penaeus monodon* juvenile. An enhance growth performance represented by increasing body weight and total length, and immunomodulating effect represented by elevating immunogenic levels of *Penaeus monodon* juvenile. This polysaccharide as also expected for protection against microbial infection, *Vibrio harveyi* and white sport syndrome virus (WSSV). The challenge method in black tiger shrimp was used to determine the level of disease resistance.

2. Review of literature

2.1 Penaeid shrimp biology

2.1.1 Taxonomy

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal body. The subphylum Crustacea is made up of 42,000, predominantly aquatic species that belong to 10 classes. In the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda as indicated in Figure 1.

2.1.2 Morphology

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum and by a segmented abdomen as illustrated in Figure 2. Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles concentrate in the abdomen. Appendages of the cephalothoraxes vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form the jaw-like structures that are involved in food uptake (Solis, 1988). In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Bell et al., 1988; Baily et al., 1992). The internal morphology of penaeid shrimp as illustrated in Figure 3. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the blood cells are called hemolymph and hemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valved hemolymph vessels leave the heart and branch several times before the hemolymph arrives at the sinuses that are scattered throughout the body, where exchange of substances takes place. After passing the gills, the hemolymph returns in the heart by means of three wide non-valved openings (Bauchau, 1981).

Penaeus monodon Fabricius, the common names is grass shrimp or giant black tiger prawn or tiger prawn or jumbo tiger prawn.

Phylum Arthroda

Subphylum Crustacea

Class Malacostraca

Order Decapoda

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1815

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Figure 1. Taxonomic definition of the black tiger shrimp, *Penaeus monodon*, Fabricius, 1798 (Brusca et al., 1990)

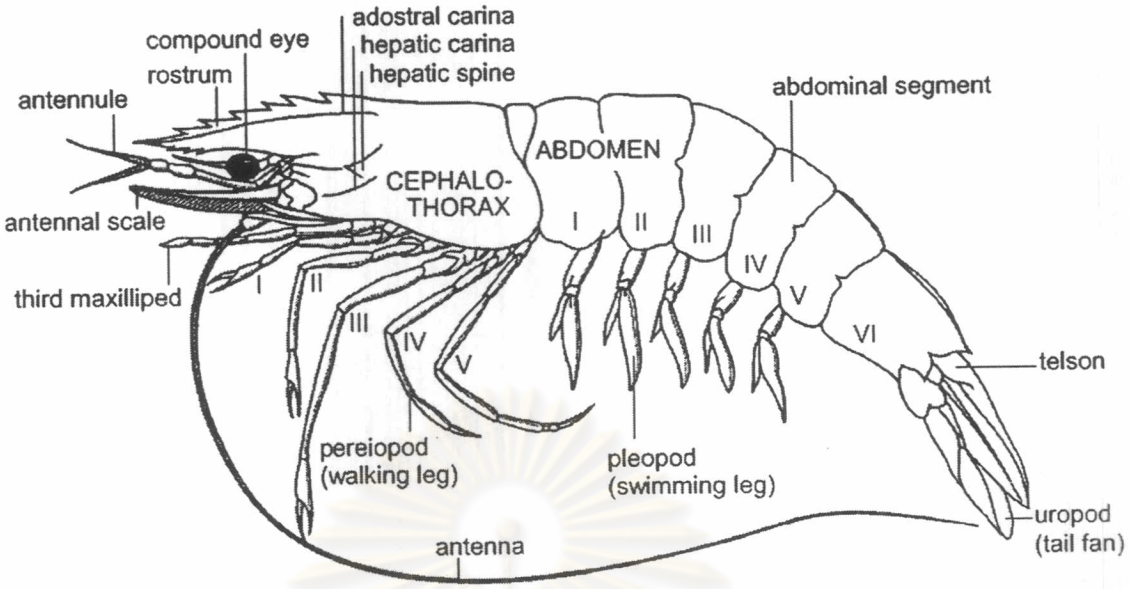


Figure 2. Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990)

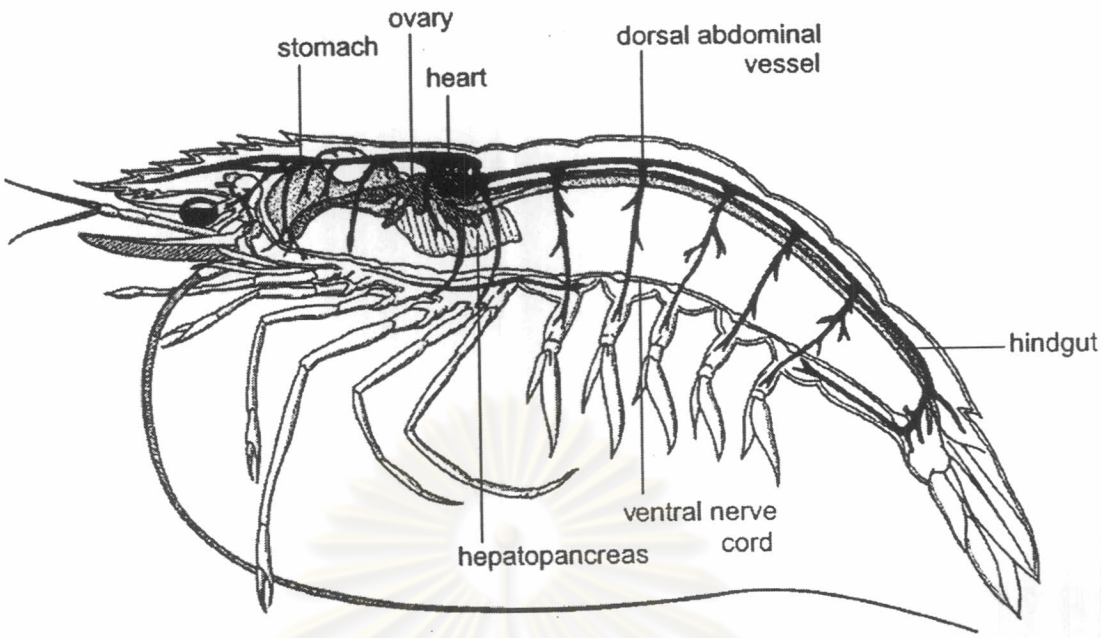


Figure 3. Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are hemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the hemolymph vessels that leave the heart ends in the lymphoid organ, where the hemolymph is filtered. This organ is located ventro-anteriorly to the hepatopancreas. The hemocytes are produced in haematopoietic tissue. This organ is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of the maxillipeds. Lymphoid organ and haematopoietic tissue are not indicated in Figure 3.

2.1.3 Distribution and life cycle

The black tiger shrimp *Penaeus monodon* is widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. The penaeid life cycle includes several distinct stages that are found in variety habitats of seawater as illustrated in Figure 4. Juvenile shrimp prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and reproduction takes place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch into the first larval stage, which is the nauplius. The nauplii feed on their reserves for a few days and develop into the protozoeae. The protozoeae feed on algae and metamorphose into mysis. The mysis feed on algae and zooplankton and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called post larvae (PLs). Larval stages inhabit plankton-rich surface waters offshore, with a coastal migration as they develop.

2.1.4 Shrimp culture

Development of shrimp farming: Shrimp farming started more than a century ago in Southeast Asia where farmers raised incidentally wild shrimp crops in tidal fish ponds (Rosenberry, 1997).

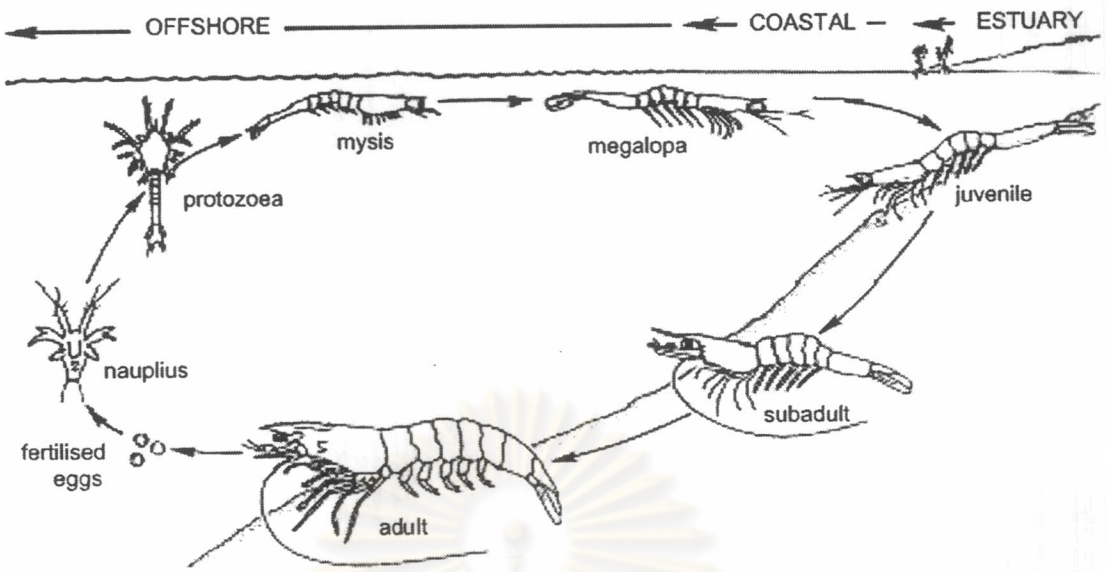


Figure 4. The life history cycle of the black tiger shrimp *Penaeus monodon*. Eggs hatch within 16 hr after fertilization. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called post larvae. Transition from juvenile to subadult takes 4.5-8.5 months and subsequently completion of sexual maturity occurs within 10 months (Motoh, 1984).

In 2000, more than 85% of the cultured shrimp production was still realized by farmers in the eastern hemisphere, with Thailand as the main shrimp farming country, followed by China, Indonesia and India (Rosenberry, 2001). A little to raise extent, shrimp are produced in Latin America, with Ecuador as the leading country. At present, shrimp farming is also substantially expanding towards the Middle East and Africa, but exact data are hardly available (Rosenberry, 2001).

Shrimp farming in Thailand, over last two decades at Thailand's coastal shrimp aquaculture industry has expanded, providing considerable economic benefits for farmers and the diverse industries which service and support shrimp farming. Export of shrimp and shrimp products has earned the country substantial foreign revenue (Jenkins et al., 1999). Intensive shrimp production was first introduced into Thailand in the mid-1980s along the coast of the upper Gulf, just south of Bangkok (Flaherty and Vandergeest, 1997). Shrimp farming in Thailand is characterized by rapid spatial mobility, and repeated boom and bust cycles due to disease and price fluctuations (Vandergeest et al., 1999). Shrimp farming has been practiced in Thailand for around 55 years (Tookwinas, 1993). The traditional method of shrimp production was extensive farming. The proliferation of shrimp farm began in 1972 when the Department of Fisheries (DOF) began to promote recently developed technologies and intensive culture (Katesombun, 1992). The expansion was partially facilitated by the development of hatchery technology in the late 1960s (Liao, 1990) which allowed the production of large number of larvae for stocking ponds.

Shrimp productions in Asian are classified three shrimp culture systems such as the extensive (2-5 PLs/m^2), semi-intensive (5-20 PLs/m^2), and intensive culture (30-150 PLs/m^2) system, according to their stocking levels and pond size (NACA, 1994). Beginning with the extensive about 3 decades ago, parts of this system were modified into a semi-intensive system in 1980, and later the intensive culture system was introduced (Lightner, 1983; Lee et al., 1992; Funge-Smith, 1997; Rosenberry, 2001; Menasveta, 2002).

Extensive or conventional culture: Extensive farms have an enormous requirement for land. Stocking relies on the shrimp larvae that are naturally

present in seawater to initially fill ponds. The extensive type evolved from the hunting and gathering of food in the near shore. This system use lower technology, requires vast, and low-lying areas, such as mangrove forested areas. In this system, shrimp are stocked at low densities (2-5 PLs/m^2) in large ponds (50-100 rai) or tidal enclosures in which a little or no management is exercised. Cultivation in crop takes 3-4 months usually produced two crops per year. Farmers depend almost entirely on natural conditions in extensive culture. Shrimp culture can not be controls the production in shrimp pond, usually low production. Shrimp will to reside eat the natural nutrition in seawater. The growth performance of shrimp pond is usually produced in low survival rate and growth rate that also depended on the rich nutrition in seawater from environment.

Intensive culture: Intensive shrimp farming has been developed steadily over the last decade in response to increasing world market demand. In system stoking the shrimp larvae from hatcheries are at high densities (30-150 PLs/m^2) in ponds (4 rai), tanks and raceways where a high level of investment is required. The production system evolved from extensive toward intensive with increasing inputs of high quality feed and water supply. This system use higher technology, requires a smaller area, and higher elevations the shrimp product. Thus, it does not require mangrove areas, but it is suitable for areas behind the mangroves. The production is quite high when compared with the two other systems. Because the high stocking density and intensive feeding, however, this system faced several problems, such as coastal pollution caused by the farm effluent and disease problems. This system was later modified to a more biosecure system, that is, a closed recirculating water system, a reduced or zero water exchange system, and shrimp culture at inland locations away from coastal influences.

Semi-intensive culture: Semi-intensive culture falls between these two systems. This system, shrimp are stocked at densities of between 5-30 PLs/m^2 in the medium pond size (15 rai), investments a medium level, use a medium level of technology and tidal enclosures. Cultivation in crop takes between 4-6 months, allowing two crops per year. The recirculation water exchange system is management and supplemented the shrimp larvae from hatchery-grown larvae for stocking shrimp larvae. Paddle aerators are used to maintain adequate oxygenation in

water and pumps facilitate water exchange. This shrimp culture system can be controls the production in shrimp pond, a lot of the production shrimps more than extensive culture but less than intensive culture.

Environment-friendly schemes in intensive shrimp farming as following parameters for stocking (Baliao, 2000):

1. Salinity	25-30	ppt
2. pH	7.5-8.5	
3. Temperature	28-32	°C
4. Alkalinity	above 80	ppm
5. Transparency	35-45	cm.
6. Water color	Brownish green	
7. Dissolved oxygen	above 4	ppm
8. Ammonia	below 0.1	ppm
9. Hydrogen sulfide	below 0.02	ppm
10. Water depth	at least 100	cm
11. Bacterial count	*Vibrio spp.* below 10^2 CFU	

In shrimp culture system has obtained products the post larvae (PLs) from commercial hatcheries, supplying for intensive production. The hatcheries vary from small-scale, low-input backyard hatcheries, to large-scale and high-tech hatcheries that can produce billions of PLs per year under strictly controlled circumstances (Rosenberry, 1997). Currently, farmers more and more rely on hatchery produced, mainly from wild caught, sexually mature females that are induced to spawn in hatcheries, and still to a very limited extent from cultured broodstock animals. The commercial use of domesticated broodstock is likely to increase together with increasing demands the PLs in shrimp culture industry (Alfaro, 2001).

2.1.5 Important culture species

The most important cultured penaeid shrimp species are the black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*L. vannamei*), kuruma shrimp (*P. japonicus*), blue shrimp (*P. stylirostris*) and Chinese white shrimp (*P. chinensis*). World shrimp production is dominated by *P. monodon* which accounted for more than 50% of the production in 1999. The black tiger shrimp, *Penaeus monodon* Fabricius, is widely distributed in the Indian Ocean and Western Pacific Ocean (Mohamed, 1970; Holthuis, 1980). Several Southeast Asian countries provide the main fishing

grounds for *P. monodon* (Motoh, 1981). *Penaeus monodon* is the largest, reaching 330 mm or more in body length, and exhibits the highest growth rate of all cultured penaeids (Lee and Wickins, 1992). *Penaeus monodon* can reach a market size up to 25-30 g within 3-4 months after PL stocking in culture ponds and tolerates a wide range of salinities (Rosenberry, 1997). Although *P. monodon* was normally considered as exceptionally tough, the rapid growth and intensification of its culture industry generated crowding and increased environmental degradation, which made the animals more susceptible for diseases (Lightner, 1983; Johnson, 1989). Nowadays, many disease problems are associated with this important culture.

2.1.6 The current impacts in shrimp culture

A combination of factors has lead to changes in productivity and economic viability of shrimp farms in Thailand. Pollution of farm water supplies with the water that containing the domestic, industrial and agricultural wastes. Self-pollution of water is supplies by the shrimp farm. In shrimp ponds there are high densities of intensive farm with inadequate separation of source water from effluent water, the quality of the water in the ponds deteriorates, leading to stress in the shrimp and consequently infectious disease (Smith et al., 1999).

The rapid expansion of the commercial industry shrimp operations may lead to over huge fishing on wild stocks shrimp larvae and broodstock animals. In addition cases destroying the local environmental by conversion of mangroves into ponds. Various types of land use change are associated with shrimp culture that the most widely reported is the conversion of mangrove forests into shrimp ponds (Folke and Kautsky, 1992; Flaherty and Karnjanakesorn, 1995). The next major environmental constraint encountered by intensive shrimp culture was the waste absorption capacity of the water bodies in pond which farm effluent discharged for often clean water that negative feedback in the occurrence of diseases outbreaks both infectious and non-infectious aetiologies (Lightner et al., 1992) and production crashes (Neiland et al., 1997). Disease outbreaks, mainly caused by viruses and bacteria and to a lesser extent by rickettsiae, fungi and parasites, may cause losses up to 100% (Johnson, 1989; Lightner et al., 1992; Lightner and Redman, 1998). The polluting effects of intensive shrimp culture pollute surrounding water bodies by

discharge of effluent from ponds. This impact on other users of the water body, including extensive shrimp farm, and even back on the polluting farm themselves. Species composition and biodiversity in ecology system impacts through habitat destruction, it cause of wild fry stocks and alteration of gene pools that introduction of exotic species (Larsson et al., 1994). Environmental problems faced by shrimp culture suffered by misuse and run-off of agricultural pesticides and fertilizers, sedimentation of canals and water inlet through soil erosion in upland areas due to deforestation and inappropriate agriculture practices, pollution of water sources through sewage from urban areas and industrial effluent especially heavy metal, oil spills and red tides of toxic algae (Liao et al., 1995; De Walt et al., 1996). Economic problems producers is the decline in the world price that falling as world supply expands faster than world demand, and rising as demand for input is expanding faster than supply thus the main economic problem simply reflects one of markets giving price signals within a process of adjustment to equilibrium (Niemeier, 1990; Anon, 1991).

The shrimp farmers used too much of several chemicals and antibiotic drugs in shrimp farms to improve water, soil quality and disease prevention that causes chemical and antibiotic residues in shrimp product and mud (Gräslund and Bengtsson, 2001; Le and Munekage, 2004) which leading to problem of export section, especially in European Union (EU) market (Shinbut, 2002). Thailand was the world's leading shrimp producer for the third consecutive years of the last decade (1991-1993). Since 1991, the imported countries like Japan, EU and USA have strictly checked antibiotic drugs residue in shrimp (Goebbels, 1991; Sermwatanakul, 1994). In addition incidence of antibiotic resistance, a variety of antimicrobial agents had been used successfully in the treatment of microorganism infections, such the widespread use of antibiotic in aquaculture, possibly resulted in the development of antibiotic resistance (Le et al., 2005). Drug resistance constitutes a serious potential problem in treatment of bacterial infections in aquatic animals. Very large quantities of antibiotics drug are fed to aquatic animals, especially in the intensive culture of shrimp. Some antibiotics are used to treat diseases in animals, in the same way they are also used to treat human diseases. Since much larger quantities are mixed into the animals' feed. There is now a very real danger that this abuse of antibiotics in animal feed is producing antibiotic resistant bacteria that can cause disease in humans and

animals. These diseases will be increasingly difficult to treat because of the antibiotic resistance (Somsiri, 1995). The development of intensive culture practices from extensive culture increases nutrient impacts on the local coastal environment. Alongside environmental changes such as eutrophication, salination and land use changes, are attendant social transformations (Chua et al., 1989; Primavera, 1992; Primavera, 1993). These can be the increased problem into traditionally poor coastal areas must be balanced against loss of job diversity, loss of independence, rising prices and growing inequity between farmers and non-farmers (Chong, 1990; Masae and Rakkheaw, 1992; Nuruzzaman, 1996). In intensive culture system consequently, waste loads from culture ponds as uneaten feed and metabolic wastes were increased, and then that laden effluent discharged from shrimp farms transfer to environment (Lin, 1995). In traditional intensive shrimp culture, the deteriorated pond water is frequently exchanged with new external water supply to maintain desirable water quality for shrimp growth (Wang, 1990; Hopkins, 1993). The nutrient laden effluent discharged from shrimp farms can cause eutrophication of coastal waters and its impact has been a major environmental concern (Hopkins, 1995; Shang, 1998).

The shrimp culture pond will be success reproduction that depends on three factors such as nutrition, hormonal control and environment. These innovations have made shrimp culture more efficient in controlling diseases, more sustainable, and more environmentally friendly. The sustainability and development of shrimp aquaculture are largely at stake as significant ecological and pathological problems are increasing in the vast majority of the shrimp producing countries. Prevention and control of diseases are now the priority for the durability of this industry. Therefore, the prevention and the control of shrimp diseases need an integrated approach in which knowledge of shrimp immunity must be improved (Bachère, 2000).

2.2 The crustacean defense system

2.2.1 Evolution of the immune system

The immune system can be divided into two systems of important defense against infectious agents that have been selected during evolution

both the innate (natural) and the acquired (adaptive) immune system. The innate immune system can be found in all multi-cellular animals and consists of cellular and humoral defenses. The most important cellular defense reactions against invading microorganisms are phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting. The humoral defense factors, such as clotting proteins, agglutinins, hydrolytic enzymes and antimicrobial peptides are often produced conjunction with stimulate the cellular defense. The acquired immune system is found only in vertebrates and operates through lymphocytes. Invertebrate animal is widely distributed and can be found in almost any kind of habitat. Their dispersal and survival mostly depend on successful defenses against various kinds of pathogens and microorganisms. The efficacy of their defense system is witnessed by their persistent survival through many years of evolution (Millar and Ratcliffe, 1994).

2.2.2 Crustacean hemolymph circulatory system

Shrimp have an open circulatory system. Hemolymph moves through a series of arteries to various organs and from there into the interstitial spaces of the haemocoel (body cavity) and subsequently to the gills (for oxygenation) and back to the heart for distribution (Cameron and Magnum, 1983). The fluid within this system is called hemolymph because there is no separation between the circulatory and lymphatic system in crustacean (Martin and Hose, 1992). The circulatory system of black tiger shrimp consists of heart, arteries, capillaries, hemal sinuses and blood cells (hyaline cell, semigranulocyte cell, granulocyte cell). The hematopoietic tissues are intimately associated with this system because of their involvement in the production of blood cells. The oka or lymphoid organ is considered to play an important role in the shrimp internal defense response (Arkarajamon, 1991). The blood circulatory system of shrimp is an open circulatory system. Blood is pumped through the body by a one-chambered heart. Veins and arteries carry blood to and from the heart, aided by contraction of the body muscular. There is no connecting of capillaries, arteries and veins, so the blood just passes through the tissue spaces. Thus, shrimp and all other arthropods have an open circulatory system (Haywood and Wells, 1989). In hemolymph of black tiger shrimp has hemocyamin pigment which function for oxygenation as for hemocytes has function for cellular immune response. The hemocytes of shrimp can divided into three different cell types following

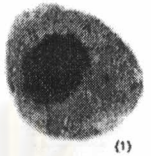
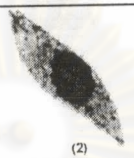
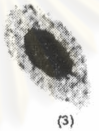
morphological distinct types, based upon the quantity and sizes of granules contain within including (Table 1):

Agranulocytes or Hyaline cell: Agranulocytes is the smallest of the hemocytes and no or few granules in cytoplasm. The hyaline shapes are discoid, spindle or crescent. Diameter of hemocyte is 6.4-13.9 x 6.4-8.3 μm (Supamattaya et al., 2000). The nucleocytoplasmic ratio, nucleus/cytoplasm, is the highest of the hemocytes that mean which the large of nucleus and scanty cytoplasm (Hose at al., 1990). The agranulocytes have also been implicated in the release of chemicals needed for tanning of the cuticle following molting (Johansson et al., 2000). The important function in immune response is in phagocytosis (Hose and Martin, 1989).

Semigranulocyte cell: Semigranulocytes are intermediate in appearance between aganulocytes and granulocytes (Bauchau, 1981; Söderhäll and Cerenius, 1992). Diameter of hemocyte is 9.0-14.2 x 4.2-6.8 μm and a few amount of granule in the cytoplasm, size of small granule is range 0.5-1.0 μm (Supamattaya et al., 2000). The nucleocytoplasmic ratio is the lower than hyaline cells (Hose et al., 1990). The important function in immune response is in cell-to-cell communication, encapsulation of particles too large to be phagocytized, and in the storage and release of the proPO system and other cytotoxic chemicals (Johansson et al., 2000).

Granulocyte cell: Granulocytes are the largest of the hemocytes of black tiger shrimp. A diameter rage of granulocyte is 12.2-14.6 x 7.2-7.8 μm and contained a large amount of granule in cytoplasm (Supamattaya et al., 2000), size of granule is range 1.3-2.0 μm (Johansson et al., 2000). The nucleocytoplasmic ratio is the lower than hyaline cells (Hose et al., 1990). The important function in immune response is primarily involved in the storage and release (degranulation) of the proPO system and lysosomal enzymes and other cytotoxic chemicals (Johansson et al., 2000).

Table 1. Characteristics of shrimp (*Penaeus monodon*) hemocytes (Van de Braak et al. 1996)

Cell type	Nucleus shape	Nucleus colour	Cell shape	Cytoplasm colour	Cell morphology (light micrographs) / Description
(1): granulocyte	round, oval or horseshoe shaped	Blue	round or oval	red	 (1) type 1 hemocyte with eosinophylic in cytoplasm
(2): semigranulocyte	oval or horseshoe shaped	Blue	elongated	colourless or light red	 (2) type 2 hemocyte with oblong shape and colourless to light eosinophylic in cytoplasm
(3): semigranulocyte	round, oval or horseshoe shaped	Blue	round or oval	colourless or light red	 (3) type 3 hemocyte is round to oval with colourless to light eosinophylic in cytoplasm
(4): semigranulocyte	not obvious	the blue round substance contains red globules			 (4) types 4 hemocyte with eosinophylic globules
(5): hyaline	round or oval	dark blue	round and small	colourless	 (5) type 5 hemocyte with low nucleus/cytoplasm ratio

2.2.3 Characteristics of the crustacean immune system

The immune systems of shrimp are innate immunity. There is no true specific immunity (no true antibodies and substantially less lymphocyte heterogeneity), though some aspects of specific immunity (inducibility) appear to be present in some cases. The major innate defense system in invertebrates is the melanization of pathogens and damaged tissues (Cerenius and Söderhäll, 2004). Immune response in shrimp and crustacean can be divided into two systems both cellular defenses and humoral defenses (Lackie, 1980; Ratcliffe et al., 1985; Smith and Chisholm, 1992).

Cellular defenses: Hemocytes play an important and central role in the cellular immune response, including clotting, non-self recognition, phagocytosis, melanization, encapsulation, cytotoxicity and cell-to-cell communication (Söderhäll, 1999). Clotting (coagulation) of hemolymph is an important mechanism to protect an animal from excessive loss of body fluids and immobilize microorganisms invaders. Hemocyte activation has effects in rapid clotting, cellular degranulation, activation the prophenoloxidase (proPO) system and subsequently the production of sticky molecules (Johansson and Söderhäll, 1992). In the presence of foreign chemical or specific foreign substances such as lipopolysaccharides (LPS) and β-glucan cause hemocyte lysis. Lysed hemocytes release enzymes that initiate clotting through the proPO system (Söderhäll et al., 1996). The each type of hemocyte in hemolymph plays differential function in immunity system as indicated (Table 2).

Humoral defenses: Humoral factors are primarity non-self recognition factors that including a variety of antimicrobial peptides, lectins, agglutinin, cytokine-like factors, modulators, other defensive enzymes and reactive oxygen intermediates are released from granules contained in the hemocytes to effect killing of microorganism invaders and pathogen prior to phagocytosis. Many humoral immune factors have been described in shrimp and other crustaceans (Smith and Chisholm, 1992). Several of these described factors that originate or reside in the hemocytes and are released during the immune response.

Table 2. Crutacean hemocytes and function in the immune response

Type of hemocyte	Function	Referents
Agranulocyte	Phagocytosis	-McKay and Jenkin, 1970 -Smith and Söderhäll, 1983 -Hose and Martin, 1989
	Hemolymph clotting	-Omori et al., 1989 -Hose et al., 1990
Semigranulocyte	Phagocytosis	-Hose et al., 1990 -Bachè et al., 1995 -Itami et al, 1998 -Gargioni and Barracco, 1998
	Storage and release of the proPO system	- Mc Kay and Jenkin, 1970 -Smith and Söderhäll, 1983 -Johansson and Söderhäll, 1985
	Cytotoxicity	-Johansson et al., 2000
	Encapsulation	-Hose et al., 1990
Granulocyte	Phagocytosis	-Bachè et al., 1995 -Itami et al, 1998 -Hose et al., 1990 -Gargioni and Barracco, 1998
	Storage and release of the proPO system	- Mc Kay and Jenkin, 1970 -Smith and Söderhäll, 1983 -Johansson and Söderhäll, 1985
	Cytotoxicity	-Johansson et al., 2000
	Encapsulation	-Hose et al., 1990

2.2.4 Immune mechanisms of the crustacean defense system

A schematic overview of the most important defense factors in the crustacean defense system is indicated (Figure 5 and 6). In invertebrates, the first immune defense process is the recognition of invading microorganisms, which is mediated by the hemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000) as indicated in Figure 6. The presence of minute amounts of compounds of microbial origins from microorganisms or parasites, such as β-1,3-glucan, lipopolysaccharides and peptidoglycans, has to take place in order to transfer the message to the cells that will synthesize the appropriate immune factors, such as antimicrobial peptides, cytotoxic, opsonic, lectin and other defensive enzymes. The foreign particle will induce activation of the prophenoloxidase-activating system (proPO-AS), and may also induce activation of other defense processes. The proPO-AS was controlled the activation of proPO into active phenoloxidase (PO) by a cascade of serine proteinases and other factors. The invertebrate immune system recognizes large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Söderhäll et al., 1996). The proPO-AS is an efficient non-self recognition system in invertebrates that can recognize and respond to the foreign particle (Cerenius and Söderhäll, 2004). The result of activation of the proPO-AS, the parasite is blackened in the host hemolymph by the deposition of melanin due to the action of phenoloxidase and oxidoreductase. This reaction is called the melanization reaction and is easily observed around parasites in the hemolymph or the exoskeleton (cuticle). The melanization reaction is a common response to parasite entry in invertebrate. The proPO-AS consists of several different protein among which are proteinases, proteinase inhibitors and recognition molecules that recognize structural features of the bacterial and fungal components. When activation of system, the associated proteins gain biological activity and participate in the cellular defense reactions of the host animal (Söderhäll and Cerenius, 1998).

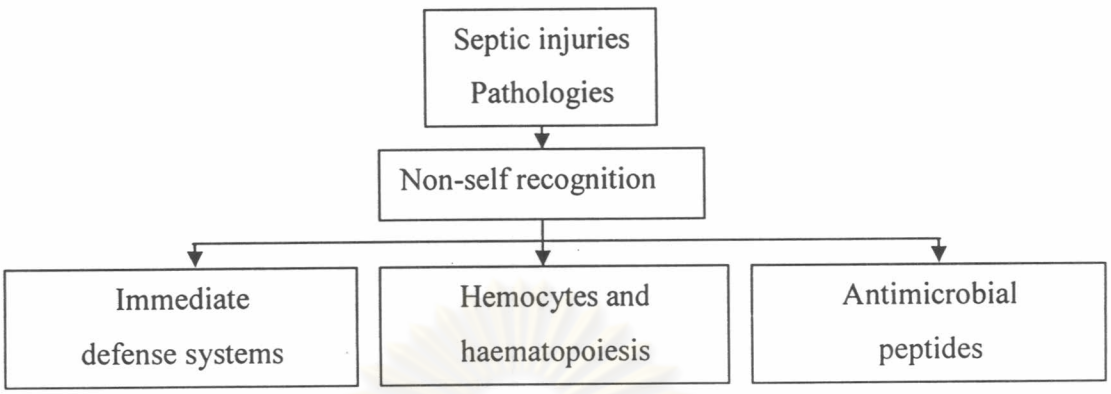


Figure 5. Immune defense in shrimp responses the pathogenic and microorganisms. The first immune process is the recognition cell wall components of invading microorganisms and pathogenic by recognition protein, which is non-self recognition of innate immune. Some of the recognition protein are β-1,3-glucan binding protein, peptidoglycan recognition protein and lipopolysaccharide binding protein, which reacts with the specific carbohydrates expressed on cell surfaces of microorganism, and form a complex molecule. The complex molecule will induces degranulation and activation of prophenoloxidase (proPO). Although these microbial components can directly activate defensive cellular functions of heamocyte: phagocytosis, melanization, encapsulation and coagulation. The non-self recognition factor activates several immediate defense systems mediated. Several components associated with the immediate defense systems, which leads to the reaction of melanization, proPO-activating system and coagulation. Some component of these has been identified such as a plasmatic clotting factor and α2-macroglobulin. The innate immune response of arthropods also relies upon the production of antimicrobial peptides that are active against a large range of pathogens such as endotoxin, tachycitins and tachystatins (Vargas-Albores, F., and Yepiz-Plascencia, 2000; Bachère, 2000).

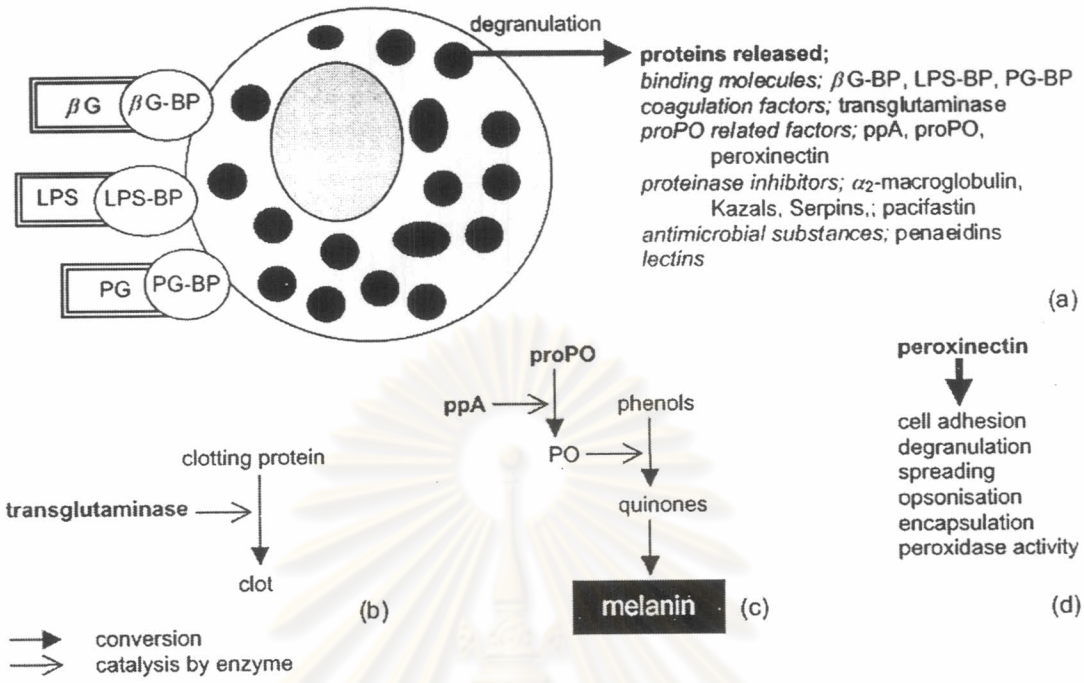


Figure 6. Simplified overview of the most important defense factors of crustaceans that are mediated by the hemocytes. Different recognition proteins in the hemolymph recognize and bind cell wall components of microorganisms. Subsequently their cellular binding is induced the immune defense responses. The hemocytes degranulate and release different proteins (a). Several proteins are pro-enzymes, others are substrates. The proteins that are released are involved in the clotting (b), the prophenoloxidase activating (c) system or in other cellular activation processes (d). The proteins involved in those processes that are released from the hemocytes are indicated in bold letters. Activation of cascade processes is regulated by different proteinase inhibitors. βG = β-1,3-glucan; βG-BP = β-1,3-glucan binding protein; LPS = lipopolysaccharide; LPS-BP = lipopolysaccharide binding protein; PG = peptidoglycan; PG-BP = peptidoglycan binding protein; PO = phenoloxidase; ppA = prophenoloxidase activating enzyme; proPO = prophenoloxidase. (Van de Braak, 2002)

Several types of modulator protein have been described that recognize cell wall components of microorganisms. The recognition protein will recognize carbohydrate moieties of cell wall components of microorganisms, like lipopolysaccharides (LPS) or peptidoglycans from bacteria, or β-1,3-glucans from fungi (Söderhäll et al., 1996; Vargas-Albores et al., 1996; 1997). Some of the recognition protein are lectins and can work directly as agglutinins or opsonins. Lectins are non-enzyme proteins that bind to specific carbohydrates expressed on cell surfaces of microorganism (Kopácek et al., 1993; Söderhäll et al., 1996). After binding of the recognition protein with the microbial component, a second site becomes active for cellular binding. Hemocyte activation is generated after this second binding step, which play an important and central role in host defense (Vargas-Albores and Yepiz-Plascencia, 2000). The proteins in defense system that have been reported in plasma of crustacean are indicated in Table 3.

After detection of foreign material, hemocytes migrate to the site of invasion. The open circulatory system demands a rapid and efficient defense, in which the proteoloytic cascades play an important role (Sritunyalucksana and Söderhäll, 2000). The hemocytes are involved in the synthesis and storage the proenzymes and substrates of the clotting and proPO cascades (Johansson and Söderhäll, 1992; Söderhäll et al., 1996; Sritunyalucksana and Söderhäll, 2000). The coagulation mechanism is an important immune defense system in crustaceans. The clotting mechanism entraps foreign material and prevents loss of hemolymph. The transglutaminase involved the clotting reaction in crustaceans which described in the freshwater crayfish *Pacifastacus leniusculus* (Kopácek et al., 1993). The clotting reaction is induced when transglutaminase is released from the hemocytes or tissues. The Ca^{2+} found increases in plasma to form a gel that involved the transglutaminase catalyses polymerization of the clotting protein (Kopácek et al., 1993; Yeh et al., 1998).

The proPO-activating system is an important immune defense system in crustaceans (Söderhäll and Cerenius, 1998). Several components associated with the proPO-activating system and induce to the reaction of melanisation (Figure 7).

Table 3. Some of proteins in plasma of crustaceans has been reported and involved in the immune defense system.

Protein	Reference
β-1,3-glucan binding protein	Sritunyalucksana et al., 2002
Lipopolysaccharide binding protein	Jomor and Natori, 1992
Mannan binding protein	
peroxinectin	Sritunyalucksana et al., 2001
Kazal inhibitor	Sritunyalucksana, 2001
transglutaminase	H. H. Song (unpublished)
clotting protein	Yeh et al., 1999
proPO	Sritunyalucksana et al., 1999
Lectin	Marques and Barracco, 2000
Agglutinin	Vargas-Albores et al., 1993
Bactrricidin	Stewart and Zwicker, 1972
Cytokine-like factor	Smith and Chisholm, 1992
α-macroglobulin	Smith and Chisholm, 1992

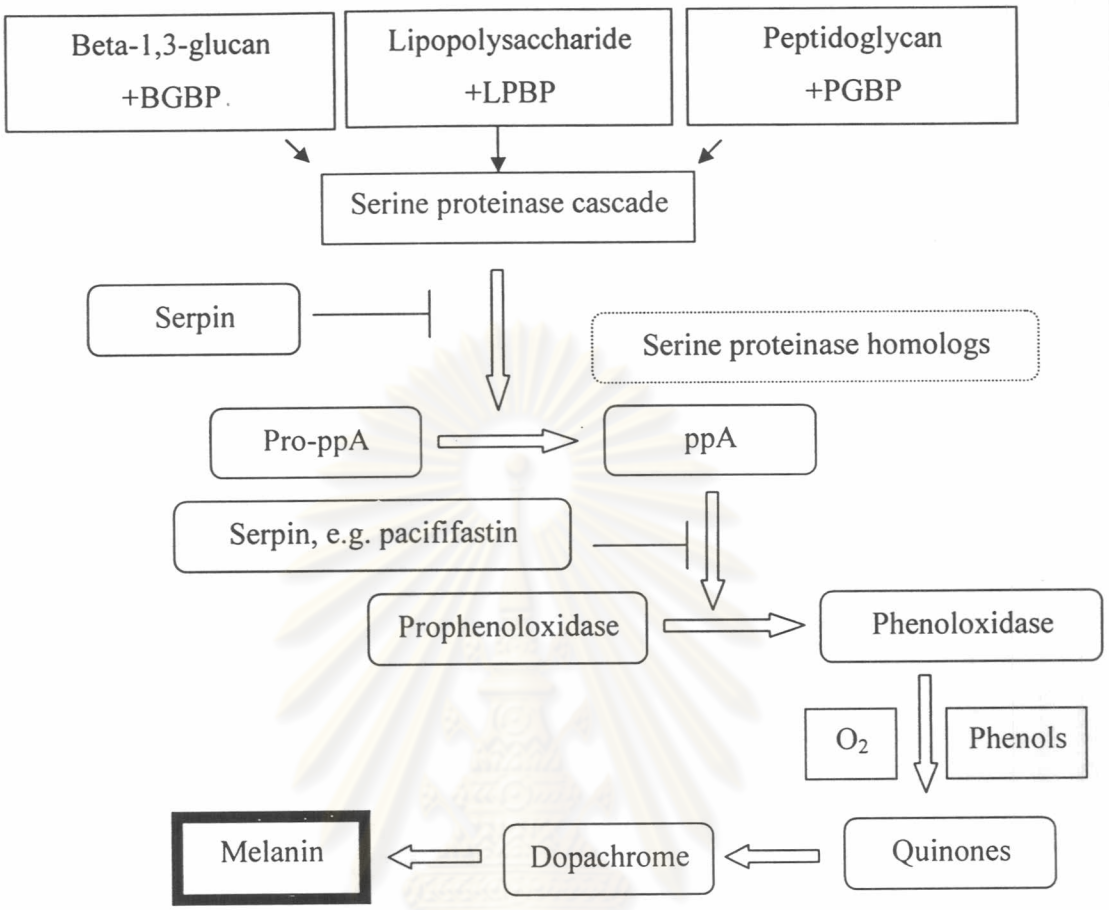


Figure 7. Overview of the arthropod prophenoloxidase-activating system. The system is activated by recognition proteins that have bound to β-1,3-glucan, lipopolysaccharides, peptidoglycans, or by other compounds such as endogenous factors produced upon tissue damage. A cascade of serine proteinases, which will cleavage to pro-form of the prophenoloxidasse-activating enzyme (pro-ppA) into active ppA. The serine proteinase homologs are some ppAs require them and some ppAs are capable of cleaving proPO directly into active phenoloxidase. BGBP = β-1,3-glucan binding protein; LPBP = lipopolysaccharide binding protein; PGBP = peptidoglycan binding protein; Pro-ppA = prophenoloxidase-activating enzyme; ppA = activated prophenoloxidase (Cerenius and Söderhäll, 2004).

Upon activation and degranulation of the hemocytes, the inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA). The PO enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin. During this formation also antimicrobial factors are formed (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). Melanin is a dark brown pigment that sequesters the pathogens, thus preventing contact with the host animals. Melanised matter can often be seen as dark spots in or under the cuticle of arthropods.

An important factor that is associated with the proPO system is peroxinectin, which was recently cloned for *P. monodon* (Sritunyalucksana et al., 2001). Peroxinectin has two different functions both cell-adhesion and peroxidase activity. Peroxinectin in crayfish is synthesised in the hemocytes, stored in the secretory granules in an inactive form, and released in response to stimuli and activated. Transmembrane receptors of the integrin family on the hemocytes play an important role in the cell-adhesion function of peroxinectin (Johansson, 1999). The cell-adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination, while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading microorganisms (Johansson and Söderhäll, 1988; Kobayashi et al., 1990). Phagocytosis is the internalization of small foreign particles by individual cells. After ingestion, also shrimp hemocytes, like vertebrate blood cells, use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994). If large amounts of particles enter the body or if they are too large to be internalized, several hemocytes will cooperate to seal off the pathogens these phenomena are called nodule formation and encapsulation, respectively (Söderhäll et al., 1996).

Enzyme inhibitors, produced together by the hemocytes, are necessary to regulate the proteinase cascades, prevent over activation and damage to the host tissue. Serine proteinase inhibitors from the Kazal and serpin families have been identified in crustaceans (Kanost, 1999), and α2-macroglobulin, which serves as a broad spectrum protease-binding protein is stored in the hemocyte granules (Armstrong and Quigley,

1999). In addition, hemocytes play an important role in the production and discharge the agglutinins (Kopácek et al., 1993), antibacterial peptides (Destoumieux et al., 2000) and cytotoxic molecules such as lysosomal enzymes: lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases (Millar and Ratcliffe, 1994).

2.3 Pathogenic diseases in shrimp

Although a large portion of the world's farmed shrimp is produced in Asia, shrimp culture operations do not succeed over the entire area. Shrimp aquaculture growth in Asia has suffered many several problems in recent years, and the major factor contributing to the problem in sustaining shrimp aquaculture are disease outbreaks, environmental degradation and poor management practice (Primavera, 1998). One of these problems is disease. Several shrimp diseases have threatened shrimp production both viral diseases, such as the most devastating ones namely yellow-head virus (YHV) and white spot syndrome virus (WSSV) (Flegel, 2001) and bacterial diseases such as filamentous bacteria and vibrios, with the latter being more important ones namely *Vibrio alginolyticus* and *Vibrio harveyi* (Lavilla-Pitogo, 1995).

The viral diseases of penaeid shrimp approximately 20 viruses have been described in shrimp culture. The white spot syndrome virus (WSSV) has had the greatest impact on shrimp culture and at present causes still the major disease problem (Rosenberry, 2001). Other important viruses are infectious hypodermal and haematopoietic necrosis virus (IHHNV), hepatopancreatic parvo-like virus (HPV), baculoviral midgut gland necrosis virus (BMNV), baculovirus penaei (BP), yellow head virus (YHV), *Penaeus monodon* baculovirus (MBV), lymphoid organ vacuolisation virus (LOVV) and Taura syndrome virus (TSV) (Lightner, 1996). Viral diseases are often accompanied by bacterial infestations (Lightner et al., 1998). Only a small number of bacterial species have been diagnosed as infectious agents in penaeid shrimp. *Vibrio spp.* is by far the major bacterial pathogens and can cause severe mortalities, particularly in hatcheries. Vibriosis is often considered to be a secondary (opportunistic) infection, which usually occurs when shrimp are weakened (Johnson and Söderhäll, 1989; Lightner

et al., 1992). Primary pathogens can kill even when other environmental factors are adequate, whereas opportunistic pathogens are normally present in the natural environment of the host and only kill when other physiological or environmental factors are poor. In practice, the differences in effects are marginal between primary pathogens, such as the white spot syndrome virus nowadays in shrimp, and opportunistic pathogens. Because (semi-)intensive shrimp culture is relatively new, basic knowledge of the interaction between the pathogens of cultivated shrimp and the reaction of the hosts is still poor (Flegel, 1997), which complicates the development of intervention strategies. Therefore, during the last decade, infectious diseases constitute a main barrier to the development of shrimp aquaculture, both in terms of product quality and regular supply, thereby threatening the continuity of the development (Meyer, 1991; Mialhe et al., 1995; Rosenberry, 2001).

Virus disease: The virus disease can cause considerable mass mortality in shrimp cultures. White spot syndrome virus (WSSV), one of the viruses in particular that have caused major disease losses to shrimp farmers at during rearing of the black tiger shrimp in the Thailand (Flegel and Fegan, 1993) and a broad host range infecting several cruatacean species such as shrimps (penaeid and non-penaeid), crabs, aquatic inset larvae and crayfishes (Lo et al. 1996; Maeda et al., 1998; Li et al., 2005). Some species are susceptible enough to become diseased and some are not so highly susceptible as to succumb to the diseae, but the latter are important as carriers able to spread the pathogen (Kanchanaphum et al., 1998; Wang et al., 1998). WSSV is considered to be primary pathogens has been reported in cultured penaeid shrimp in the Asia-Pacific region since 1993 (Inouye et al., 1994; Wongteerasupaya et al, 1995). WSSV is a virus group provisionally named whispovirus (Van Hulten et al., 2000 and Tsai et al., 2000). WSSV virions are DNA parvovirus particles, a circular double-strand DNA with an estimated size of 305 kb, enveloped with a distinctive tail-like appendage at one end, rod-shaped, nucleocapsids with a bacilliform to ovoid shape about 275 nm in length and 120 nm in width (Wongteerasupaya et al., 1995; Durand et al., 1997; Yang et al., 2001). The virions contain one nucleocapsid with a typical striated appearance and five major and at least 13 minor proteins (Van Hulten et al., 2000). WSSV virions have found primarily in the

intranuclear inclusion bodies of the affected cells. In shrimp farming system can be transmitted by horizontal transmission of virus via oral ingestion and via water borne (Chou et al., 1998; Corsin et al., 2001). In hatcheries can betransmitted by vertical transmission as the primary route from infected spawners to eggs (Mushiake et al., 1999). The viral epizootics in crustaceans caused by these viruses are characterized by lack of an inflammatory response in moribund shrimp. Crustaceans do not possess immunoglobulin and T cell receptor and thus do not seem to have a true adaptive immune response although immunoglobulin-like proteins have been found in some insects (Söderhäll and Thornqvist, 1997). In shrimps are a lack of inflammatory response to viral pathogens and the occurrence of single to multiple persistent viral infections. The principal clinical sign of WSSV is presence of white spots in the exoskeleton such as carapace (Chou et al., 1995). WSSV-infected shrimps have a loose cuticle with white spots of 0.5–2.0 mm in diameter in the cuticular epidermis but are most apparent on the inside surface of the carapace (Lightner 1996). The body colour of diseased shrimp becomes pale or reddish. The histopathological feature of WSSV is the hypertrophied nuclei of curticular epidermis, connective tissue, lymphoid organ, antennal gland, hematopoietic tissue and nervous tissue. WSSV infected cells are observed first in the stomach, gill and cuticular epidermis in shrimp after this, it subsequently spreads systemativally to other tissues of mesodermal and ectodermal origin (Chang et al., 1996) and cause a cumulative mortality up to 100% within 3 to 10 days from the onset of the disease (Takahashi et al., 1994).

Bacterial disease: Bacterial diseases have become limiting factors in shrimp culture systems. Various bacterial have been associated with shrimp disease, although only two groups have been implicated in major loses both filamentous bacteria and vibrios, with the latter being more important. Major *Vibrio* species have been reported in penaeid shrimps such as *Vibrio alginolyticus, V. anguillarum, V. cholerae, V. fluvialis, V. damsela, V. vulnificus, V. parahaemolyticus* and *V. harveyi.* Vibriosis disease is found to be the main cause of serious production losses in penaeid shrimp farms (Nash et al., 1992) and penaeid larval in the hatchery (Zafran et al., 1992). Bacterial infection of shrimp with *Vibrio spp.* (Vibriosis) is often related to injury, stress or diseases caused by other pathogens. *Vibrio harveyi* can cause mortalities from inconsequential to 100% that

depending on the associated stress conditions (Lightner, 1983). *Vibrio harveyi*, one of bacterial flora in the aquatic environment that the most persistent problems are effecting in shrimp culture especially to larval penaeids in the hatchery (Lavilla-Pitogo et al., 1990). *Vibrio harveyi* is a natural inhabitant of the seawater environment where it can be found in low numbers (Orndorf and Colwell, 1980). *Vibrio harveyi* is considered to be opportunistic and secondary pathogens. *Vibrio harveyi* is a gram-negative, luminescent, marine bacterium isolated both in a free-living state and as a commensal organism in the enteric contents of marine animals (Jiravanichpaisal et al., 1994). Symptom of sickness shrimps are showed erratic or disoriented swimming behaviour near the surface of the pond water or lay on the pond bottoms at the edges without exhibiting escape reflexes, empty gut and a dark, slow response to stimuli and discolouration of the appendages. Shrimp infected with *Vibrio harveyi* is exhibits a continous greenish luminescence. Light microscopy revealed densely packed bacteria in the hemocoel of luminescent moribund larvae (Lavilla-Pitogo et al., 1990). The infected larvae appear luminescent in darkness and suffer heavy mortality. The infection hepatopancreas showed necrosis of tubular hepatopancreatic cells with bacteria inside the lumens and subsequent granulomatous lesions enclosing the invaded tubules. Shrimp have a per-cuticular infection and exhibited bacteraemia resulting in hemolytic aggregation in response to the bacteria in the subcuticular connective tissue, lymphoid organ, interstitial tissue of the hepatopancreas and other tissues. Induce necrosis of hepatopancreatic cells and the thickened basal lamina, subsequent granulomatous encapsulation of the invaded tubules, and production of granulation tissue around granulomatous lesions and necrosis in the heart and lymphoid organ (Jiravanichpaisal and Miyazaki, 1994). The initial histopathological change that occurs in the tissues of shrimp with vibriosis is poorly vacuolated hepatopancreatocytes. Subsequent edema, hemocytic infiltration and fibrosis in the hepatopancreatic interstitial tissues ensue (Anderson et al., 1988). More advanced stages of the infection are manifested by granulomatous hepatopancreatitis effecting the entire tubular system of the digestive organ (Nash et al., 1992). In shrimp juveniles, systemic bacterial infection was manifested by increasing opaqueness of abdominal muscles, anorexia and lethargy (Ruangpan et al., 1991). Virulence in *Vibrio harveyi* has been reported the production of an extracellular protein referred to as toxin T1 with a

molecular mass of approximately 100 kDa. The extracellular protein is produced during the mid-exponential phase of growth and has sequence similarity to virulence-associated proteins in *Salmonella*, *Shigella* and *Bacillus* species (Harris and Owens, 1999).

The internal defence mechanisms during vibriosis are often involved utilize haemocytes that regarded as a central and important way to eliminate micro-organisms or other small particle. Hemocytes are migrated to the site of injury and most immediately degranulated the bacterium. After this, process of wound sealing and bacterial degradation will begin before melanisation occurs. Many bacteria are encapsulated near the injury site. Bacterial clearance in the haemolymph has induced by humoral factors by agglutinated bacteria and followed by uptake in different places in the body. Bacteria is accumulated in the lymphoid organ where their degradation. The hemolymph, including the antigens, migrates from the central tubular lumen through the wall of lymphoid organ, where the bacteria are arrested and their degradation is started. Lymphoid organ revealed the presence of many phagocytic cells that morphologically resemble small-granular hemocytes. It is mean the hemocytes settle in the tubule walls before they phagocytose. Lymphoid organ is a filter for virtually all foreign material encountered in the hemolymph (Van De Braak et al., 2002).

2.4 Polysaccharides

Polysaccharides are carbohydrate of the most abundant organic molecules in nature. More than half of all 'organic' carbon is found in polysaccharide. The majority of these substances contained long chain units of monosaccharide which has simple elemental formula of $C_n(H_2O)_n$ hence the name 'hydrate of carbon' (Mckee et al., 1999). The structure of polysaccharide chains is still in its premature state with respect to the structure in solid and solution. The structural analysis may offer the most fundamental knowledge to understand the functions of polysaccharides, but the diversity and irregularity of polysaccharide chains make the structural analysis a formidable task. Polysaccharide chains are partly organized but are considered to be mostly amorphous. Thus, the crystallographic analysis of polysaccharide chains has been performed by either

using the small oligosaccharide single crystals or the x-ray fiber pattern diffraction. They have been adapted for a wide variety of biological functions, which include energy sources and structural elements (Kajiwara and Miyamoto, 1998).

Classification: Carbohydrates are biopolymers classified as monosaccharide, disaccharide, oligosaccharide and polysaccharide according to the number of simple sugar units they contain. The polysaccharides can be divided into the following:

- Monosaccharides for example; ribose and glucose
- Oligosaccharides for example; sucrose and saccharose
- Polysaccharides for example; starch and cellulose

A monosaccharide unit is the backbone of common to many polysaccharides, its linkage mode varies and characteristic functions/properties will appear accordingly. The conformation analysis of polysaccharide chains involves two aspects: (1) the characterization of a single-chain conformation and (2) the analysis of the chain assembly of polysaccharides.

Monosaccharides: Monosaccharides or simple sugars are defined as polyhydroxy aldehydes or ketones. Monosaccharides with an aldehyde functional group are call aldoses, while those with a ketone group are called ketoses. The simplest aldose and ketose are glyceraldehydes and dihydroxyacetone, respectively. Sugars are also classified according to the number of carbon atoms they contain. For example, the smallest sugars are called trioses, pentoses and hexoses, respectively. The most abundant monosaccharides found in living cell are the pentoses and hexoses. In class name of sugar will be describe monosaccharides by combine information about carbon number and function groups. Example for some class sugars, aldohexoses in this name is referred to containing a six-carbon number and function groups of aldehyde.

Oligosaccharides: Oligosaccharides are often used for polysaccharides that consist of two to ten monosaccharide units. Disaccharides are composes of two

monosaccharide units linked by a glycosidic linkage. Found in abundance in nature, disaccharides provide a significant source of calories in many human diets. Examples of important diet disaccharides include lactose and sucrose. Oligosaccharides are most often found attached to polypeptides in glycoproteins and to some lipids in glycolipids. Among the best-characterized oligosaccharide groups are those attached to membrane and secretory proteins found in the endoplasmic reticulum and Golgi complex of various cells.

Polysaccharides: Polysaccharide molecules are used as storage forms of energy or as structural materials. They are composed of large numbers of monosaccharide units connected by glycosidic linkages. Most common polysaccharides are large molecules containing from hundreds to thousands of sugar units. These molecules may have a linear structure like that of cellulose or amylose, or they may have branched shapes like those found in glycogen and amylopectin. Unlike nucleic acids and proteins, which have specific molecular weights, the molecular weights of many polysaccharides have no fixed values. The size of such molecules is a reflection of the metabolic state of the cell producing them. For example, glycogen molecules in a well-fed animal many have molecular weights as high as $2x10^7$ daltons.

Polysaccharides may be divided into two classes: homopolysaccharides, which are composes of one type of monosaccharide, and heteropolysaccharides, which contain two or more types of monosaccharides.

The homopolysaccharides found in abundance in natural are starch, glycogen and cellulose. All of these substances yield D-glucose when they are hydrolyzed. Starch and glycogen are glucose storage molecules in plants and animals, respectively. Cellulose is the primary structural component of plant cells.

The heteropolysaccharides are high-molecular-weight carbohydrate polymers that contain more than one kind of monosaccharide. It may contain many of the sugar residues of amino acid derivative, these substances are often referred to as

glycosaninoglycans (GAGs). GAGs, the principal components of the proteoglycans, are classified according to their sugar residues, the linkages between these residues, the presence and location of sulfate groups. Five classes are distinguished: hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparin and heparin sulfate and keratin sulfate.

Carbohydrates are important in crustaceans as an energy source in the Krebs cycle, in glycogen storage, in chitin synthesis, in the formation of steroids and in fatty acid synthesis. In addition carbohydrates have a protein sparing effect (Capuzzo, 1982). Carbohydrate digestion in crustacean has many of carbohydrases such as α and β-amylase, maltase, saccharase, chitinase and cellulose (Kooiman, 1964). Various dietary carbohydrates have been reported that carbohydrate being incorporated into shrimp diet for the growth and feed efficiency. Use of dietary carbohydrates, in addition to immunostimulants agents, has been reported and wildly accepted by shrimp culture industry. Some of dietary carbohydrates have reported the improving of the immune defense system of shrimp as an immunostimulant. Shrimp have a powerful ability to respond non-specifically to a variety of compounds, most of which are apparently carbohydrate-based. For example of dietary carbohydrates used in shrimp culture as following:

Glucans: Glucans are carbohydrate polysaccharides, a commonly structural component of the cell walls of fungi, yeast, some algae and some bacteria. They are also found in a variety of other organisms including in many plants. Glucans are polymer of glucose molecules consisting of glucose molecules with different types of linkages. The most common the specific type of chemical bond linkage associated with immune stimulating properties is the β-1,3-glucan linkaged. Beta glucan are being sold in many different forms such as yeast glucan and peptide-glucan β-1,3-glucan, most of the commercially available form are derived from the common yeast *Saccharomyces cerevisiae*. Some forms of β-1,3-glucan such as lentinan is (1→3)-β-D-glucan (Chihara, 1988) and schizophyllan is β-1,3-glucan (Matsuyama et al., 1992). The structure of glucan is demonstrated in Figure 8.

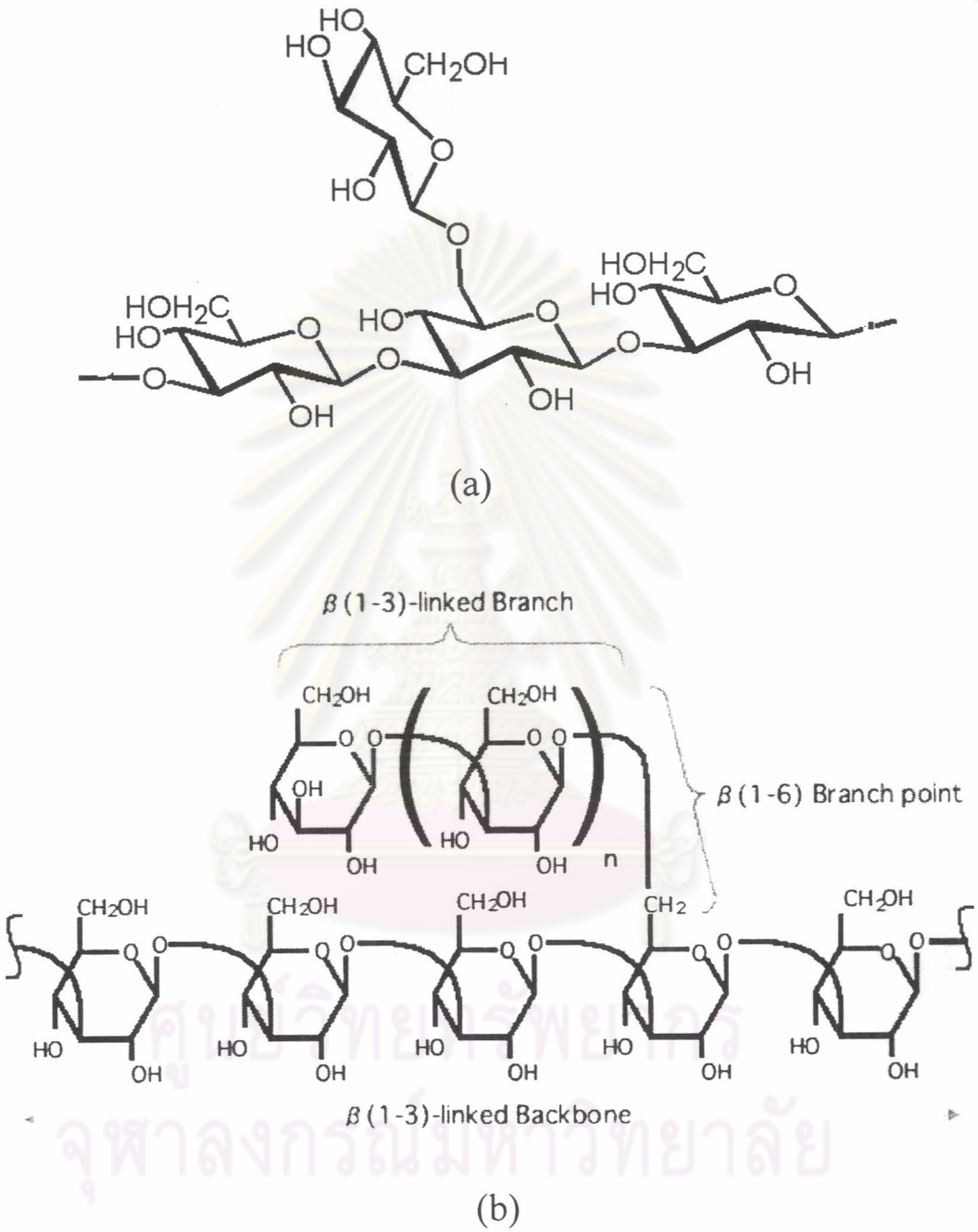


Figure 8. Structure unit of β-1,3-glucan. (a) Conformation structures of β-1,3- glucan, D-glucoses backbone are linked with (1→3)-β-glycosidic bond and (1→6)-β-glycosidic bond forming branch point. (b) Detail forming of β-1,3-glucan with β-(1→6)-linked of branch point.

They are essentially yeast extracts with varying degrees of purity. Some studies reported that glucan (but not all) exert a short-term effect on the immune system of shrimp and can provide non-specific protection against bacteria (Sung et al., 1994; Liao et al., 1996) and viral diseases (Chang et al., 1999). Glucan have been reported the effects in shrimp to increase the tolerance to stress (Song et al, 1997).

Lipopolysaccharides: Lipopolysaccharides (LPS) is an important structural component of the cell wall which a specific type of gram-negative bacteria, though they can also be found in algae and other types of bacteria as well. Lipopolysaccharides are composes of lipids and carbohydrates. Lipopolysaccharide structures molecules have a lipid region (lipid A), a core oligosaccharide and an 'O-specific' chain, which is the principle determinant of the immunological reactivity of bacterium. Lipopolysaccharides of some bacteria are toxic to humans and other animals. The structure of lipopolysaccharides is demonstrated in Figure 9.

Lipopolysaccharides (LPS) are usually the first structures that invading bacteria and present to the immune system in animal. Lipopolysaccharides are exerts both specific and non-specific effects on the immune system of all animals, and potent non-specific effects in crustaceans (Söderhäll and Häll, 1984; Sung et al., 1996). Vibrios, the most common bacterial pathogens effecting shrimp, present these structural components to the immune system of animal. LPS have been reported to be a potent non-specific antiviral (Takahashi et al., 2000; Takahashi, Kondo, et al., 2000) and antibacterial effects (Sritunyalucksana et al., 1999). In addition, LPS have been reported that increased the survival rate (Itami et al, 1991), enhanced the growth performance and improved feed conversion and growth in shrimp (Song and Sung, 1990).

Peptidoglycans: Peptidoglycan is component of the cell walls of many different bacteria and microorganisms, though it is found in greater amounts in gram-positive bacteria. Peptidoglycans are a mixture of amino acids and sugars. Peptidoglycan of bacterial cell walls is a heteropolymer of alternating β-(1→4)-linked of N-acetylglucosamine and N-acetylmuramic acid residues.

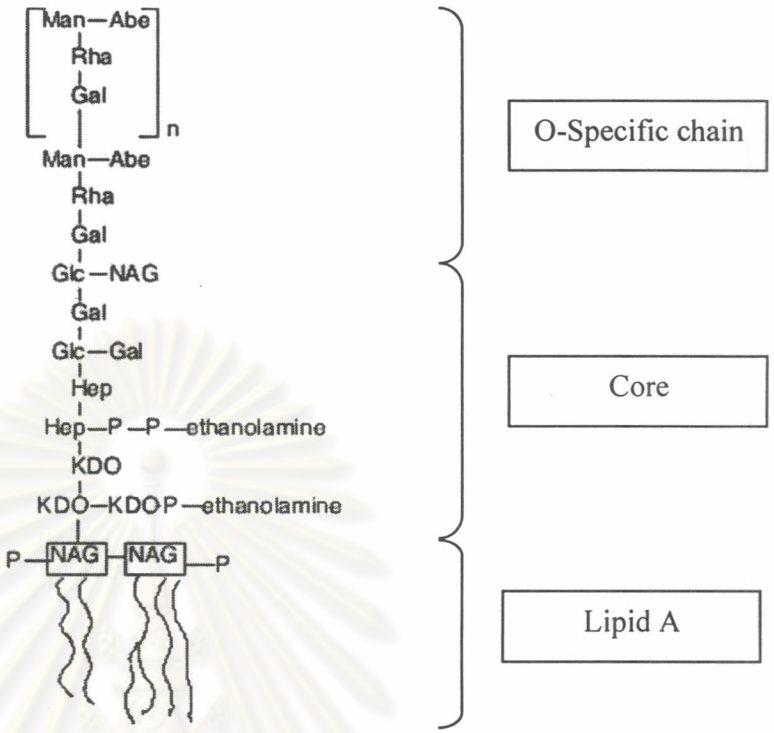


Figure 9. Structure unit of lipopolysaccharide. The lipopolysaccharide are the dominant surface of the outer membrane of gram-negative bacteria. The lipopolysaccharide component in common gram-negative bacteria have the lipid A, fatty acids, core region, oligosaccharide compound and O-specific chain, which the principal determinant of the serotype (immunological reactivity) of the bacterium. NAG = N-acetyl-D-glucosamine; KDO = 2-keto-2-deoxyoctonic acid or 2-keto-3-deoxyoctonic acid; Abe = 3,6-dideoxyhexose.

The linear polymers lie side by side in the cell wall, cross-linked by sort peptide of a tetrapeptide (L-Ala-D-Glu-L-Lys-D-Ala) that attach in amide link to the carboxyl group of lactate in N-acetylmuramic acid residues. The heteropolymer strands are covalently connected by pentaglycine chain bridge through the terminal D-Ala residue of one tetrapeptide strand and a neighboring of the L-Lys on another tetrapeptide chain. The structure of peptidoglycan is demonstrated in Figure 10.

Peptidoglycans are active on the non-specific immunity of shrimp by presence a compound which is one of the first things that the shrimp immune systems will response to an invading pathogen (Sritunyalucksana et al., 1999). Peptidoglycan have been reported the effects in black tiger shrimp to increases the tolerance to stress, growth rate, survival and feed conversion rate (Boonyaraptalin, et al., 1995). Peptidiglycan have also been reported the enhancing of the disease resistant against virus infectious including white spot syndrome virus WSSV (Itami et al., 1998).

Sodium alginate: Sodium alginate is a polysaccharide. Sodium alginate is found in algae cell wall such as in brown seaweed (*Phaeophyceae*), fungus and yeast. Sodium alginate are linear copolymer, salts of aginic acid, consists of (1→4)-β-D-mannuronate and (1→4)-α-L-guluronate, which different from that of fungus and yeast-derived polysaccharide (Dumitriu, 1987). Sodium alginate has gelling polysaccharide property. The structure of sodium alginate is demonstrated in Figure 11.

Sodium alginate has been reported to enhance the resistance of common carp *Cyprinus carpio* against *Edwardsiella tarda* infection that extracted from *Undaria pinnatifida,* a brown algae (Fujiki et al., 1994). Sodium alginate has been reported to increase the non-specific defense system of common carp *Cyprinus carpio* that extracted from giant kelp *Macrocystis pyrifera* (Fujiki and Yano., 1997). Sodium alginate has been reported to increase the resistance and immune ability of white shrimp *Litopenaeus vannamei* against *Vibrio alginolyticus* infection that extracted from giant kelp *Macrocystis pyrifera* (Cheng et al., 2004).

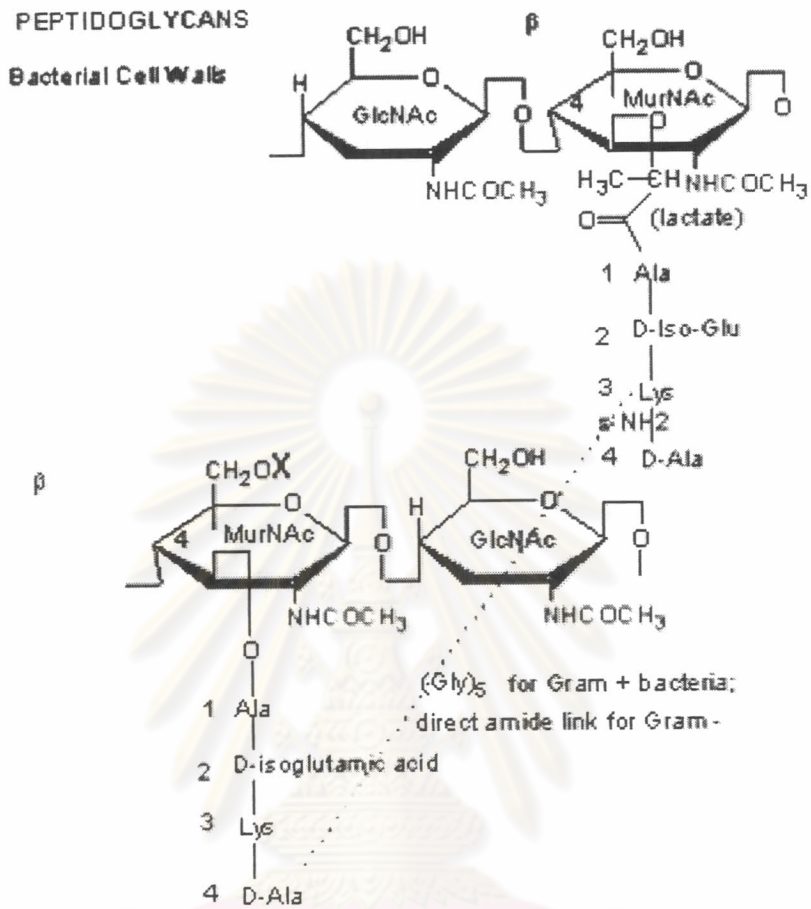


Figure 10. Structure unit of peptidoglycan. Peptidoglycan component is constituted of the two aminosugars, N-acetylglucosamine and N-acetylmuramic acid residues, alternate by β-(1→4)-linked. The peptide units are linked through the lactic acid carboxyl group of N-acetylmuramic acid residues to the amino terminus of a tetrapeptide. D-alanine is always the linkage unit between peptidoglycan chains. Ala = L-alanine; D-Ala = D-alanine; D-Iso-Glu = D-isoglutamic acid; Lys = L-lysine; GlcNAc = N-Acetylglucosamine; MurNAc = N-Acetylmuramic acid.

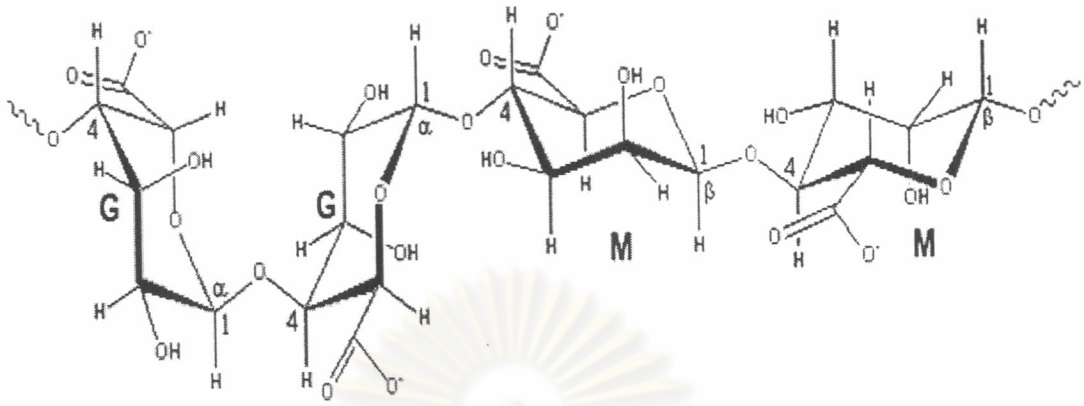


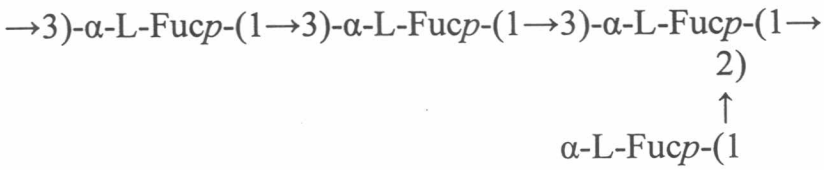
Figure 11. Structural unit of alginates, alginates are linear unbranched polymers containing β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) residues.

Fucoidan: Fucoidan is found in algae cell wall such as in brown seaweed (*Phaeophyceae*) which produces families of sulfated fucoidan and other polysaccharide. Fucoidan is located in the intercellular tissue and most strikingly in droplets that exude from surface of the fronds (Doner and Whistler, 1973). Fucoidan is a sulfated polysaccharide, a class of sulfate-containing polysaccharides. In main chain of fucoidan consists of a sulfated L-fucose sugar. Some type of fucoidans is the complex sulfated polysaccharide from the brown seaweed (Sugawara et al., 1989).

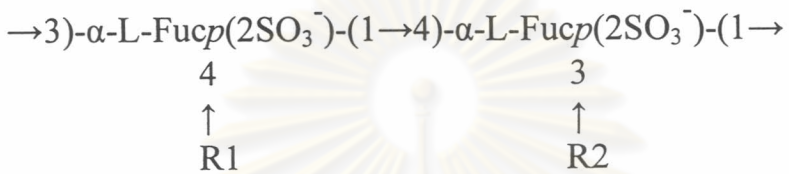
Fucoidan has been reported in shrimp that it reduce the impacted of the enveloped virus WSSV (Takashi et al., 199). Chotigeat et al., 2003 has reported that oral administration of crude fucoidan extracted from brown seaweed can reduce the impact of WSSV in black tiger shrimp, inhibit the growth both gram positive and gram negative bacteria, and increase the phagocytic activity these parameters infer that fucoidan may stimulate immunity in shrimps. The some structure of fucoidan is demonstrated in Figure 12.

Some type of fucoidans was found to inhibit human immunomodeficiency virus (HIV) *in vitro*. These activities presumably resulting from a direct interaction of the polysaccharide with the binding site of virus on the target cell or inhibit the adsorption of enveloped viruses into host cell (Witvrouw and De Clercq, 1997). Some effect of fucoidan has antibacterial activity (Rao and Parekh, 1981), antiviral activity (Fabregas et al., 1999).

Chitin: Chitin is a polysaccharide, cellulose-like biopolymer that found in the component of shells or walls of invertebrates such as in the main component of crustacean exoskeletons, gut lining, peritrophic membrane of most crustacean species, the cell walls of fungi and yeasts. Chitin is an unbranched polymer of glucosamine molecules. Chitin is consisting of glucosamine molecules with the β-(1→4)-N-acetyl-D-glucosamine linkage. The structure of chitin is demonstrated in Figure 13.



(a)



R1 = SO3-, Acetate or H

R2 = Acetate or H

(b)

Figure 12. Structure unit of fucoidan from brown seaweeds. (a) Fucoidan structure of *Chorda filun* is a linear backbone which consisting linked with (1→3)-α-glycosidic bond and (1→2)-α-glycosidic bond forming branch point (Chizhov et al., 1999). (b) Structure of fucoidan from *Fucus evanescens* which consisting of L-fucose, sulfate and acetate. The fucoidan is a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues (Bilan et al., 2002).

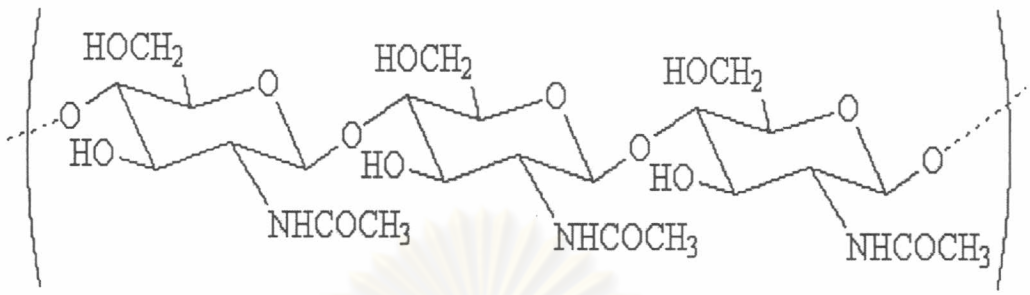


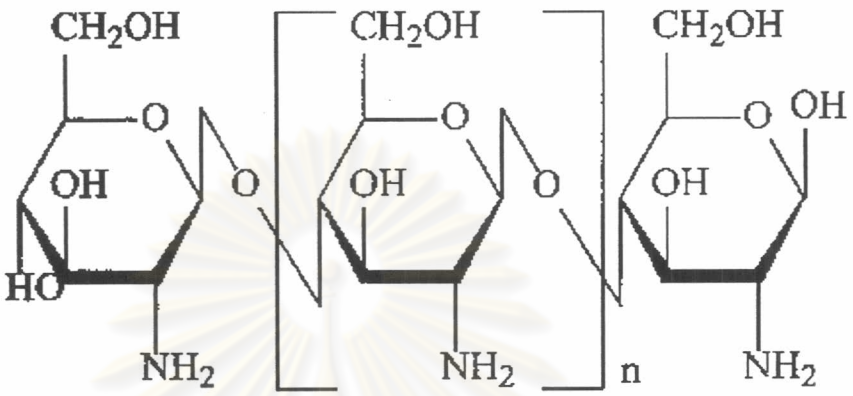
Figure 13. Structure unit of chitin consists with a linear backbone of poly-β-(1→4)-N-acetyl-D-glucosamine.

Chitin is highly acetylated about 15-21% acetyl content and insoluble in common solvents (Fillar and Wirick, 1978). Shiau and Yu, 1997 has reported the effects of chitin in black tiger shrimp in enhancing the growth rate in contrast with the finding of Fox (1993), increasing levels of dietary chitin did not significantly effect promote weight gain, feed conversion ratio and survival rate. Dietary carbohydrate source from glucosamine has been found to be a growth promoting factor in shrimp (Kanazawa et al., 1970). This growth effect indicates the shrimp ability to absorb and utilize dietary sources of glucosamine (Kitabayashi et al., 1971).

Chitosan: Chitosan is an aminopolysaccharide which prepared from chitin by deacetylation with alkali treatment. Chitosan is a polymer of glucosamine molecules which prepared from deacetylation reaction by treatment the chitin with alkali. The deacetylation of chitin will provided a polysaccharide that consisting of glucosamine molecules with the β-(1→4)-N-acetyl-D-glucosamine linkage, behaves as a weak anion exchange resin and has viscosity properties similar to those of certain water soluble dietary fiber such as guar, gum and pectin (Furda, 1983). The structure of chitosan is demonstrated in Figure 14.

Chitosan is lowly acetylated about 3-5% acetyl content, in contrast with chitin (Fillar and Wirick, 1978). Chitosan has been reported the immunostimulatory effects to increase in brook trout fish *Salvelinus fontinalis* that provided the protection against *Aeromonas salmonicida* infection (Anderson and Siwicki, 1994). Rainbow trout treated with chitosan by injection or immersion showed the increasing of the immunostimulatory effects (Anderson et al., 1995).

Carrageenan: Carrageenan is sulfated polysaccharide and found in algae cell wall such as in various red seaweeds (*Rhodophyceae*). Carrageenan has been identified into three major carrageenan fractions: κ (gelling), ι (gelling), λ (non-gelling). All fractions are composed of galactose residues sulphated to different degree and alternate β-(1→4)-linked (Blanshard and Mitchell, 1979). The structure of carrageenan is demonstrated in Figure 15, 16, 17 and 18.



Chitosan

Figure 14. Structure unit of chitosan, an aminopolysaccharide, consists with a linear backbone of poly-β-(1→4)-N-acetyl-D-glucosamine that prepared from deacetylation reaction of chitin by treatment with alkali reagent.

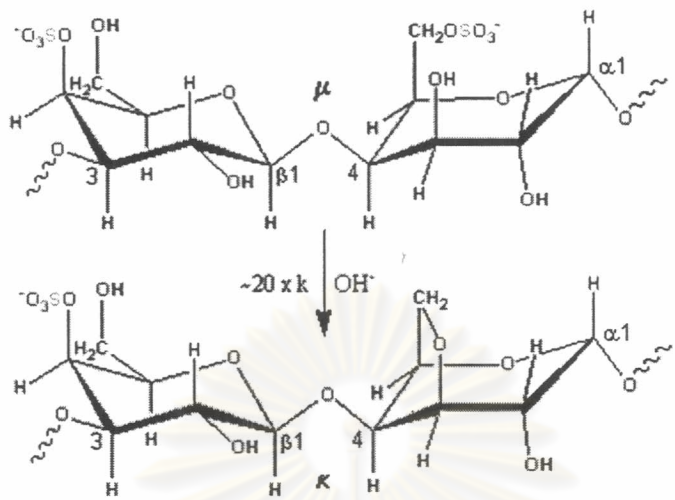


Figure 15. Structure of *k*-carrageenan (kappa-carrageenan) [-(1→3)-α-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-D-galactopyranose-(1→3)-]. k-Carrageenan is produced by alkaline elimination from *μ*-carrageenan isolated mostly from the tropical seaweed *Kappaphycus alvarezii* (also known as *Eucheuma cottonii*)

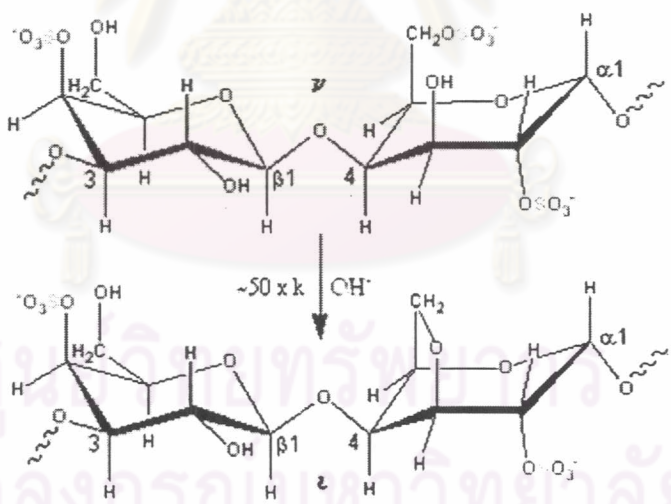


Figure 16. Structure of *i*-carrageenan (iota-carrageenan) [-(1→3)-α-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-D-galactopyranose-(1→3)-]. i-Carrageenan is produced by alkaline elimination from *ν*-carrageenan isolated mostly from the Philippines seaweed *Eucheuma denticulatum* (also called Spinosum).

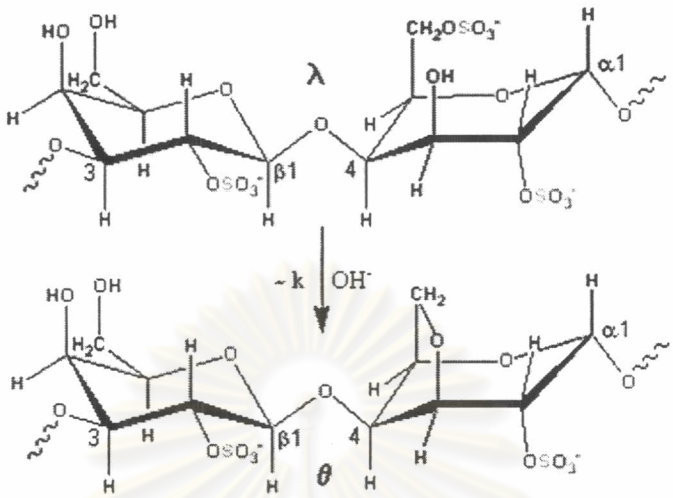


Figure 17. Structure of λ-carrageenan (lambda-carrageenan) [-(1→3)-α-D-galactopyranose-2-sulfate-(1→4)-D-galactopyranose-2,6-disulfate-(1→3)-]. λ-Carrageenan (isolated mainly from *Gigartin pistillata* or *Chondrus crispus*) is converted into θ-carrageenan (theta-carrageenan) by alkaline elimination, but at a much slower rate than causes the production of *i*-carrageenan and *k*-carrageenan.

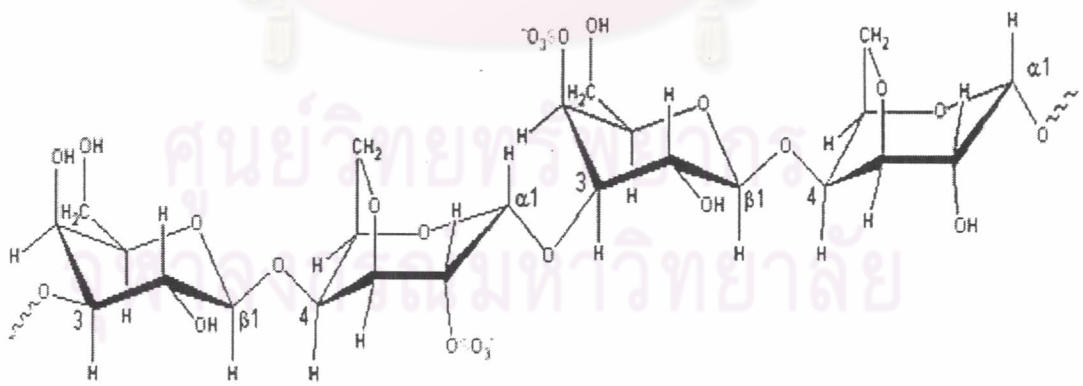
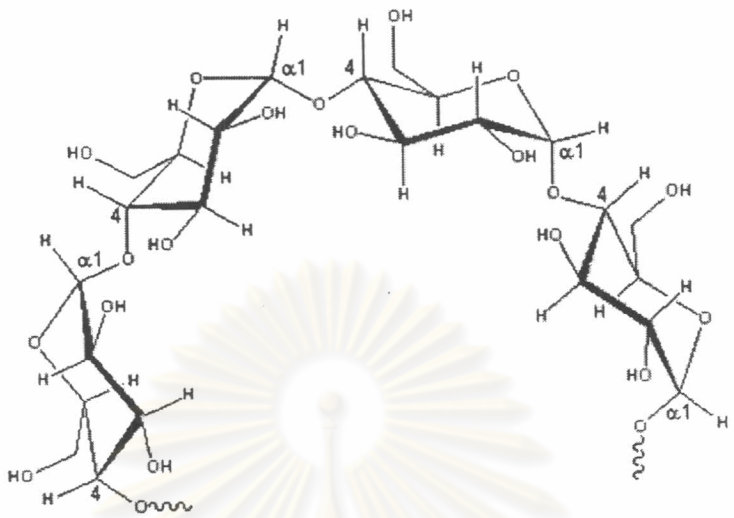


Figure 18. Structural unit of carrageenan consists of alternating (1→3)-α-D-galactopyranose and (1→4)-β-D-galactopyranose units

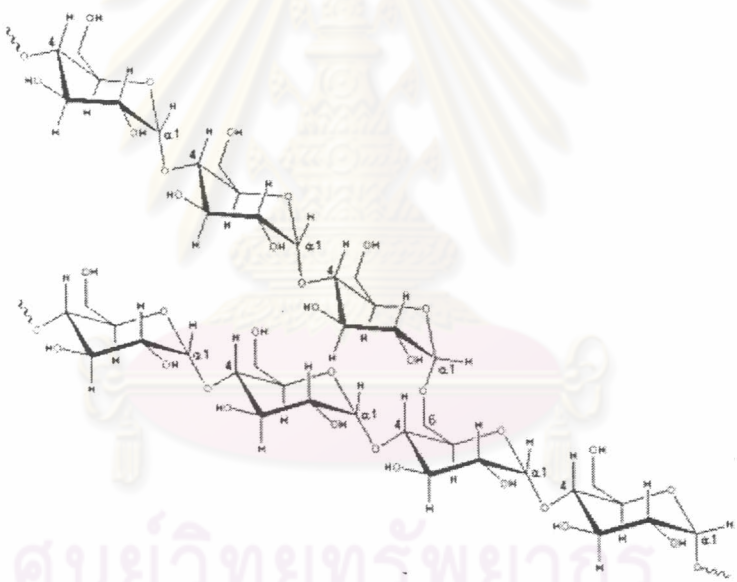
Carrageenan has been reported the use as seaweed meals from red seaweed *Kappaphycus alvarezii* and *Gracilaria heteroclada*, as binders in shrimp diets to promote growth in black tiger juvenile shrimp *Penaeus monodon* such as increase the weight gain and survival rate, improve the feed conversion ratio (FCR) and reduce organic waste from the feed (Peñaflorida and Golez, 1996).

Starches: Starches are the most important storage polysaccharide and found in general plant. Starch occurs intracellular as large granules. Starch is made in the chloroplast from D-glucose formed photosynthesis mechanism. Starch in plant is especially abundant in tubers and seed. Starches are carbohydrate polymer of D-glucose. Starch is heavily hydrated because they have many exposed hydroxyl groups available to hydrogen bond with water. Starch usually contains two components of glucose polymer, amylose and amylopectin. Amylose, a linear, straight chain, (1→4)-α-D-glucopyranose polymer. Such chains vary in molecular weight from a few thousand to over a million. Amylopectin also has a high molecular weight (up to 100 million) but unlike amylose is its highly branched. The glycosidic linkages joining in amylopectin chains are (1→4)-α-D-glucopyranose with periodic branching of side chains with α-(1→6) linkages.

Starches have been reported the effect of promoting growth in brine shrimp *Balanus balanoides* (Barnes et al. 1978), white shrimp *Litopenaeus vannamei* (Gaxiola et al., 2005). Increasing starch level from 10 to 40% in shrimp diets of *Penaeus merguiensis* and *Penaeus idicus* will promotes growth and feed conversion ratio, decreases the protein-to-carbohydrate ratio, increases the survival rate better than monosaccharides, which seem to inhibit growth (Boonyaratpalin, 1998). Gelling properties of starches are dependent upon the amylose component. The structure of starch is demonstrated in Figure 19.



Representative partial structure of amylose



Representative partial structure of amylopectin

Figure 19. Structural unit of amylose and amylopectin. Starch consists of two types of molecules, amylose (normally 20-30%) and amylopectin (normally 70-80%). Both consist of polymers of α-D-glucose units in the $^4C_1$ conformation. In amylose these are α-(1→4)-linked whereas in amylopectin about one residue in every twenty or so is also α-(1→6)-linked forming branch points. The relative proportions of amylose to amylopectin and α-(1→6) branch points both depend on the source of the starch.

2.5 Nutritional requirement of shrimp

The shrimp diets are an important requirement for growth and healthiness of shrimp. The essential nutritional composition of shrimp diets must be contained proteins, carbohydrates, lipids and sterols, fibers, minerals and vitamins. Each ingredient has its specific role of importance for growth accelerator.

2.5.1 Proteins: The major content in shrimp diet is protein. Proteins are a large class of nitrogenous substances of a complex polymer of amino acids containing carbon (50%), oxygen (22%), hydrogen (0.7%), nitrogen (16.0%) and other elements such as sulphur, phosphorus, iron and iodine. Nitrogen (N) is the most important element of protein. The basic units of protein are amino acids. There are some essential amino acids can not be synthesized by aquatic animals and have to be provided by feeding. Amino acids are not only the building blocks of protein but also involved in growth, maintenance of body tissue and being an energy source for the well-being of the animal (Pandian, 1989).

In the shrimp digestive tract, dietary proteins are split into free amino acids and short peptide chains by enzymes, and then amino acids absorbed through the gut-wall, protein appears in the form of amino acids in blood circulation (Hepher, 1988). Utilization of dietary protein depends on digestibility of proteins which is effected by a number of factors (Paulraj, 1995). The black tiger shrimp *(Penaeus monodon)* required about 40% protein in diet to produce a maximum growth when reared in seawater (Shiau et al., 1991). The ten essential amino acids in diet for shrimp compose of arginine, histidine, isoleucine, lecine, lysine, methionine, phenyalanine, threonine, tryptophan and valine (Alava and Lim, 1983).

The principles for providing crustaceans with dietary protein are similar to those for fishes. In addition to species, food-habits, age, water temperature, sources of protein and energy level of diets as well as stocking rate effects the protein requirement of crustaceans (Kamazawa, 1995). For intensive shrimp culture commercial

feed should contain not less than 35 % protein while semi-intensive shrimp culture requires in a rage between 20-35% (Lim and Persey, 1988). The percent protein commonly cultured crustaceans is requires in a rage between for black tiger prawn (*Penaeus monodon)* 40-50, white shrimp (*Litopenaeus vannamei)* 30-35, northern brown shrimp (*Penaeus aztecus*) 25-45, indian white prawn (*Penaeus indicus*) 36-63, kuruma prawn (*Penaeus japonicus*) 35-60, banana prawn (*Penaeus merguiensis*) 34-50, northern white shrimp (*Penaeus setiferus*) 28-32, blue shrimp (*Penaeus stylirostris*) 30-35, pink shrimp or common prawn (*Palaemon serratus*) 30, giant river prawn (*Macrobrachium rosenbergii*) 25-45, fresh water prawn (*Macrobrachium nobilis*) 30 and american lobster (*Homarus americanus*) 11-60 (Pandian, 1989). A guideline for amino acid requirement of crustaceans is the amino acid composition of the whole shrimp as demonstrated in Table 4.

2.5.2 Carbohydrates: The second major content in shrimp diet is carbohydrates. Carbohydrates or polysaccharides are generally composed of carbon, hydrogen and oxygen. Carbohydrates provide energy nutrients for all organisms. Globally carbohydrates form the major volume of all biological material.

Carbohydrates are simply classified as mono-, oligo- and polysaccharides as illustrated in Figure 20. Monosaccharides are the small molecules of basic compound of simple unit, linking of monosaccharide units to form oligosaccharides and polysaccharides, respedtively.

The major carbohydrates of feed ingredients for aquatic animals are oligo- and polysaccharides (starch, cellulose, pectin, etc.). Starch from plants, a major source of carbohydrate for shrimp, is a macromolecule commonly used in feed formulation composed of 2,000 to 8,000 β-glucose units (New, 1987).

Table 4. Amino acid profile of prawns (g/16 g N) (Pandian, 1989)

Amino acid	Tiger prawn *Penaeus monodon*	Indian prawn *Penaeus indicus*	Kuruma prawn *Penaeus japonicus*
Arginine	9.16	9.94	8.16
Histidine	1.52	1.91	2.40
Isoleucine	3.86	4.01	4.58
Leucine	8.04	7.24	8.04
Lysine	6.83	6.62	8.46
Methionine	2.01	1.82	2.90
Phenylalanine	4.02	4.01	4.83
Tyrosine	3.05	2.93	4.20
Valine	3.70	3.09	4.44

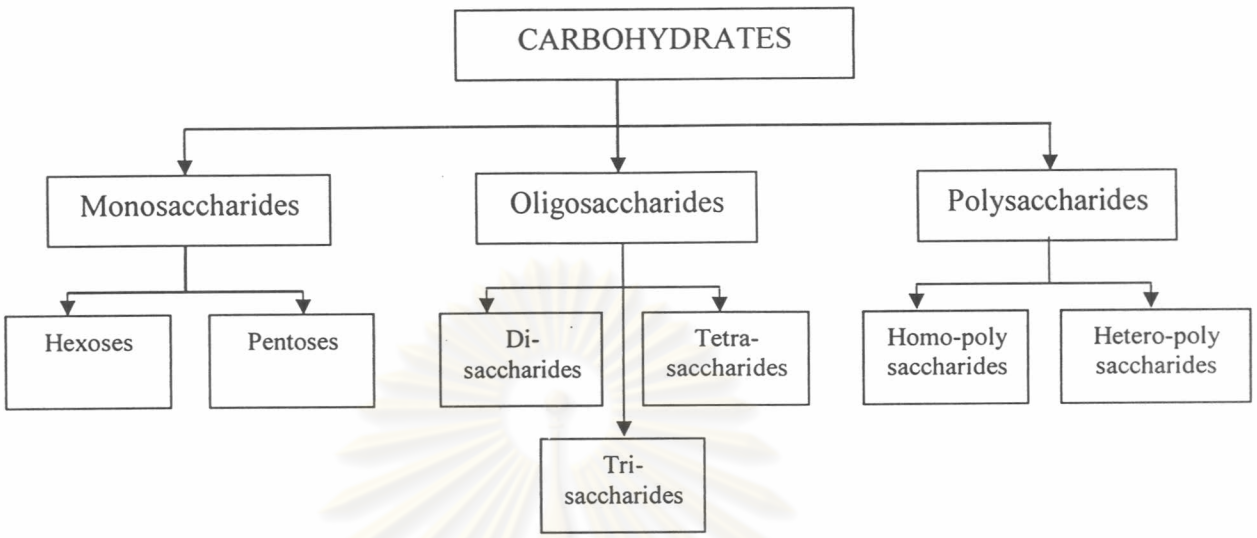


Figure 20. Classification of carbohydrates (saccharides)

In general, polysaccharides in the digestive tract are split into monosaccharides by enzymatic hydrolysis and then absorption. The enzyme for splitting cellulose such as cellulases is only produced by microorganisms. The indigestible lignin is the fibrous material of plant cell wall which obstructs the utilization of carbohydrates by the bacteria in digestive tract. The animal starch is glycogen, which primarily is the carbohydrate storage molecule that deposited in the liver (Hertrampf, 1991).

The analysis of composition is generally indirectly determined for carbohydrate. They are the difference between the sum of moisture, crude protein, crude fat, ash, crude fiber content and 100. Protein values were calculated from the level of total nitrogen, named the 'nitrogen-free extract' (N-free extract). It does not encompass cellulose, lignin and pentosanes which are included in the crude fiber content (Felwell et al., 1978). Gross energy, digestible energy and metabolicable energy are instruments for the determination of the energy value of feed ingredients. Metabolicable energy is difficult to determine with aquatic animals and the digestible energy from experiments are not readily available.

In crustaceans carbohydrates are important as an energy source for chitin synthesis and synthesis of non-essential fatty acid. In addition carbohydrates have a protein sparing effect (Capuzzo, 1982). However, there are differences in the utilization of the various carbohydrate sources. The disaccharides like sucrose, maltose and trehalose, and polysaccharides such as dextrin and starch have a high nutritive value as carbohydrate sources (Chen et al., 1988). Crustaceans are incapable of digesting oligosaccharides from seeds of legumes and pulses due to the absence of specific enzymes for digestion of non-α or β-(1$\rightarrow$4) linkage of these oligosaccharides (Chow et al., 1980). Chitin is a linear polysaccharide with the principle formula $(C_6H_{13}O_5)_n$ and contained in the major component of the exoskeleton of crustaceans. Due to moulting this polysaccharide has to be steadily replaced. It is synthesized from glucose via glucosamine, a precursor of chitin usually found in aquatic arthropods (Kanazawa, 1982). Although dietary crude fiber may stimulate the microbial gut flora, its composition in commercial diets should not be more than 5.0% (Fair et al., 1980).

2.5.3 Lipids: Lipids are fatty acid esters of glycerol and the primary means by which animals store energy. Dietary lipids have two major functions. They are sources of energy and fatty acids. Dietary lipids have also give palatability to the feed and serve as a vehicle for the absorption of fat-soluble vitamins and sterols and in addition play a very important role in the structure of biological membranes such as phospholipids and sterol esters (Dupree et al., 1984; Chuang, 1990). Lipids with high content of unsaturated fatty acids particularly polyunsaturated fatty acids (PUFA) are very liable to oxidation. Metabolites of lipid oxidation may react with other nutrients and reduce their availability. Malonaldehyde, a product of fat oxidation can be toxic to fish (Castell, 1979). Crustaceans have to be provided with exogenous lipids. The requirement is lower than that of fish. Recommendations in diet are in the range of 4.0 to 10.0% for marine shrimps and 3.0 to 6.0% for freshwater shrimps. In juvenile Kuruma prawn (*Penaeus japonicus*) the n-3-series of fatty acids is more effective than the n-6-series. The reproductive performance of shrimp broodstock is insufficient when there is lack of essential fatty acids (EFA) (Millamena et al., 1984). In the presence of phospholipids larval *Penaeus japonicus* required less PUFA than juveniles. The requirement is estimated to be 1.0 to 1.6%. Crustaceans have a dietary requirement for sterols because they are incapable of synthesizing *de novo* sterols from acetate and mevalonic acid. Dietary cholesterol is the most effective lipid ingredient. Very likely sterols other than cholesterol have to be converted to cholesterol in the body (Teshima et al., 1986). Diet supplement with 0.25 to 1.0% cholesterol meets the requirements of *Penaeus japonicus*. juvenile shrimp (Petriella et al., 1984).

2.5.4 Vitamins: Vitamins are organic nutrients, essential to man and animals. Insufficient dietary supply results in deficiency symptoms. Although vitamins occur naturally all of them can be synthesized by chemical or microbiological processes. The characteristic of a true vitamin is the co-enzyme function (Cody, 1984). There are 14 vitamins which are classified as fat-soluble and water soluble vitamin as showed in Table 5. Vitamins are important for good health and high growth performance.

Table 5. Major natural sources of vitamins.

Vitamins	Other name for vitamin	Sources
1. Fat-soluble Vitamins		
Vitamin A	Retinol	Fish oils
Vitamin D_3	Calciferol	Fish oils
Vitamin E	Tocopherol	Vegetable oils
Vitamin K_3	Menadion	Leaf meals, alfalfa
2. Water-soluble Vitamins		
Vitamin B_1	Thiamin	Legumes, bran, yeast
Vitamin B_2	Riboflavin	Yeast, liver, milk, soya beans
Vitamin B_6	Pyridoxine	Yeast, cereals, liver
Vitamin B_{12}	Cyanocobalamin	Fish meal, fish viscera, slaughterhouse wastes
Vitamin C	Ascorbic acid	Fresh fish tissue, insects
Biotin	Vitamin H	Liver, yeast, milk products
Folic acid	Pteroylglutamic acid	Yeast, fish tissue, fish viscera, leaf meal
Nicotinic acid	Vitamin PP	Yeast, legumes, forage
Antithetic acid	Vitamin B_3	Yeast, bran, animal offal, Fish tissue
Choline	Vitamin B_4	Wheat germ, legumes

Vitamins have been described such as enhancing immunity of the organism by vitamin A and the detoxifying effect of vitamin E when there is an over-supply of selenium (Hertrampf, 1985). Generally, fat-soluble vitamins and vitamin C are less stable than other water-soluble vitamins. The stability of vitamins are effected by moisture, oxidation, reduction, trace minerals, heat, light, pressure, pH and the storage time (Coelho, 1991).

The primary sign of vitamin deficiencies in crustaceans are growth retardation and high mortality rate. However, in most cases vitamin deficiency symptoms are due to insufficient availability of more than one vitamin (Hepher, 1988). Fat-soluble vitamins, particularly vitamin A, can be deposited in the liver while water soluble vitamins are hardly deposited in the organism. Deficiencies of water-soluble vitamins therefore occur much faster than fat-soluble vitamins. Vitamin requirements have been categorized and defined as the: minimum vitamin requirement, optimum vitamin requirement and suboptimal scope. Stress situations such as high salinity or poor water quality require higher vitamin fortification. Since crustaceans are slow eaters, their diets require higher vitamin levels to counteract the loss of water-soluble vitamin through leaching.

Vitamin toxicity, too high vitamin supply can be harmful to animals. Tolerance levels are not the same for each vitamin that of the requirement (Combs, 1988). The toxicity of vitamins is classified as follows: vitamins with high toxic potential (vitamin A, vitamin D and choline), vitamins with moderate toxic potential (vitamin B_2, nicotinic acid, pantothenic acid) and vitamins with low toxic potential (vitamin E, vitamin K_3, vitamin C, vitamin B_1, vitamin B_6, vitamin B_{12}, biotin, folic acid). The excess vitamin supply (hypervitaminosis) has been observed in fish (Tacon, 1987).

2.5.5 Minerals: Minerals are essential nutrients for aquatic animals. For optimum performances they must be provided in proper amounts and in biologically available form. In contrast to terrestrial animals, aquatic animals can absorb part of the required minerals from the water through their gills or even through their entire body

surface. Minerals are not only required for the formation of bones, scales, teeth and exoskeletons, but are also needed in physiological processes and functions (Piedad, 1989). The mineral content of the whole body depends on the age of the animal and the mineral supply. Both essential and almost all non-essential minerals are found in the animal body. But the general opinion that marine aquatic animals are highly contaminated with heavy metals, has been proven to be incorrect. Mineral absorption is effected by the aquatic species and certain environmental factors such as mineral content of the water, water temperature and pH of the water. The bio-availability of minerals, as effected by a number of factors, is comparable to the digestibility of nutrients of organic (Lall, 1989). The basic of mineral function and requirements for crustaceans are similar to that of fish. Marine crustaceans are able to absorb mineral from the aquatic environment. The more intensive shrimp culture which less the physiological mineral requirement can be met by the water (Kurmaly, 1994). Due to the frequent moulting of crustacean large quantities of minerals are lost and have to be replaced. Prior to ecdysis, minerals must be removed from the old exoskeleton to soften it. Only a certain portion of the minerals can be stored in tissues. Oversupply of minerals might be just as harmful as deficiencies as indicated by experiments under laboratory conditions (Kanazawa et al., 1984). There are 23 minerals, classified as macro and micro (trace) minerals, which are essential to aquatic animals and guidelines for the macro and trace mineral requirement as shown in Table 6 (Ewart, 1982; Tacon, 1987; Davis, 1990). Minerals required at levels of more than 100 mg/kg diet (basis: dry matter) are macro elements. All others are micro (trace) minerals.

2.5.6 Other Feed Supplements

2.5.6.1 Chemo-attractants

Gustation and Olfaction: Food attraction and feeding stimulation are significant consideration in the formulation and ultimate acceptance of aquatic feeds. The absence or presence of a minimal concentration of ingredients elicits a positive stimulatory response in a particular aquatic species (Meyers, 1986).

Table 6. Classification of minerals in feed for cultured aquatic animals and requirements of shrimp.

Macro minerals (%)		Trace minerals (mg/kg)		Trace minerals[1]	
Calcium	Ca (0.5-1.25)	Cobalt	Co (5-10)	Arsenic	As
Phosphorus	P (1.0-2.0)	Copper	Cu (10-35)	Chromium	Cr
Potassium	K (0.7-0.9)	Iodine	I (30)	Nickel	Ni
Magnesium	Mg (0.1-0.3)	Iron	Fe (70-300)	Silicon	Si
Sodium	Na (0.2-0.6)	Manganese	Mn (20-45)	Tin	Sn
Sulfur	S	Selenium	Se (0.1-0.2)	Vanadium	V
Chloride	Cl	Zinc	Zn (90-110)		
		Aluminium	Al		
		Fluorine	F		
		Molybdenum	MO		

[1] Biological function still uncertain

The pattern of food search behavior is induced by the taste (gestation) and the smell (olfaction). The sense of taste and smell are both chemical senses. The taste is the 'sense at close range' while the smell is the 'sense at a distance'. Receptors of the taste are the taste buds which are chemo-receptors. The total number of taste buds varies from species to species (Nørhede, 1991). In aquatic animals the sense of test is more important than the sense of smell. Taste buds not only can be found in the mouth but also in the lips. They also may cover the head as well as the whole body including the tail.

Phagostimulatory substances: To make the search for food by cultured aquatic animals more efficient, formulated feed is fortified with phagostimulatory substances, also named chemo-attractants. They provide the proper 'signals' that allow aquatic animals to recognize the pellet as a potential food source. To activate the feeding behavior of aquatic animals is quite complex (Ward, 1991). An important activator of feeding behavior is glycine (Meyers, 1986). Mixtures of amino acids have proven to be more effective than individual amino acids (Adron et al., 1978). The essential amino acids like lysine may be has no or only limited chemo-attractant effect (Mearns, 1985). The lipid fraction of the feed also may have chemo-attractant properties. Phospholipids and their derivatives were found to be attractive for yellowtail *Seriola quinqueradiata* and abalone *Haliotis spp.* (Harada, 1987; Fallu, 1991). Glycine content is found in relatively large concentration in various marine invertebrates as crab 35-711, shrimp 251-961 and krill 106 mg/100 g raw muscle (Meyer, 1986). The acceptance preference for certain chemo-attractants is differs between aquaculture species.

2.5.6.2 Antibiotics

Performance promotion: Antibiotics are bacteriostatic or bactericidal substances in the wide range of microorganisms. They are priority effective against bacterial infections but not for viral infections. Antibiotics are somewhat selective in their antibacterial actions. They are, therefore, divided into effectiveness against

'gram negative bacteria' and 'gram positive bacteria'. For instance, streptomycin is mainly effective against gram-negative bacteria while penicillin is most effective against gram-positive organisms. Antibiotics such as chlortetracycline and oxytetracycline have a wider rage of activity and are effective against both of bacteria, called 'broad-spectrum' antibiotics, the former called 'narrow-spectrum' antibiotics (Siegmund et al., 1961). The performance promoting effect of antibiotics in mammals and poultry is well while in fish and shrimp cultures were less successful. It is presumed that, contrary to warm-blooded animals, fish do not have a permanent bacterial flora in the intestine because the empty gut of healthy fish is more or less sterile (Bardach et al., 1972). In cultured aquatic animals signs of resistance to pathogenic bacteria have been observed after feeding diets containing antibiotics (Fallu, 1991). The use of antibiotics as performance promoter is limited in many countries. An antibiotic which should be approved by the EU as a performance promoter has to meet the following essential criteria: The antibiotic is not to be used for human and veterinary medicine, after feeding of an antibiotic there are no residues in the animal that is produced and there should be no development of any kind of resistance against pathogenic germs. Thus only few antibiotics meet the above mentioned requirements. The number of feed antibiotics approved by the EU is very short period such as avilamycin, avoparcin, flavophospholipol, monensin-sodium, salinomycin-sodium, spiramycin, tylosin-phosphate, virginiamycin and zinc bacitracin (Weinreich et al., 1994), and will be re-considered due to popular objections.

Prevention and cure: A wide rang of antibiotic is important for the prevention and treatment of diseases in aquatic animals. However, this is no longer a nutritional matter. The application, therefore, has to be supervised by veterinarians or fish pathologists. After application of the antibiotic, withdrawal periods have to be observed so that the consumable parts of the aquatic animals are free of residues. Due to this concern some countries have enforced strict regulation (JWH, 1995).

2.5.6.3 Antioxidants

Autoxidation and chemical reactions: Feed and feedstuffs readily undergo oxidation when exposed to air. The oxidation reaction is irreversible and results in chemical changes causing losses in nutrients and a reduction in the shelf-life of feed and feedstuffs. This oxidation reaction is referred to as 'autoxidation' or 'rancidity'. Predominantly, substances which have unsaturated carbon (C) atoms (double bonds) in their molecular structure are very susceptible to autoxidation. Ingredients commonly effected by autoxidation are fat, oils, vitamins and pigments. Rancidity causes palatability problems in feed, loss in vitamin potency and colour strength of pigments. Protein and carbohydrates are not prone to autoxidaion because they do not contain the oxygen labile double bonds. It can be prevented by chelating of metallic ions that are responsible for catalyzing the initiation phase of autoxidation and by scavenging of free radicals by antioxidants which serve as electron donors. Substances that prevent autoxidation are classified as: natural antioxidants and synthetic antioxidants. Some natural chelating agents available are citric acid, tartaric acid, phosphoric acid and their salts. Damaging effects of oxidation: Major physiological and pathological damaging effects of feeding oxidized fat and oils are: dark colouration, anemia, lethargy, brown-yellowish pigmented livers, abnormal kidneys and gill clubbing.

The products of lipid oxidation will react with other nutrients, e.g. protein and vitamins, and reduce the available dietary levels. Oxidation products may also be toxic, e.g. malonalaldehyde is toxic to rats (Rasheed, 1963). Higher levels of vitamin E and selenium in diet may detoxify oxidized dietary fat. Antioxidants used as feed additives have to be safe for man and animals. The number of antioxidant has to be approved by the EU such as L-ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin, calcium-L-ascorbate, 5,6-Diacethyl-ascorbic acid, dodecyl gallate, sodium-L-ascorbate, octyl gallate, propyl gallate, synthetic alphatocopherol, synthetic deltatocopherol, synthetic gammatocopherol and natural extract with high content of tocopherols (Weinreich et al., 1994).